Changes in Serum Adhesion Molecules, Chemokines, Cytokines, and Tissue Remodeling Factors in Euthyroid Women Without Thyroid Antibodies Who Are at Risk for Autoimmune Thyroid Disease: A Hypothesis on the Early Phases of the Endocrine Autoimmune Reaction

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Background: The target glands in spontaneous animal models of endocrine autoimmune disease show, prior to the autoimmune reaction, growth and connective tissue abnormalities, whereas the autoimmune reaction is initiated by an early accumulation of macrophages and dendritic cells in the target glands.

Aim: The aim of the study was to test the hypothesis that serum factors related to these growth and connective tissue abnormalities and the early accumulation of immune cells, ie, tissue growth/remodeling factors, adhesion molecules, chemokines, and pro- and anti-inflammatory cytokines, are related to thyroid peroxidase autoantibodies (TPO-Abs) seroconversion in subjects at risk to develop autoimmune thyroid disease (AITD).

Design: A controlled study on 64 TPO-Ab-negative euthyroid female relatives with at least 1 first- or second-degree relative with documented autoimmune hyper- or hypothyroidism, 32 of whom did and 32 who did not seroconvert to TPO-Ab positivity in 5-year follow-up. The relatives were compared with 32 healthy controls. In all subjects we measured serum levels of chemokine (C-C motif) ligand (CCL)-2, CCL3, CCL4, soluble vascular cell adhesion molecule, soluble intercellular adhesion molecule-1, thrombospondin-1, vascular endothelial growth factor-A, angiopoietin 1 receptor-2, metalloproteinase-13, platelet-derived growth factor-BB, fibronectin, IL-1β, IL-6, TNF-α, IL-10, and growth differentiation factor-15 by multiplex (cytometric bead array) or a single commercial ELISA.

Results: Both seroconverting and nonseroconverting family members showed an up-regulation of fibronectin and a down-regulation of platelet-derived growth factor-BB and the adhesion and migration factors CCL2, CCL4, soluble vascular cell adhesion molecule-1, angiopoietin 1 receptor-2, and metalloproteinase-13. The seroconverters differed from the nonseroconverters by an up-regulation of the proinflammatory compounds Il-1β, IL-6, and CCL3.

Conclusion: This study shows that euthyroid females within AITD families show a characteristic pattern of abnormalities in serum levels of tissue remodeling factors, growth factors, chemokines, (vascular) adhesion molecules, and cytokines prior to the occurrence of TPO-Abs in serum. The results provide proof of principle that preseroconversion stages and seroconversion to AITD might be predicted using serum analytes related to growth/connective tissue abnormalities and migration/accumulation abnormalities of macrophages and dendritic cells. **(*J Clin Endocrinol Metab* 98: 2460–2468, 2013)**

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Abbreviations: AITD, autoimmune thyroid disease; BB-DP, biobreeding diabetes-prone; CCL, chemokine (C-C motif) ligand; DC, dendritic cell; FN, fibronectin; FT_4, free T_4; GDF-15, growth differentiation factor-15; HC, healthy control; IQR, interquartile range; MMP, metalloproteinase; NOD, nonobese diabetic; NSC, nonseroconverter; PDGF, platelet-derived growth factor; SC, seroconverter; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule 1; TBII, thyrotrophin binding inhibiting immunoglobulin; T1D, type 1 diabetes; THBS1, thrombospondin-1; TIE-2, angiopoietin 1 receptor-2; Tg-Ab, thyroglobulin antibody; TPO-Ab, thyroid peroxidase autoantibody; VEGF, vascular endothelial growth factor.

The etiology of autoimmune thyroid disease (AITD), encompassing Graves' hyperthyroidism and Hashimoto's thyroiditis, is multifactorial (1). Genetics play an important role in the development of AITD, illustrated by the concordance rate for Graves' disease and Hashimoto's hypothyroidism in monozygotic twins, which is higher than in dizygotic twins (2, 3). In addition, many patients with AITD have family members affected by this disorder. However, it has been estimated that at best 79% of the liability to develop AITD can be attributed to genetics (3), and therefore, environmental risk factors must also be involved (4).

In the early 2000s, we initiated a follow-up cohort study (the Amsterdam AITD cohort) to determine risk factors involved in newly incident cases of AITD. To increase the likelihood of diagnosing new patients during a 5-year follow-up, we included only women who had at least 1 relative with documented AITD and who were in self-proclaimed good health. Evidence for thyroid autoimmunity, ie, presence of thyroid peroxidase autoantibodies (TPO-Abs), was found at baseline in 24% of the cohort (5). In the 5-year follow-up 10.2% of participants developed de novo TPO-Abs [so-called seroconverters (SC)], whereas 6.5% progressed to overt autoimmune hypothyroidism or hyperthyroidism, particularly in the TPO-Ab-positive subjects. Taken together, our findings in the Amsterdam cohort are thus in agreement with the generally accepted view that subjects with a family history of AITD are at increased risk for autoimmune thyroid disease and that TPO-Abs are acceptable markers to predict progression to clinical disease (5–7).

