Early stages of thyroid autoimmunity: follow-up studies in the Amsterdam AITD cohort

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Chapter 9

General discussion
The studies presented in this thesis are all done within the Amsterdam AITD cohort. As outlined in chapter 1, the Amsterdam AITD cohort consists of euthyroid women between 18-65 years in self proclaimed good health who had in common that they all had one or more 1st or 2nd degree relatives with documented evidence of autoimmune thyroid disease, either Hashimoto’s disease or Graves’ disease. The cohort was followed up for 5-years with annual assessments. The uniqueness of the cohort is its follow up. Although abnormalities in thyroid function and regulation in relatives of patients with autoimmune thyroid disease has been studied before (including some early studies [1]) we were unable to detect in the literature previous studies (apart from the Amsterdam AITD cohort [2]) in which relatives of autoimmune thyroid disease patients had been followed in time.

**Natural history of autoimmune thyroid disease**

Although many studies have investigated the progression rate from subclinical to overt autoimmune hypothyroidism or hyperthyroidism, a careful review of the literature however did not detect studies on the progression from euthyroidism to overt autoimmune hypothyroidism or to overt hyperthyroidism. The data of chapter 2 suggest that progression towards overt autoimmune hypothyroidism is a gradual process taking several years, but that in contrast overt autoimmune hyperthyroidism develops faster in terms of months. This conclusion was based on the fact that the 38 hypothyroid cases had already higher TSH and lower fT4 concentrations than the 76 controls at baseline, whereas in contrast neither TSH nor fT4 values differed between the 13 hyperthyroid cases and their 26 controls at baseline. This was also true one year before the hyperthyroid event. It means that in the hypothyroid cases a minor degree of thyroid failure already existed at baseline and that the transition to autoimmune hypothyroidism had already started in the past. Although TPO-Ab were present in 79% of hypothyroid cases and 77% of hyperthyroid cases at baseline, TSH receptor antibodies were not demonstrable in hyperthyroid cases at baseline and one year before the event, but became detectable in Graves’ hyperthyroid cases at the time of the event. In line, we observed that the transition from euthyroidism to overt hypothyroidism in our study is on average 4 years, whereas the mean transition time from euthyroidism to overt hyperthyroidism of 2 years was shorter.

The development of overt hypothyroidism or overt hyperthyroidism can be viewed as the final stage of autoimmune thyroid disease. The immediate previous stage is the subclinical phase when serum TSH is abnormal but serum fT4 is still within the reference range: subclinical hypothyroidism or subclinical hyperthyroidism. Subclinical hypothyroidism is not a rare disorder with a prevalence around 5-10% in the general population [3-6]. Many studies have investigated the progression rate from subclinical to overt autoimmune hypothyroidism or hyperthyroidism. We have not studied specifically the transition from subclinical hypothyroidism or subclinical hyperthyroidism to overt hypothyroidism or hyperthyroidism in our cohort because the sample size was
too limited. However, literature studies indicate that in subclinical hypothyroidism there is frequently a spontaneous normalization of TSH (varying from 4% up to 52% in various studies [6-15]). Progression to overt hypothyroidism ranges from 7.8% to 17.8% in various studies [6,8,10]. According to the initial serum TSH concentrations (TSH 4-6, >6-12, >12 mU/L), Kaplan-Meier estimates of the incidence of overt hypothyroidism in subclinically hypothyroid women were 0%, 42.8%, and 76.9% respectively after 10 years (or 0%, 3%, and 11% respectively per year) [13]. The incidence of overt hypothyroidism was higher in patients with TPO-Ab (58.5% vs 23.2%). The importance of thyroid antibodies is also evident from a Dutch study: 9.6% of 55-year old women with TPO-Ab had raised TSH levels 10 years later, in contrast to 3.2% of women without antibodies [16]. The most extensive data are from a 20-year follow-up in the participants of the Whickham survey [17]. The incidence of overt hypothyroidism was 4.1 per 1000 women per year and 0.6 per 1000 men per year. Odds ratio’s for development of spontaneous hypothyroidism in surviving women are 14 (95% CI: 9-24) for raised TSH regardless of thyroid antibody status, 13 (95% CI: 8-19) for positive thyroid antibodies regardless of TSH, and 38 (95% CI: 22-65) for raised TSH and positive thyroid antibodies combined. Odds ratio’s for men are higher: 44 (95% CI: 19-104) for raised TSH regardless of thyroid antibody status, 25 (95% CI: 10-63) for positive thyroid antibodies regardless of TSH, and 173 (95% CI: 81-370) for raised TSH and positive thyroid antibody status combined. Most interestingly, the risk for developing hypothyroidism in the Whickham survey already starts at TSH levels of 2.0 mU/L. Similar cutoff values of 2.5 mU/L for predicting hypothyroidism from baseline TSH values have been reported in the Amsterdam AITD cohort [2], and in an Australian population-based study with a 13-years follow-up [18].