Markers to predict the risk for AITD in very early stages of the autoimmune disease and before TPO-Abs are detectable in serum are limited and comprise the genetic polymorphisms of immune-related genes (major histocompatibility complex class II molecules, cytotoxic T-lymphocyte antigen 4, protein tyrosine phosphatase, non-receptor type 22) and genes related to thyroid antigens (the TSH receptor and thyroglobulin) (1, 8). For this study we hypothesized that other early prediction markers might be found in a set of molecules related to the accumulation and activation of macrophages and dendritic cells (DCs) in the thyroid gland just prior to thyroid autoimmunization. In animal models of spontaneously developing AITD, ie, the nonobese diabetic (NOD) mouse and biobreeding diabetes-prone (BB-DP) rat, macrophages and DCs play a key role in the initiation of the thyroid autoimmune reaction (9, 10). Extensive data gathered in these animal models show that the actual process starts with the accumulation and clustering of macrophages and DCs in the target organs, shortly followed by a T and B cell reaction in the draining lymph nodes.

The initial accumulation of macrophages and DCs in the target glands-to-be is thought to occur via infiltration of monocytes from the blood stream. Diapedesis of monocytes from the blood stream via endothelium into and through the tissues requires chemokines (11), such as eg, chemokine (C-C motif) ligand (CCL)-2, CCL3, and CCL4. Apart from chemokines, the attachment of the cells to the endothelium is important for diapedesis, and after diapedesis an interaction of the cells with extracellular matrix proteins is essential (12). Vascular factors and adhesion molecules, such as integrins, vascular cell adhesion protein 1, and thrombospondin-1 (THBS1) (13), play important roles in these adhesive interactions. Evidence of the important role of chemokines and integrins has been found previously in AITD in the up-regulation of CCL2 (14) and that of soluble intercellular adhesion molecule-1 (sICAM-1 or soluble cluster of differentiation 54) in the serum of patients (15, 16). There are also indications that the angiopoietin-1 receptor (TIE-2) system has a role in the recruitment of monocytes to the thyroid gland in AITD (17).

Apart from adhesive processes, a remodeling of the connective tissue is a prerequisite for monocytes, macrophages, and dendritic cells to travel through the tissues. Matrix metalloproteinases (MMPs) and platelet-derived growth factors (PDGFs) produced by the cells of the mononuclear phagocyte system are instrumental in this remodeling (18).

Finally, to initiate the thyroid autoimmune response, DCs and macrophages need to switch to a proinflammatory nontolerogenic state. Cytokines, such as IL-1β, IL-6, TNF-α, IL-10, and growth differentiation factor 15 (GDF-15) secreted by these proinflammatory mononuclear phagocytes are positive and negative regulators of such inflammatory set point change of the cells.

In this study we investigated the putative abnormalities in the above-listed chemokines, adhesion molecules, tissue remodeling factors, growth factors, and pro- and antiinflammatory cytokines in humans at risk for the development of AITD, taking advantage of the Amsterdam AITD cohort (5). We compared serum levels of CCL2, CCL3, CCL4, soluble vascular cell adhesion molecule (sVCAM)-1, sICAM-1, THBS1, vascular endothelial growth factor (VEGF)-A, TIE-2, MMP13, PDGF-BB, fibronectin (FN), IL-1β, IL-6, TNF-α, IL-10, and GDF-15 between the 3 groups: 32 euthyroid family members of AITD patients who developed de novo TPO-Abs during the 5-year follow-up (SCs), 32 euthyroid family members of AITD patients who did not develop TPO-Abs in the follow-up

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[nonseroconverters (NSCs)] and 32 healthy controls (HCs); the latter 2 control groups of subjects were matched to the SCs for age, smoking habits, and current estrogen medication. We previously reported that current smoking prevented the development of TPO-Abs in the cohort (19); the use of oral contraceptives was also negatively associated with the development of TPO-Abs (20).

Subjects and Methods

Subjects

The present studies were carried out among the 803 subjects from the Amsterdam AITD cohort. The cohort has previously been described in detail (20). In short, the cohort consisted of women between 18 and 65 years of age in self-proclaimed good health without a history of thyroid disease, who had at least 1 first- or second-degree relative with documented autoimmune hyper- or hypothyroidism. Results of thyroid function tests at study entrance revealed overt hypothyroidism in 10 subjects and overt hyperthyroidism in 3 subjects, leaving 790 subjects to be included in the present study. Subjects were followed up for 5 years, or less time when overt hyper- or hypothyroidism had occurred (defined as TSH $<$ 0.4 mU/L in combination with free T_4 (FT_4) $>$ 20.1 pmol/L or TSH $>$ 5.7 mU/L in combination with FT_4 $<$ 9.3 pmol/L, respectively). At each annual visit to our institution blood samples were collected to measure TSH, FT_4, TPO-Abs, thyroglobulin antibodies (Tg-Abs), and thyrotrophin binding inhibiting immunoglobulin (TBII).

All subjects were asked to fill in questionnaires on smoking habits (current and past) and use of oral contraceptives or other estrogens (current and past). Current pregnancy is an exclusion criterion for the present studies. Current smoking is defined as smoking now or having stopped smoking within 1 year before visiting our institution clinic. Current estrogen usage is defined as presently on exogenous estrogen medication.

The population who qualified for this study was selected from the inception cohort of the 790 euthyroid subjects after excluding subjects who had any serological sign of AITD at baseline, ie, abnormal TSH and/or thyroid antibodies at baseline (TPO-Abs of $\geq$ 100 kU/L, Tg-Abs of $\geq$ 100 kU/L, or TBII of $\geq$ 12 U/L). Consequently, 521 euthyroid participants without any serological signs of AITD at baseline were thus enrolled.

The actual selection of subjects for the present study from these 521 participants was performed as follows: at first 81 euthyroid subjects who were TPO-Ab and Tg-Ab negative at baseline but developed TPO-Abs during follow-up (so-called SCs) were identified. The end point for a seroconverter was the time at which she had become positive for the first time for TPO-Abs without developing abnormal TSH. Each selected SC was matched with a euthyroid subject who was TPO-Ab and Tg-Ab negative at baseline and did not develop TPO-Abs up to the time at which the seroconverter to whom they were matched had received her end point. SCs and NSCs were matched for age, current smoking use, current estrogen use, and duration of follow-up. Because the cost of measuring all the compounds in all samples is very high, for reasons of cost-effectiveness, we then randomly selected 32 seroconverters and their corresponding 32 NSCs. The mean age of the selected subjects was 36.8 years (range 20–58 years).

As a control group, we used 32 female subjects between 20 and 69 years of age, who were recruited through advertisements in local newspapers, to participate in an ongoing program within our institution for delineating reference values of endocrine function tests. They were also in self-proclaimed good health, had no family or personal history of thyroid disease, and had normal TSH and no thyroid antibodies. Blood samples were collected over the same period of time as those of the Amsterdam AITD cohort and were processed in the same manner.

All subjects gave informed written consent and the Medical Ethics Committee of the Academic Medical Center in Amsterdam and the Medical Ethics Committee of Erasmus Medical Center in Rotterdam approved the study.

TSH, FT_4, and TPO-Ab determinations

Serum samples were stored at −20°C until determination of the study parameters. Serum TSH and FT_4 were measured using time-resolved fluoroimmunoassay (Delphia, Turku, Finland). Reference values are 0.4–5.7 mU/L for TSH and 9.3–20.1 pmol/L for FT_4. TPO-Abs and Tg-Abs were measured by chemiluminescence immunoassays (LUMI test anti-TPO and LUMI test antithyroglobulin, respectively; Brahms, Berlin, Germany). Improved versions of both assays became available during follow-up: the detection limits of these new assays were 30 kU/L for TPO-Abs and 20 kU/L for Tg-Abs. The TPO-Ab concentrations obtained with the old assay were multiplied by a factor 0.72 to obtain comparative values in the new assay. TPO-Ab and Tg-Ab concentrations were considered to be positive at values greater than 100 kU/L. TSH receptor antibodies were determined as TBIIs using the TRAK assay (Brahms); the detection limits in the first- and second-generation TRAK assays were 5 and 1 IU/L, respectively, and values above 12 and 1.5 U/L, respectively, were considered to be positive.

Serum analytes

The levels of TNF-α, IL-10, IL-6, IL-1β, MMP-13, CCL3, CCL4, VEGF-A, PDGF-BB, CCL2, sVCAM-1, and sICAM-1 were measured using the FlowCytomix multiple analyte detection system (eBioscience, San Diego, California), a bead-based multiplex immunoassay, according to the manufacturer's protocol. Samples were analyzed with a BD LSR II flow cytometer (BD Biosciences, San Diego, California), and the raw data were further converted with FlowCytomix Pro 3.0 software (eBioscience). ELISA technology was used for single-analyte detection of FN and TIE-2 (platinum ELISA; eBioscience) and GDF-15 and THBS1 (quantikine colorimetric sandwich ELISA; R&D Systems, Abingdon, United Kingdom) according to the manufacturer's protocol. Undetectable serum analyte levels were considered as 0 pg/mL and were included in the statistical analysis.

Statistics

Statistical analysis was performed using the SPSS 20 package for Windows (SPSS Inc, Chicago, Illinois). Data were tested for normal distribution using the Kolmogorov-Smirnov test. Depending on the distribution pattern, parametric (Student's *t* test and 1 way ANOVA) or nonparametric group comparisons (Mann-Whitney *U* and Kruskal-Wallis *H* tests) were applied. Correlations were determined by Spearman correlation. Dendrograms were constructed by SPSS using hierarchical cluster analysis of the serum analytes using the between-groups linkage method. The heat map was designed using Java Treeview (by

Table 1. Characteristics of Healthy Controls and Female Euthyroid Relatives of AITD Patients Grouped for TPO Antibody Conversion During 5 Years of Follow-Up

	HCs	NSCs	SCs	HC vs NSC *P* Value	HC vs SC *P* Value	NSC vs SC *P* Value
n	32	32	32			
Age, y, mean (range)	35 (22–61)	33 (19–62)	33 (18–61)	.401	.399	.999
BMI, kg/m^2, mean (range)	23.8 (18.1–33.7)	24.0 (18.7–42.1)	24.2 (19.1–40.8)	.840	.744	.902
TSH median (IQR)	1.50 (1.20–2.00)	1.20 (1.00–1.65)	1.40 (1.20–2.00)	.473	.141	.265
FT_4 median (IQR)	13.0 (12.2–14.7)	13.5 (12.3–14.6)	12.0 (11.9–14.5)	.894	.533	.653
Current smoking, %	15 (47%)	15 (47%)	15 (47%)			
Current estrogen use, %	6 (19%)	12 (38%)	12 (38%)			

Alok Saldanha, Department of Genetics, Stanford University School of Medicine). Levels of significance were set at $P = .05$ (2 tailed). The specific tests and group size are mentioned in table footnotes and in the figure legends. Graphs were designed with GraphPad Prism 5.04 (GraphPad Software, San Diego, California) for Windows.