Subclinical hyperthyroidism has a reported prevalence of 0.63-3.2% [3,19-22]. In contrast with subclinical hypothyroidism, subclinical hyperthyroidism has less frequently an autoimmune origin; most cases are due to multinodular goiter. Subclinical Graves’ disease has been identified in a retrospective follow-up study in 9% of 96 patients with endogenous subclinical hyperthyroidism [23]. Subclinical hyperthyroidism can spontaneous normalize [20,21]. Spontaneous remission of subclinical hyperthyroidism is rare if the cause is a nodular goiter or toxic adenoma [24] but not so rare when the cause is Graves’ disease [25]. Follow-up studies have estimated that the progression rate from subclinical hyperthyroidism to overt hyperthyroidism is 1–5% per year [6,26-29]. However, these studies did not differentiate autoimmune subclinical hyperthyroidism from other causes of TSH suppression. The reason why in some instances subclinical thyroid dysfunction due to AITD disappears spontaneously but in other instances, progresses to overt thyroid dysfunction is incompletely understood. It could well be that continuation or discontinuation of exposure to environmental factors is involved. Alternatively, it could reflect the well-known dynamic nature of autoimmune diseases with many remissions and exacerbations in their natural history.

Before the stage of subclinical thyroid dysfunction, another stage in the natural history of AITD has generally been recognized, characterized by the presence of thyroid antibodies in serum and normal TSH and normal fT4. The prevalence of TPO-Ab is 10-13% in the general population [3,17], 27% in women with at least one 1st or 2nd degree relative with AITD [30]
and 43-48% in first-degree relatives of AITD patients [31,32]. In the Amsterdam AITD cohort the 5-year probability of seroconversion to TPO-Ab was 14.5% (chapter 3). The presence of TPO-Ab is a strong risk factor for the development of both autoimmune hyper- and hypothyroidism as reported in two previous studies of the Amsterdam AITD cohort [2,30]. The relevance of TPO-Ab in this respect was confirmed by a recent case-control study in which thyroid antibodies were measured at four time points: between 7 and 5 years, between 5 and 2 years, between 2 and 0.5 years before and at the time of the clinical diagnosis of Hashimoto’s hypothyroidism or Graves’ hyperthyroidism [33]. In Hashimoto’s thyroiditis, TPO-Ab were found in about 66% of the cases at all time points while in Graves’ disease the presence of TPO-Ab gradually increased from 31% at 5–7 years before the diagnosis to 57% at diagnosis. In agreement with our data, in this study TPO-Ab precede by years the development of overt autoimmune thyroid disease. It has been suggested that an hypeoechogenic pattern at thyroid ultrasound indicating thyroid autoimmunity might be detected in even earlier stages, before the thyroid antibodies are present in serum [34-36].

The relationship between AITD and antibodies bears similarities with other autoimmune diseases. It has been reported that at least one antibody specific for SLE was present before the diagnosis of SLE in 88% of 130 patients [37]. Adrenal cortex autoantibodies and/or 21-hydroxylase antibodies have been proposed as indicators of the development to adrenal failure in Addison’s disease [38] although a great variability in their predictive value was reported [39-44]. Moreover, like in subclinical AITD, the progression from subclinical Addison’s disease to overt adrenal failure will not necessarily happen in all subjects. Adrenal cortex autoantibodies positive subjects with subclinical adrenal cortical failure can spontaneously restore their adrenal function [45,46].

As outlined in figure 1 of chapter 2, in the stage prior to the occurrence of TPO-Ab in serum there are no serum markers to identify susceptibility to AITD; there are just particular genetic polymorphisms. However it might well be that this situation will change. It is likely that the immune process associated with the development of AITD is initiated early, before the occurrence of thyroid antibodies in serum. It is known that lack or blockade of CD4+CD25+ T regulatory cells (Treg) breaks the immunological tolerance [47] and induces the development of multiple autoimmune disorders [48,49]. Euthyroid women from the Amsterdam AITD cohort exhibit various signs of a reduced T-cell activation, irrespective of their TPO-Ab status as they have reduced serum levels of sIL-2R and reduced proportions of circulating CD4+CD25+ lymphocytes [50]. It suggests that reduced expansion of putative CD4+CD25+Treg is associated with an increased risk of developing thyroid autoimmunity. It may well be that changes in immunological surveillance are evident in this early stages of AITD from aberrant genetic fingerprints in monocytes. Such fingerprints may have to do with activation of chemotactic pathways, the attachment to endothelial cells and the interaction with extracellular matrix proteins of the monocytes in AITD [51]. If these changes in immunocompetent cells are already detectable in very early stages of AITD, one may consider the possibility that apart from genetic factors the susceptibility for AITD already starts during pregnancy. Some studies indeed suggest that low birth weight increases the risk to develop TPO-Ab [52,53], but other studies failed to confirm this association [54,55]. Thyroid autoimmunity has also been associated with fetal microchimerism in
observational studies [56,57]. However, the exact role of fetal microchimerism remains unclear as population studies failed to detect this association [58,59].

SMOKING

In chapter 3 we investigated the relationship between smoking behaviour and de novo development of thyroid antibodies in the 521 women of the Amsterdam AITD cohort who at baseline had a normal TSH and no thyroid antibodies in serum. Kaplan-Meier analysis indicated a 5-year probability of seroconversion of 20.1% for TPO-Ab and/or Tg-Ab and 14.5% for TPO-Ab only (see Supplemental Figure 1, Chapter 3). In the nested case-control study the frequency of smokers among cases was comparable to that in controls at study entrance, but at 1 year before seroconversion and at the time of seroconversion there were less current smokers in cases than in controls. Current smoking thus appears to be protective against the development of TPO-Ab and Tg-Ab with odds ratio’s between 0.52 and 0.62 (see Supplemental Figure 2, Chapter 3).

In contrast to our longitudinal study, all other studies on the relationship between smoking and thyroid antibodies have been cross-sectional. Nevertheless, three large studies all report a lower frequency of smokers in subjects with thyroid antibodies: odds ratio’s were 0.69 (95% CI, 0.48–0.99), 0.57 (95% CI 0.48 – 0.67) and 0.66 (95% CI 0.55–0.79) in the NHANES III study [60], the Amsterdam AITD cohort at baseline [30] and the Danish population study [61] respectively. A recent community based study from Iran likewise observed a higher frequency of TPO-Ab in never smokers than in ever smokers (13.5% vs. 6.7%, p<0.001) [62].

We did not study the relationship between smoking and the development of subclinical hypothyroidism in our cohort due to the limited number of subjects with subclinical hypothyroidism. But a large cross-sectional population-based study from Norway [63] revealed a lower prevalence of subclinical hypothyroidism among current smokers compared to never smokers (odds ratio’s 0.54, 95% CI 0.45–0.66 in women, and 0.37, 95% CI 0.26-0.52 in men). A population based study from Korea likewise reports that current smoking is inversely related with subclinical hypothyroidism [64]. These studies however did not distinguish between autoimmune and non-autoimmune causes of subclinical hypothyroidism.

With regard to the relationship between smoking and the development of overt hypothyroidism, our nested case-control study described in chapter 2 suggests again a protective effect of smoking. Whereas smoking status at study entrance did not differ between the 38 hypothyroid cases and their 76 controls, during follow-up there was a trend toward less smokers in the hypothyroid cases (p=0.08). The failure to reach statistical significance is likely due to the limited sample size. A recent large prospective study from Norway [65] with 11-year follow-up reports that the risk of future hypothyroidism increases gradually with higher TSH values (still within the reference range) at baseline, in complete agreement with the THEA score [2]. This Norwegian study also states that in women the association of baseline TSH with hypothyroidism at follow-up did not substantially differ by
smoking behaviour at baseline (P=0.08 for interaction). The study does not report whether smoking behaviour changed during the 11-year follow-up. Remaining studies on this topic are all cross-sectional. The NHANES III study [60] as well as the Danish [66] and Norwegian [63] population based studies all found a low prevalence of overt hypothyroidism among smokers compared with nonsmokers (odds ratio’s 0.6 (95% CI 0.5-0.8); 0.47 (95% CI 0.33-0.67); and 0.6 (95% CI 0.38-0.95) for women and 0.51 (95% CI 0.15-1.73) for men, respectively). Again, the studies do not analyze separately autoimmune hypothyroidism and non-autoimmune hypothyroidism.

It is thus evident that smoking protects against the development of TPO-Ab as well as against the development of (autoimmune) subclinical and overt hypothyroidism. A previous meta-analysis published in 2002 [67] did not find a relationship between smoking and hypothyroidism. Probably the follow-up study design and the much larger sample size of the more recent studies account for the discrepancy.

The protective role of smoking on the development of TPO-Ab and hypothyroidism is in sharp contrast to the well established risk of smoking for developing Graves’ disease. There are no studies investigating the relationship between smoking and the de novo development of TSH receptor antibodies. In the cross-sectional Norwegian study [63] it was found that smoking increases the risk of subclinical hyperthyroidism (OR 1.83, 95% CI 1.10-3.06) and overt hyperthyroidism (OR 2.37, 95% CI 1.34-4.20) in women. The odds decreased significantly the longer the time period was since smoking cessation. This study refers to all causes of hyperthyroidism. However, it is beyond any doubt that smoking is a risk factor for the development of Graves’ hyperthyroidism as evident from a meta-analysis (OR 3.3, 95% CI 2.1-5.2) [67]. In a particular study the odds ratio of smoking for the development of overt Graves’ hyperthyroidism was 1.9 (95% CI 1.1-3.2) and for the development of Graves’ ophthalmopathy was 7.7 (95% CI 4.3–13.7) [68]. Cessation of smoking is associated with a lower risk of Graves’ ophthalmopathy [69]. In our own nested case-control within the Amsterdam AITD cohort we failed to detect a risk of smoking for the development of autoimmune hyperthyroidism (chapter 2), which is not surprising in view of the very limited sample size of our hyperthyroid cases (n=13).