Results

To select subjects without serological signs of thyroid autoimmunity at baseline, we included women from the inception cohort who were in self-proclaimed good health, had a normal TSH and normal FT_4, and were negative for TPO-Abs, Tg-Abs, and TBII at study entrance. With regard to the SCs, 6 of the 32 women (19%) who developed TPO-Abs during follow-up were also Tg-Ab positive at the time of seroconversion to TPO-Abs (Tg-Ab levels were 235, 180, 135, 120, 115, and 100 kU/L). With regard to the NSCs, just 2 of the 32 women (6%) were Tg-Ab positive (levels 150 and 125 kU/L) at the time their corresponding SC developed TPO-Abs. As expected and as a result of the randomization and stratification procedure, SCs, NSCs, and HCs were not different regarding age, current smoking behavior, and current estrogen medication (Table 1).

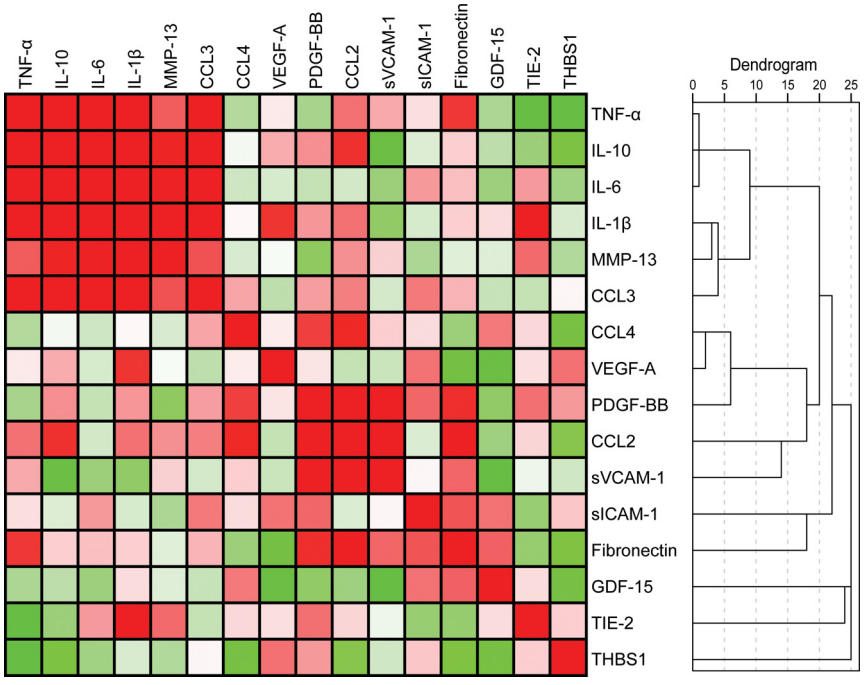

Figure 1. Heat map of hierarchical cluster analysis of the tested serum levels of cytokines, chemokines, adhesion molecules, and tissue remodeling factors. Color-coded correlation matrix illustrates Spearman's correlation coefficients between the serum levels of the indicated compounds. Significant positive correlations ($P < .05$) are given in the red scale (darkest red are correlation coefficients >0.50), and significant negative correlations are given by the green scale. Lighter fields are not significant. In addition, a dendrogram is presented as a result of the hierarchical clustering. The dendrogram and heat map show in essence 3 major clusters of mutually correlating compounds: 1 cluster of inflammatory compounds (cluster A: TNF-α, IL-10, IL-6, IL-1β, MMP13, and CCL3); a second cluster of vascular adhesion and growth and migration factors (cluster B: CCL4, VEGF-A, PDGF-BB, CCL2, and sVCAM-1) and a small cluster (cluster C) containing sICAM1 and FN.

Figures 2–4, and Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org, show the results of the analyte measurements in the serum of the SCs, NSCs, and HCs. But first, we performed a cluster analysis (Figure 1) and found in essence 3 clusters of mutually in-expression-level correlating compounds, ie, a cluster A containing most of the inflammatory cytokines/chemokines (TNF-α, IL-10, IL-6, IL-1β, MMP-13, and CCL3), a cluster B containing the vascular adhesion, growth, and migration factors (CCL4, VEGF-A, PDGF-BB, CCL2, sVCAM-1), and a small cluster C containing FN and sICAM-1. The other tested compounds (GDF-15, THBS-1, and TIE-2) did barely correlate to the expression levels of the other compounds. Results in Figures 2–4 and Supplemental Table 1 are presented