It appears that smoking behavior may determine to a certain extent the clinical phenotype of AITD: smokers are less likely to develop Hashimoto’s hypothyroidism but more likely to get Graves’ hyperthyroidism. The mechanism behind these opposite effects of smoking is poorly understood. The effects of smoking could be mediated indirectly via the immune system, or there could be direct effects of smoking on the thyroid gland. Cigarette smoking affects the immune system in multiple ways. It has pro-inflammatory effects like increased free radical burden, elevated neutrophil count, elevated circulating T cells, elevated CD4 activity, release of intracellular antigens, augmented auto-reactive B cell activity and increased acute phase and pro-inflammatory reactants. It results in increased Th2 regulated allergic diseases and an increased rate of autoimmune diseases. Smoking has also immuno-suppressive effects like reduced IFN response, reduced antigen presenting activity, reduced circulating immunoglobulins, reduced T-cell activity, reduced neutrophil activity and inhibition of inflammatory cytokines; it results in decreased innate and acquired immunity.
and increased infection rates [70]. Cigarette smoking thus affects both cell-mediated and humoral immune responses, but it is still obscure how smoking may inhibit immune pathways leading to Hashimoto’s thyroiditis while at the same time enhancing immune pathways leading to Graves’ disease. Nicotine induces sympathetic activation which can increase thyroid hormone secretion and it may also have a direct thyroid stimulatory action along with other components of tobacco smoke like benzpyrene [71]. Serum thiocyanate concentration is higher in smokers than non-smokers. Thiocyanate, a major component of smoke, derived from hydrogen cyanide, leads to increased excretion of iodine, inhibits iodine uptake by the thyroid, competes with iodide in the organification process, and inhibits thyroid hormone synthesis [72]. However, exposure to thiocyanate would then make subjects susceptible to hypothyroidism whereas the observation is that smoking protects against hypothyroidism. Significant interactions between thyroidal effects of smoking and ambient iodine intake may exist [64,66]: smoking had a negative interaction with iodine intake (OR 0.930, 95% CI 0.869-0.996) [64]. Whereas TPO and Tg are both intracellular antigens, the TSH receptor is exposed at the plasma membrane. One might hypothesise that smoking in a nonspecific manner (via e.g. increased cotinine levels in the blood) stimulates shedding of the extracellular A-subunit of the TSH receptor, thereby provoking an immune response against the TSH receptor protein. This hypothesis is supported by recent findings that the free (shedded) A-subunit of the TSH receptor rather than the holoreceptor initiates or enhances the immune response leading to Graves’ hyperthyroidism [73].

A considerable body of literature deals with the effect of smoking on other autoimmune diseases. Smoking has found to be a risk factor for some autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, Goodpasture’s syndrome, primary biliary cirrhosis, multiple sclerosis, Crohn’s disease) but a protective factor for other autoimmune conditions (Behçet’s disease and ulcerative colitis) [70]. Of particular interest is the similarity of smoking effects on phenotypic appearance of AITD (protective for Hashimoto’s disease but risk-bearing for Graves’ disease) and on phenotypic appearances of inflammatory bowel disease: protective for ulcerative colitis and risk-bearing for Crohn’s disease. The relative risk of developing Crohn’s disease is 4.8 in those who smoked before disease onset [74], and the odds ratio is 0.41 (95% CI 0.34-0.48) for current smokers to develop ulcerative colitis in comparison with lifetime non-smokers [75].

**Alcohol**

In contrast to smoking, alcohol intake has not been extensively studied for its relation with autoimmune thyroid disease. Some recent studies suggest a protective role of alcohol in particular autoimmune diseases [76-78]. Therefore we hypothesized that alcohol consumption reduces the risk for the development of AITD. In order to test this hypothesis, we performed two case control studies nested in the Amsterdam AITD cohort, presented in chapter 4.
In the first study we evaluated whether alcohol consumption was associated with de novo occurrence of TPO-Ab in euthyroid women. No association could be found: alcohol consumption did not differ between cases and controls at study entrance, one year before seroconversion and at the time of seroconversion. There are no other studies in the literature investigating the relationship between alcohol and thyroid antibodies. The same is true for the relationship between alcohol and subclinical hypothyroidism.

In the second study we evaluated an association between alcohol consumption and the development of overt autoimmune hypothyroidism. Here, an association was observed: alcohol consumption at study entrance did not contribute to the risk of developing overt hypothyroidism in the 5-year follow-up, but less cases than controls consumed >10 units of alcohol per week at one year before and at the time of event (respective odds ratio’s 0.54 (95% CI 0.14-2.06), 0.23 (95% CI 0.05-1.04), and 0.23 (95% CI 0.05-1.06)). Our results are in agreement with a cross-sectional case-control study from Denmark [79] which provided evidence for a protective role of alcohol consumption in the development of overt autoimmune hypothyroidism (OR 1.00 as reference for alcohol abstainers, OR 0.58, 95% CI 0.35-0.96 for alcohol consumers of 1-10 units/week, OR 0.40, 95% CI 0.21-0.78 for alcohol consumers of >11 units/week).

We did not study an association between alcohol consumption and overt hyperthyroidism in our cohort due to the limited sample size. The only report in the literature we could detect on this topic, is a study from Sweden twenty years ago [80]. In this paper alcohol was evaluated as a possible confounding factor in the relationship between stress and Graves’ disease. As controls consumed alcohol more often than cases, alcohol consumption appeared to be protective against Graves’ disease (OR 0.4, 95% CI 0.2-0.8 for consuming alcohol once weekly or more often). Although these data have to be confirmed, it could mean that alcohol consumption protects against both overt hypo- and hyperthyroidism. This would be in contrast with the opposite effect of smoking on AITD.

The effect of alcohol could be mediated indirectly via the immune system, or there could be a direct effect of alcohol on the thyroid gland. Alcohol suppresses all branches of the immune system, both in male and females [81]. Acute moderate alcohol ingestion in humans results in modulation of the monocyte derived inflammatory cytokine production [82]. Chronic alcohol intake is associated with impaired delayed-type hypersensitivity and decreased host defense against infections [83]. Moderate consumption of either wine or beer has been associated with lower levels of systemic inflammation [84]. Some experts suspect that alcohol exerts an “all-or-none” effect on immune responses —that is, the presence or absence of alcohol, rather than its amount— dictates the immune response. Other researchers believe that low doses of alcohol—the amount equivalent to a glass of wine—can confer health benefits, including protection against damage to the immune system [81]. With regard to the protective effect of alcohol against the development of overt autoimmune hypothyroidism, our study presented in chapter 4 observed a trend to a dose response relationship (just failing to reach statistical significance due to the small sample size), but a dose response relationship was observed in the Danish study [79]. If one assumes that the effects of alcohol are mediated via the immune system, then one would...
expect that alcohol would also have a protective effect on de novo occurrence of TPO-Ab, which we did not observe. Consequently a direct effect of alcohol on the thyroid gland might be involved as well. However, a direct toxic effect of alcohol on the thyroid gland independent of liver disease as reported by Hegedüs et al. [85] is difficult to reconcile with protection of alcohol against overt hypothyroidism. A third explanation for the effect of alcohol is derived from the peculiar coincidence that both smoking and alcohol to a certain extent seem to protect against autoimmune hypothyroidism. There is good evidence that alcohol and nicotine dependence both are strongly influenced by genetic factors [86]. It may be hypothesized that genes predisposing to addiction behaviour are also involved in the pathogenesis of thyroid autoimmunity.

With regard to other autoimmune diseases, alcohol consumption protects against the development of rheumatoid arthritis [77,78] but the protective effect against SLE is controversial [76,87]. Alcohol consumption does not protect against multiple sclerosis [88]. Regular consumers of alcohol had a decreased risk for progression of disability in relapsing onset of multiple sclerosis (not in progressive onset multiple sclerosis); interestingly, smoking was associated with an enhanced risk for progression [89]. The data imply that the effect of alcohol consumption and smoking might be disease specific and specific for the stage of the disease.

**Vitamin D**

There has been a recent surge of interest in the role of vitamin D in the development of autoimmunity. Vitamin D deficiency has indeed been related to some autoimmune diseases. Therefore, we tested the hypothesis that AITD is associated with vitamin D deficiency in two nested case-control studies in the Amsterdam AITD cohort (chapter 5). In both studies we matched cases and controls for factors known to affect 25-hydroxy-vitamin D (25(OH)D) status. 25(OH)D deficiency is defined as concentrations of <20 ng/mL and insufficiency is defined as concentrations of 20-30 ng/ml [90].

In the first study we investigated the very early stages of AITD characterised by genetic susceptibility to AITD while serum TSH is still normal and thyroid antibodies in serum are absent. We compared vitamin D levels in euthyroid subjects of the Amsterdam AITD cohort without thyroid antibodies at baseline with healthy controls. Contrary to our hypothesis, cases had higher serum 25(OH)D concentrations than controls (21.0±7.9 ng/ml vs. 18.0±6.4 ng/ml, p=0.01). The prevalence of 25(OH)D deficiency in cases was also lower and not higher than in controls.

In the second study we investigated women who at baseline had normal TSH and no thyroid antibodies, and contrasted vitamin D levels in women who during follow up developed TPO-Ab (cases) or who remained without TPO-Ab (controls). In this study we matched cases and controls also for the duration of follow-up until seroconversion in cases. We did not
observe significant differences in 25(OH)D between cases and controls (baseline, 22.6±10.3 ng/ml vs. 23.4±9.1 ng/ml; follow-up 21.6±9.2 ng/ml vs. 21.2±9.3 ng/ml). The frequency of 25(OH)D deficiency (<20 ng/ml) at baseline and at the time of seroconversion was comparable in cases and in controls. Additional analysis in this study of 1,25(OH)_{2}D levels did not reveal differences between cases and controls. Taken together the results of the two studies indicate we had to reject our hypothesis that the very early stages of thyroid autoimmunity are associated with low 25(OH)D levels.

In agreement with our results, a cross-sectional study from India [91] also found no differences in the prevalence of TPO-Ab between subjects with \( \leq 10 \) ng/ml or \( >10 \) ng/ml 25(OH)D. However, another cross-sectional study from Turkey (performed between October 2008 and February 2009) [92] observed lower vitamin D levels in euthyroid subjects with thyroid antibodies (Hashimoto’s thyroiditis) than in age and sex matched controls (25(OH)D 17.4±12.9 vs 29.6±25.5 ng/ml, p<0.006), as well as higher prevalence of 25(OH)D deficiency and insufficiency (25(OH)D <30 ng/ml, 86% vs. 63%, p<0.001). This study might be biased by seasonal variation in vitamin D levels: samples were collected over two seasons (autumn and winter), vitamin D levels are lower in winter than in autumn, and healthy controls were matched for age and sex but not for months of blood collection.

We did not examine the relationship between the late stages of AITD (overt hypothyroidism or overt hyperthyroidism) and vitamin D deficiency. Two recent cross-sectional studies have reported on this relationship. The first is the Turkish study [92] which has found lower vitamin D levels and higher prevalence of 25(OH)D deficiency and insufficiency in overt or subclinical autoimmune hypothyroidism than in controls. The second is a study from Hungary [93] (with blood sampling in March) which observed higher prevalence of 25(OH)D deficiency among AITD patients (both Hashimoto’s and Graves’ disease) as compared to healthy controls (72% vs. 30%). It is unclear if controls in this study had their blood samples taken in the same season as the patients. Furthermore, as there are no TSH and fT4 values reported in this paper, it is also unclear whether blood sampling in the patients were done in the treated or untreated state of Hashimoto’s or Graves’ disease. The latter point constitutes another bias because thyroid hormone deficiency and excess both alter vitamin D metabolism.