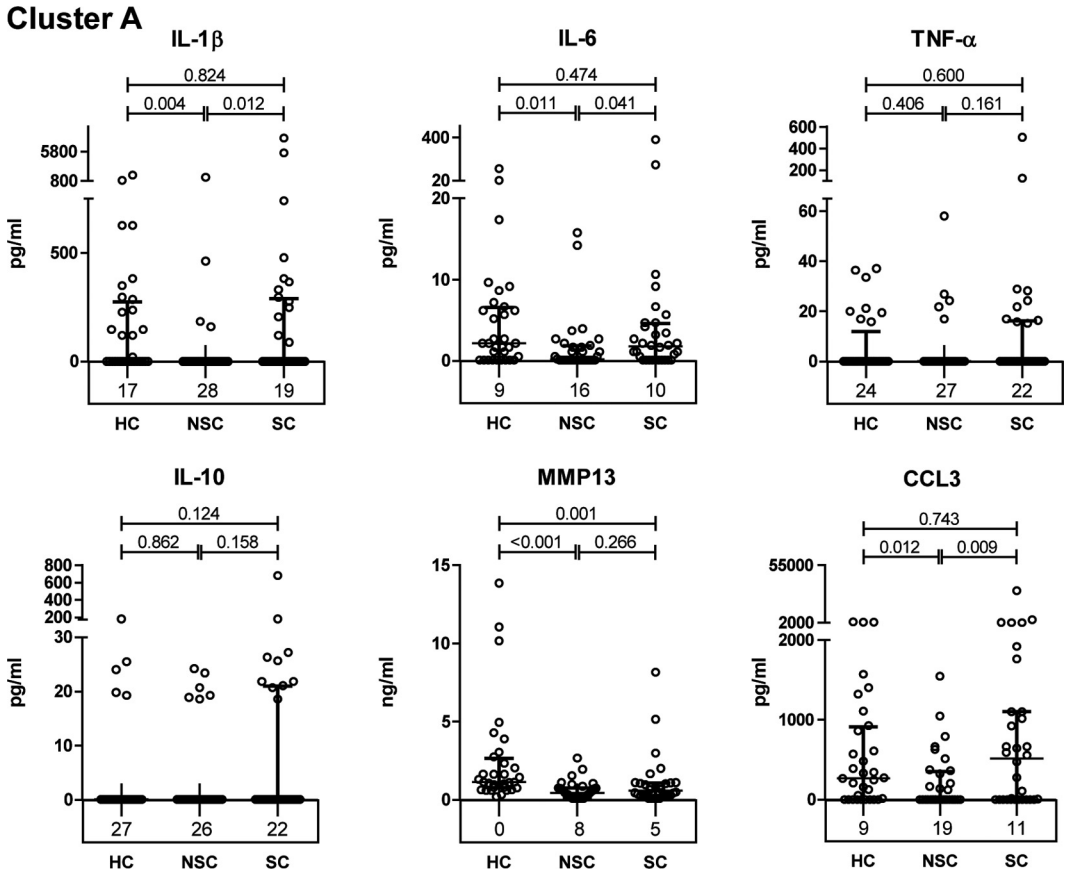

Figure 2. The figure represents the outcomes of serum measurements of all cluster A compounds. Medians with IQR are shown for each group of serum measurement in HCs and NSC and SC family members. Bars represent *P* values between groups (see also Supplemental Table 1); the number of undetectable (0) concentrations is shown below the dots for each study group.

according to the cluster analysis as groups of compounds.

In NSC subjects, as compared with HCs, an up-regulation of cluster C compounds was found, namely a statistically significant strong up-regulation of FN (see also Figure 4). The up-regulation of FN occurred in the vast majority of the relatives, ie, in 84% of subjects (defined as the proportion of values above the mean ± SD of the HCs, Figure 4). With regard to cluster B compounds, we found in NSC subjects a strong down-regulation (Figure 3). These reductions were particularly evident for PDGF-BB (94% of the values below the interquartile range (IQR) of the HCs of subjects), sV-CAM-1 (97% of subjects values below the mean ± SD of the HC of subjects), and CCL2 (81% of subjects values below the IQR of the HCs of subjects). With regard to cluster A compounds, the levels of the inflammatory cytokines/chemokines IL-1β, IL-6, and CCL3 were also reduced and most frequently not detectable in our cytometric bead array (Figure 2). With regard to the compounds found outside the clusters (Figure 4), the TIE-2 levels were strongly reduced as well in the group of NSCs (100% of subjects values below the IQR of the HCs of subjects).

Comparing SCs with HCs, the picture was the same as in the NSCs for cluster C compounds (namely, FN up-regulated in 84% of individuals above the mean ± SD of the HCs of subjects) and for cluster B compounds (down-regulated in 85% to 100% of cases below the IQR of the HCs of subjects) (see Figures 4 and 3, respectively). When comparing SCs and NSCs, SCs, however, differed from the NSCs for cluster A compounds (Figure 2): these inflammatory compounds were not down-regulated but were at the same level as in healthy controls but significantly up-regulated as compared with the NSC subjects for the proinflammatory cytokines Il-1β and IL-6 and the proinflammatory chemokine CCL3. There were no differences observed between SCs and NSCs in the other clusters of compounds.