The effects of vitamin D on the immune system were discussed in recent reviews [94-96]. Studies indicate that 1,25(OH)_{2}D, the biologically active form of vitamin D, is a modulator of both the innate and adaptive immune system. Monocytes/macrophages and dendritic cells (DC) express the vitamin D-activating enzyme CYP27B1 and the vitamin D receptor (VDR). Therefore they can utilize 25(OH)D via localized conversion to active 1,25(OH)_{2}D. 1,25(OH)_{2}D influences innate immune responses by enhancing the chemotaxis and phagocytosis by macrophages, but also the production of anti-microbial proteins. 1,25(OH)_{2}D further modulates antigen presenting cells like DCs by inhibiting the surface expression of MHCII-complexed antigen, costimulatory molecules and the production of the cytokine IL-12 while increasing the production of IL-10. The result is an indirect shift in T cell polarization from a Th1 and Th17 phenotype toward a Th2 phenotype. T helper (Th) cells appear to be the principal target for 1,25(OH)_{2}D. VDR are expressed in
T-cells and activated upon binding of its ligand. $1,25(\text{OH})_2\text{D}$ can suppress Th cell proliferation as well as modulating cytokines production by these cells. Overall, the net result of $1,25(\text{OH})_2\text{D}$ action on T cells is to block the induction of Th1-cell cytokines, particularly IFN\text{\greek{y}}, while promoting Th2-cell responses, an effect mediated both indirectly by decreasing IFN\text{\greek{y}} production and directly by enhancing IL-4 production. $1,25(\text{OH})_2\text{D}$ suppresses B-cell proliferation and immunoglobulin production indirectly via Th cells but also via direct effects of $1,25(\text{OH})_2\text{D}$ on B-cells. Consecutively four mechanisms by which serum $25(\text{OH})\text{D}$ can influence T-cell function has been proposed: (i) direct effects on T cells mediated via systemic $1,25(\text{OH})_2\text{D}$; (ii) indirect effects on antigen presentation to T cells mediated via localized DC expression of CYP27B1 and intracrine synthesis of $1,25(\text{OH})_2\text{D}$; (iii) direct effects of $1,25(\text{OH})_2\text{D}$ on T cells following synthesis of the active form of vitamin D by CYP27B1-expressing monocytes or DCs – a paracrine mechanism; (iv) intracrine conversion of $25(\text{OH})\text{D}$ to $1,25(\text{OH})_2\text{D}$ by T cells.

In recent years, many studies reported on a possible link between the vitamin D deficiency and autoimmune diseases. Lower serum $25(\text{OH})\text{D}$ has been reported in patients with type 1 diabetes at the time of diagnosis [97] and vitamin D supplementation may contribute to the prevention of type 1 diabetes [98]. High circulating levels of vitamin D are also associated with a lower risk of multiple sclerosis [99]. Rheumatoid arthritis [100], SLE [101,102], Crohn’s disease [103,104] have also been linked to low vitamin D status. However it is not known which vitamin D levels are sufficient to improve the immune regulatory function and a more effective immune response.

**STRESS**

We evaluated exposure to stress as a risk for the development of AITD, again by nested case control studies in the Amsterdam AITD cohort.

In the first study (chapters 3 and 6) we evaluated the de novo occurrence of TPO-Ab in 521 women who at baseline had a normal TSH and no thyroid antibodies. We observed no differences in stress exposures between cases (seroconverters) and controls (non-seroconverters) at baseline, one year before the event or at the time of the event. In chapter 3 seroconversion was defined as development of TPO-Ab $>100\text{kU/L}$, in chapter 6 as $\geq 100\text{kU/L}$; the results in both chapters are the same. Two previous studies on this topic (all cross-sectional in nature) likewise failed to observe a relationship between TPO-Ab and stress exposure [105,106].

In the second study (chapter 6) we evaluated stress exposure prior to the development of overt autoimmune hypothyroidism or overt autoimmune hyperthyroidism. Again, scores on stress questionnaires did not differ between cases and controls at baseline and at one year before the event. Similar negative results with regard to the development of autoimmune hypothyroidism were observed in two previous cross sectional studies [106,107]. Our failure to observe higher exposure to stress in subjects who developed autoimmune
hyperthyroidism is likely explained by the very limited sample size of n=13. The literature however abounds with studies [80,108-113] reporting increased exposure to stress in the year prior to the diagnosis of Graves’ hyperthyroidism as compared to controls. Although these studies were retrospective in nature and thus subject to recall bias, the circumstantial evidence is rather good that stress indeed is a risk factor Graves’ disease.

The possible role of stress and of the major stress related hormones as etiological factors in the pathogenesis of autoimmune diseases have been reviewed extensively [114-117]. Stress may affect the immune system either directly or indirectly through the nervous and endocrine systems. Stress hormones, acting on antigen-presenting immune cells, may influence the differentiation of bipotential helper T-cells away from a Th1 phenotype and towards a Th2 phenotype. This results in suppression of cellular immunity and in potentiating of humoral immunity. The different phenotypic expressions of thyroid autoimmunity are largely dependent on the balance of Th1 versus Th2 immune response. A predominantly Th1-mediated immune activity may promote apoptotic pathways on thyroid follicular cells, leading to thyroid cell destruction and Hashimoto’s thyroiditis. Conversely, predominance of Th2-mediated immune response may induce antigen specific B lymphocytes to produce TSH receptor antibodies, causing Graves’ disease.

Stress has been implicated in a number of other autoimmune diseases. In an uncontrolled study SLE patients indicated stress as trigger of their disease in 76%. In primary antiphospholipid syndrome 45% of patients considered prolonged stress to be the lead cause of their disease, as did 43% of patients with rheumatoid arthritis [118]. Cross-sectional associations suggest a mutual impact of disease activity and psychological distress in rheumatoid arthritis, but the first prospective study was published in 2011 [119]. While some support was found for the idea that a higher level of disease activity is a risk factor for an increase in psychological distress the results did not support the notion that psychological distress is a risk factor for future exacerbation of disease activity. Two review papers investigated the literature on stress as a risk factor for multiple sclerosis. The conclusion of one paper is that a growing body of evidence supports an association between stressful life events and an increased risk of multiple sclerosis exacerbations. However, the nature of this relationship remains unclear because of the lack of agreement of the definition of stress and because of research design problems [120]. A systemic review incorporated only observational longitudinal studies, five for multiple sclerosis onset and 12 for multiple sclerosis relapse [121]. All studies, with only two exceptions, were in favour on the stress-multiple sclerosis relationship, but due to marked stress measurement heterogeneity, no secure conclusions could be drawn. The most recent study on this topic is the prospective Nurses’s Health Study [122]. The results did not support a major role of stress in the development of the disease, but the authors stated repeated and more focused measures of stress are needed to firmly exclude stress as a potential risk factor for multiple sclerosis.

The longitudinal observations in the Amsterdam AITD cohort allowed us also to describe the course of scores on stress questionnaires in women who at baseline and at each subsequent annual assessment remained euthyroid (TSH within the reference range) and without
thyroid antibodies in serum (no signs of thyroid autoimmunity) (chapter 7). In a way the results might be considered as the natural history of stress exposure in a healthy female population. It became evident that recent life events and daily hassles in general decrease with advancing age. This was most clear in the cross-sectional analysis of baseline data, whereas the longitudinal analysis with the follow-up data indicated a temporary increase in stress questionnaires scores up to the age of 40. The limitation of this study is that subjects above the age of 60 were very few. The strength of the study is the complete data set collected in a prospective manner in a large sample size which is likely representative for the Dutch healthy adult female population. Available studies in the literature are mostly limited in sample size and done in a retrospective manner. The cause of the age dependent change in exposure to stress is beyond the scope of this thesis.

YERSINIA

Infections have been implicated in the pathogenesis of several autoimmune diseases including AITD [123]. The best studied infection with regards to the possible association with AITD is that with Yersinia enterocolitica [124]. In chapter 8 we evaluated prospectively any association between Yersinia enterocolitica infection (YE) and AITD in two nested case-control studies in the Amsterdam AITD cohort.

First, we evaluated YE serological status in 388 euthyroid women without thyroid antibodies at baseline. The YE serological status was compared between subjects who developed TPO-Ab and/or Tg-Ab at 4 years follow-up and those who remained thyroid antibodies negative. Neither persistence nor emergence of YOP (YE outer membrane protein) IgG and IgA at 4 years follow up was associated with the TPO-Ab or Tg-Ab seroconversion. All previous studies reporting on the association between YE infection and thyroid antibodies were cross-sectional in nature and concerned patients who had been already treated for Hashimotos’s thyroiditis or Graves’ disease [125-127]. However, in agreement with our study, they also observed that YE infection does not confer an increased risk of thyroid antibodies.

Second, we evaluated YE serological status between cases (subjects who developed overt hypo- or hyperthyroidism) and controls. The proportion of subjects with YOP-IgG and YOP-IgA did not differ between cases and controls at baseline or at 1 year before the event, except a higher frequency of YOP IgA in controls 1 year before the event. If YE infection would play a causative role, one would expect a higher and not lower frequency of IgA (YOP-IgA status is linked to more recent stages of YE infection, YOP-IgG status to chronic YE infection). When hypothyroid and hyperthyroid cases with their respective controls were analyzed separately, no significant differences in YOP IgG and IgA were observed either. Our results thus indicate that YE infection does not play a causative role in the development of overt autoimmune hypo- or hyperthyroidism. Studies in the past have shown conflicting results, some reporting a higher rate of seropositivity against YE in patients with Hashimoto’s or Graves’ disease.
than in controls [128-132], whereas other studies didn’t find any relationship [133,134]. Again these previous studies were all retrospective.

Of particular interest is the paper from Denmark reporting an association between Graves’ disease and YE in a case-control study, and in twin pairs discordant for Graves’ disease [132]. The authors conclude “that YE infection plays an etiological role in Graves’ disease or vice versa. Future studies should examine the temporal relationship in more depth”. Our chapter 8 has examined the temporal relationship, and results are in the negative. The Danish findings however are in good agreement with a previous cross-sectional study in the Amsterdam AITD cohort in which the prevalence of YOP IgG and IgA in AITD relatives was higher than in controls [125]. As discussed in that paper the increased prevalence of YOP antibodies (not related to higher prevalence of TPO-Ab) suggest a higher rate of persistent YE infection in AITD relatives. Susceptibility genes for AITD may also confer a risk to YE infection.