We also investigated the analyte patterns of the smoking/nonsmoking and oral contraconceptives, using non-oral contraconceptives, using women separately, because we previously reported that smoking and the usage of oral contraceptives decreased the vulnerability for AITD (19, 20). Smoking did not have any effect on the levels of the analytes tested here. However, the use of oral contraceptives did but only for FN. Figure 5 shows that, with regard to FN, the use of oral contraceptives led to a smaller increase in the level of FN in all groups (the SCs, NSCs, and HCs, although in the latter, it was just not significant at $P = .06$).

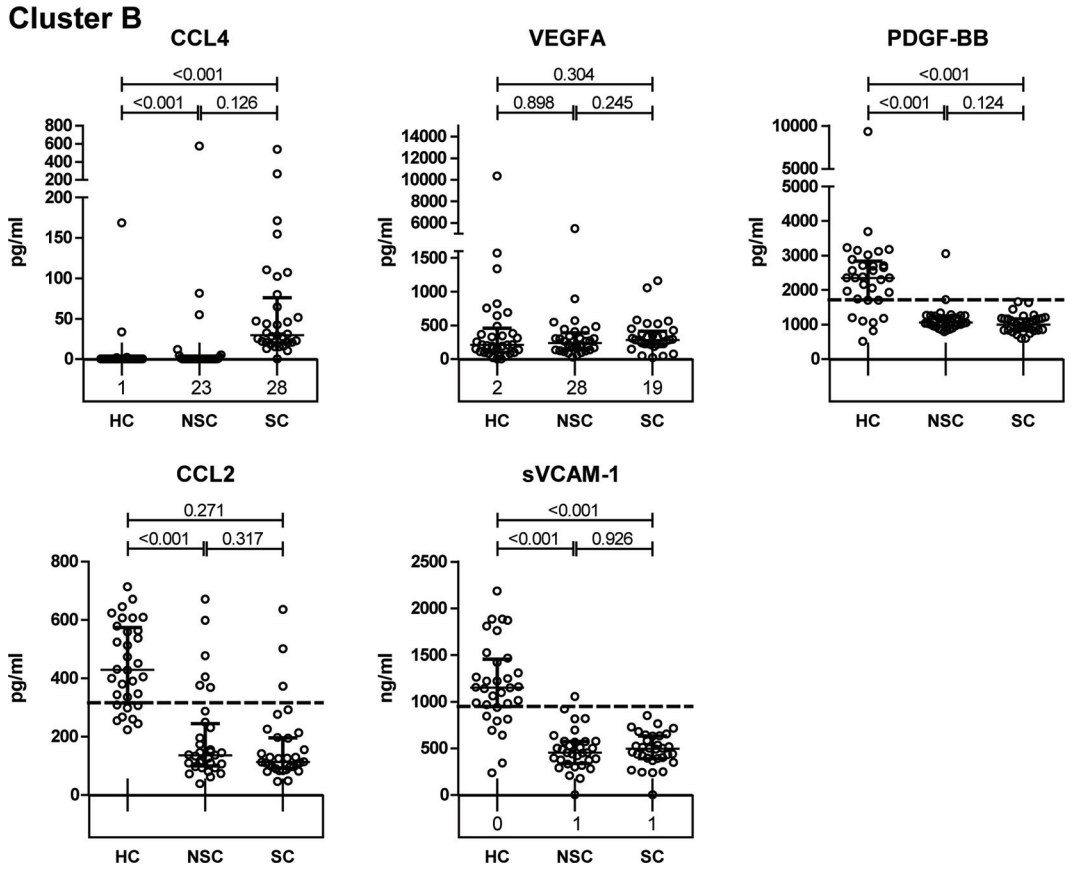

Figure 3. The figure represents the outcomes of serum measurements of all cluster B compounds. Medians with IQR are shown for each group of serum measurement in HCs and NSC and SC family members. In addition, sVCAM-1 was normally distributed; therefore, the mean with SD is shown. Bars represent *P* values between groups (see also Supplemental Table 1); the number of undetectable (0) concentrations is shown below the dots for each study group.

Discussion

This study shows that euthyroid females with at least 1 first- or second-degree relative with a documented autoimmune hyper- or hypothyroidism show a characteristic pattern of abnormalities in serum levels of tissue remodeling factors, growth factors, chemokines, (vascular) adhesion molecules, and cytokines, giving the proof of principle that very early stages of AITD (still no TPO-Abs in serum) are detectable in individuals at risk by testing peripheral blood for these compounds.

The levels of FN (up-regulated in more than 80%) and PDGF-BB (down-regulated in more than 90%) were strong determinants characterizing the first- or second-degree relatives, irrespective of later seroconversion to TPO-Ab positivity. The abnormalities suggest growth and connective tissue abnormalities in individuals with an inborn risk for AITD. Interestingly, there is an early report (21) on abnormalities in the growth of skin fibroblasts of first-degree relatives of individuals with type 1 diabetes (T1D; these individuals are also known to have a higher risk for AITD) (6, 22).

The observed high FN and reduced PDGF-BB levels in subjects at risk for AITD are reminiscent of the connective tissue and endocrine growth abnormalities that have been observed in the pre-autoimmune stage in animal models of spontaneously developing endocrine autoimmune disease, such as the obese strain chicken, the BB-DP rat, and the NOD mouse. Pre-thyroiditis abnormalities in the BB-DP rat involve a smaller thyroid volume (prior to lymphocytic infiltration) and a hampered growth of thyrocytes (23). Similar thyrocyte growth defects have been described for the obese strain of chicken, even as early as in fetal life (24). The thyroids of NOD mice have only accidentally been studied for abnormalities in growth, and in these studies a high frequency of intrathyroidal ectopic thymus tissue was found (25). However, the preweaning NOD pancreas has been studied in more detail. Irregularly shaped islets are a hallmark of the preweaning NOD pancreas (26), and these irregularly shaped islets show in addition an excessive FN content predominantly at the islet vascular pole (27). Interestingly, autoimmune insulitis starts in this model with an accumulation of macrophages and DCs essentially at these sites of high FN expression (27).

In the study reported here, we additionally found that the use of oral contraceptives resulted in a reduced increase

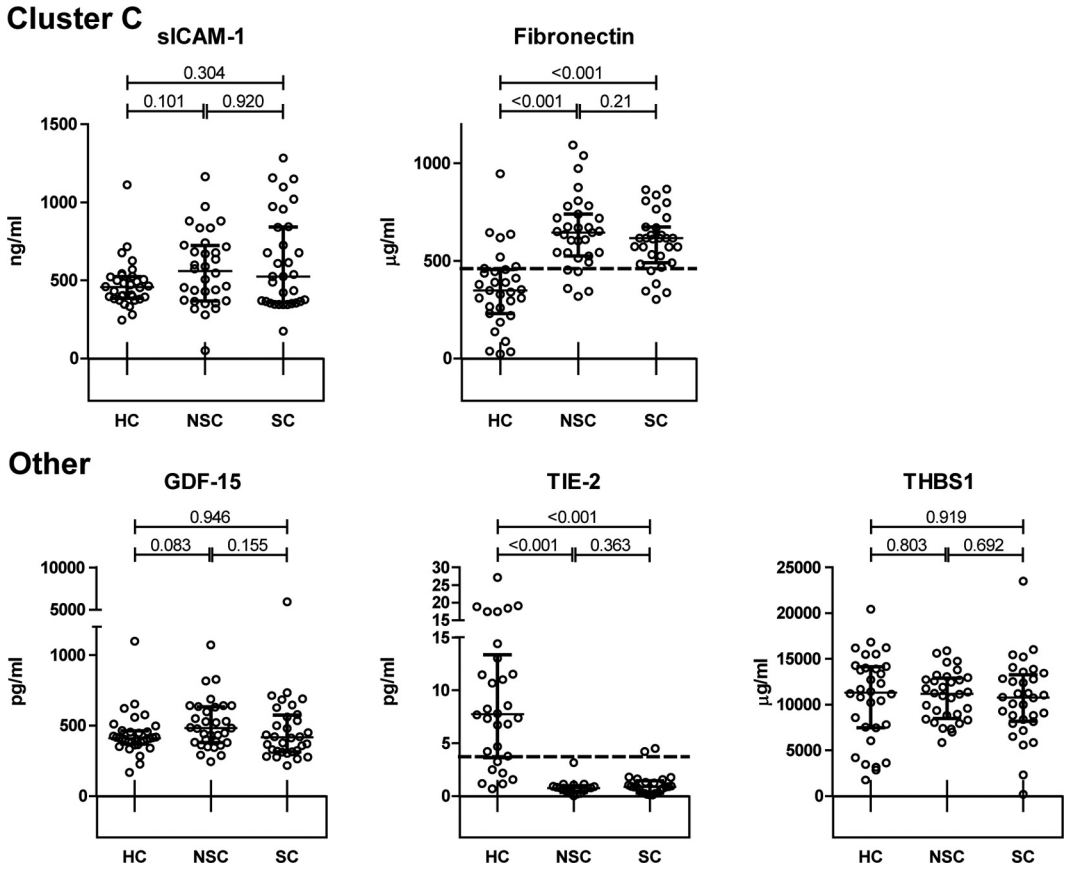

Figure 4. The figure represents the outcomes of serum measurements of all cluster C and other compounds. Medians with IQR are shown for each group of serum measurement in HC and NSC and SC family members. In addition, FN and THBS1 were normally distributed; therefore, the mean with SD is shown. Bars represent *P* values between groups (see also Supplemental Table 1).

in the serum level of FN. Because oral contraceptives also reduced the risk for TPO-Ab seroconversion in the Amsterdam cohort (20), it is tempting to speculate that oral contraceptives reduce the FN content in the target glands, thereby reducing the macrophage and DC accumulation, leading to a reduced incidence of seroconversion.

With regard to the chemokines, adhesion, and migration factors CCL2, CCL4, sVCAM-1, MMP-13, and TIE-2, we found these reduced in the serum of first- or second-degree relatives, irrespective of later seroconversion. In particular, the levels of sVCAM-1, CCL2, and TIE-2 were strong determinants and were reduced in 80%–100% of relatives. Interestingly, reduced CCL2 levels have also been reported in individuals genetically at risk for T1D (28).

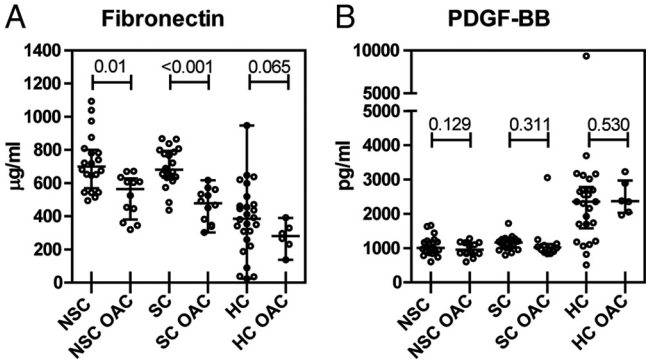

Figure 5. Means with SD are shown for the serum FN (A) and median with IQR for the serum PDGF-BB (B) levels in the tested groups, ie, HCs and NSC and SC family members, grouped according to the use of oral contraceptives (OACs; using oral contraceptives). Bars represent *P* values between groups. As can be seen, serum FN levels are reduced in subjects using oral contraceptives.

The reduced levels of chemokines, adhesion, and migration factors suggest a reduced general infiltration and migration of immune cells into and through the tissues of individuals with an inborn risk to develop an endocrine autoimmune disease. This is in contrast to patients with clinically overt AITD or T1D, who have been reported to have raised serum levels of CCL2 (14) and normal or even higher expression levels of soluble adhesion molecules and immune cellular infiltrations in their glandular tissue (29, 30).

The data on generally reduced levels of chemokines, adhesion, and migration factors in relatives of AITD patients reported here are again reminiscent of the reduced migration we have observed in the prephases of the autoimmune process in the animal models of spontaneously developing endocrine autoimmune disease. In particular, NOD macrophages show a hampered migration in these stages (31), and it is difficult to recruit (inflammatory)

monocytes from the circulation to the sites of inflammation in the NOD mouse (32).

The study reported here finally suggests that the levels of the proinflammatory cytokines and chemokines might make a distinction possible between SCs and NSCs because IL-1β, IL-6, and CCL3 did differ between NSCs and SCs (although it must be admitted that the sensitivity of the IL-1β assay was low). Reduced levels of the proinflammatory cytokines/chemokines as compared with healthy controls were measured in the family members who did not seroconvert in a follow-up of 5 years, and levels of IL-1β, IL-6, and CCL3 were reduced. In family members who did seroconvert to TPO-Ab positivity, this pattern was different, and IL-1β, IL-6, and CCL3 were significantly raised as compared with NSC subjects and reached equal values as found in comparison with healthy controls.

We like to explain this pattern of differences by assuming the following: 1) that in NSC family members of AITD patients, leukocytes are not only systemically down-regulated with regard to adhesion and migration (see previous text) but also with regard to the production of proinflammatory cytokines/chemokines, and 2) that in SC family members, the autoinflammation in the thyroid had already started and that intrathyroidal proinflammatory macrophages and DCs secrete proinflammatory cytokines, raising the levels above the systemically down-regulated levels generally found in the NSC family members.

Again, parallels with the NOD mouse model are striking. There are indeed indications that DCs and macrophages in the endocrine tissues of the NOD mouse are prior to the actual autoimmunization at a reduced maturation set point producing less chemokines and inflammatory cytokines but that at the time of seroconversion and the start of the autoimmune reaction, this reduced set point of DCs and macrophages turns over to a high inflammatory set point (33–38). In addition, there is evidence that these abnormal inflammatory DCs and macrophages do not support T cell tolerance mechanisms sufficiently, thus tipping the balance toward autoimmunization (39–42).

In conclusion, the study reported here is a limited study on a relatively small group of family members of AITD patients. The study also has its technical limitations. A limitation of the cytometric bead array test that we used for this study is that for some of the compounds sensitivity was low, such as for IL-1β, TNF-α, and IL-10. For these compounds the serum levels of many patients were below the detection limit. Also, because this is an explorative study, we did not take type I errors into account. Applying Bonferroni correction will result in a loss of significance between the subject groups for some of the cluster A compounds. Indeed, further validation studies in a larger cohort of subjects with more sensitive detection methods are needed.

Despite these limitations, our study represents a first report on the levels of tissue remodeling factors, adhesion molecules, chemokines, and cytokines in individuals at risk for thyroid autoimmune disease, and data suggest a characteristic pattern of abnormalities in the growth and composition of endocrine tissues and in the immune activation state prior to the autoreactive state and reminiscent of the preautoimmune state found in the animal models of endocrine autoimmune disease. An early identification of individuals at risk for a thyroid autoimmune disease and an exquisite knowledge on the abnormalities in immune-endocrine functioning in the very early stages are the basis for future rational approaches to prevent endocrine autoimmune disease. Therefore, the outcomes of this study urge, on the one hand, for a further profiling of the serum of individuals at risk in a multianalyte approach using assays with sufficient sensitivity and, on the other hand, for the determination of the size of the thyroid gland prior to the autoimmunization as well as the patterns of infiltration with macrophages and DCs using ultrasound and novel imaging techniques, respectively (43).

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