The postulated mechanism behind an association between YE infection and AITD is quite attractive because a number of studies provide a sound biological rationale for a causal relationship. YE has specific binding sites for TSH [135]. These binding sites are recognized by TSH receptor antibodies isolated from humans with Graves’ disease [136]. Conversely, immunization of mice with YE leads to the induction of TSH receptor antibodies [137]. YOP antibodies stained thyroid epithelial cells in immunochemistry [138,139]. Cellular immunity is also involved because YE inhibits the migration of lymphocytes from patients with Graves’ disease [139] and YE acts as a super antigen [140]. The YE protein cross reacting with TSH receptors has recently been identified: the YOP membrane porin F shared cross-immunogenicity with a leucine-rich domain of TSH receptor. The plausible theory then is that YE ompF is involved in the production of TSH receptor antibodies and the pathogenesis of Graves’ disease through molecular mimicry [127]. However, the most reliable clinical epidemiological studies (chapter 8) do not support this theory. The proteins cross reacting with TSH receptors do not seem to be YE specific, since TSH binding sites are also found in other intestinal pathogens [141].

We have not found convincing data on associations between YE infection and other autoimmune diseases. An increased prevalence of antibodies to the β-subunit bacterial RNA polymerase (ARPA) using the protein of YE O:3 (a highly conserved non-species-specific bacterial protein) was found in primary biliary cirrhosis and autoimmune hepatitis. [142]. These findings do not add an argument for a bacterial trigger of autoimmune disease, but rather suggest that ARPA belong to the pool of natural antibodies up-regulated in autoimmune liver diseases. Alcoholic cirrhosis was also associated with higher frequency of IgG and IgA antibodies against plasmid encoded proteins from YE. The data indicated that the O-polysaccharides as strong antigens are physiologically exposed to the immune system [143]. Autoimmunity in reactive arthritis might be mediated by antigen mimicry between evolutionarily conserved epitopes of ribosomal proteins and their host analogs [144].
Exogenous estrogens

In the prospective nested case-control study presented in *chapter 3* there was a just not significant association (p=0.06) between the current estrogen use and de novo occurrence of TPO-Ab and/or Tg-Ab (although not for TPO-Ab alone) during follow-up, suggesting a protective effect of estrogens. These results are in line with a previous cross-sectional analysis of the Amsterdam AITD cohort at baseline, indicating a lower frequency of ever and current estrogen use in euthyroid women with TPO-Ab as compared to TPO-Ab negative women (OR are 0.58, 95% CI 0.35-0.97 for ever estrogen use and 0.81, 95% CI 0.56-1.19 for current estrogen use) [30]. A cross-sectional population based study from Denmark [58] did not find any association between the use of oral contraceptives and thyroid antibodies in women 18-45 years old; however, postmenopausal women 60-65 years old who used in the past or were still using hormone replacement therapy had a lower prevalence of Tg-Ab but not of TPO-Ab. Another study reports that the frequency of TPO-Ab and/or Tg-Ab is similar in postmenopausal women with or without hormone replacement therapy [145].

The longitudinal nested case-control study (*chapter 2*) indicated that estrogen use had no effect on the development of overt autoimmune hypo- or hyperthyroidism. However, these results are in contrast to previous studies. In the cross-sectional analysis of baseline data of the Amsterdam AITD cohort, the ever use of estrogens seemed to protect against the development of autoimmune subclinical or overt hyperthyroidism (OR 0.17, 95% CI 0.05-0.52) but not against autoimmune hypothyroidism [30]. A large early study among 46,000 women found that the incidence of hypo- and hyperthyroidism together was lower in oral contraceptive users than in controls (RR 0.68, 95% CI 0.52-0.85) [146]. This study did not discriminate between hypo- and hyperthyroidism. The large population based Danish study concluded that the use of oral contraceptives had a protective effect for the development of Graves’ disease (OR 0.68, 95% CI 0.49-0.93), but not for Hashimoto’s disease [147]. Taken together it looks that exogenous estrogens to a certain extend may protect against thyroid antibodies (more so against Tg-Ab than to TPO-Ab) and against Graves’ hyperthyroidism.

Estrogens may enhance the immune response (mainly humoral immunity). Estrogens receptors have been found on immune cells, supporting a direct effect [148]. Depending on the relative preponderance and stimulation of different receptors in various tissues, estrogens may demonstrate pro-inflammatory or anti-inflammatory properties [149]. The use of combined oral contraceptives might be associated with an increased risk of systemic lupus erythematosus. On the other hand, exogenous estrogens, may have a favorable effect on rheumatic diseases [149].

Pregnancy

In the nested case-control study (*chapter 3*), parity did not differ between euthyroid women who developed and those who did not develop thyroid antibodies, neither at baseline,
one year before, or at the time of seroconversion. These results are in agreement with two previous cross-sectional studies [30,58].

In the other case-control study on this topic (chapter 2), parity did not differ between hypothyroid cases and controls at study entrance and at one year before the event. However, at the time of the event more cases were in the postpartum period than controls. Also, women who developed overt autoimmune hyperthyroidism were more frequently in the postpartum period than the controls at one year before occurrence of event and at the time of event. It indicates the postpartum period as a risk for developing overt AITD. Hyperthyroid cases had experienced pregnancies more often as the proportion of women who had never been pregnant was lower in hyperthyroid cases than in their controls (8% vs 38%), both at one year before the event and at the time of the event. In agreement, previous studies found that the risk for developing Graves’ hyperthyroidism is higher in the postpartum period [150,151]. Postpartum thyroiditis puts the woman at risk for permanent autoimmune hypothyroidism [152].

Large population based studies report conflicting results between parity and AITD. An Australian study found (after adjustment for age) that women who had previously been pregnant did not have a significantly increased risk of positive thyroid antibodies or a raised or reduced TSH compared with women who had never been pregnant [59]. A Danish study failed to demonstrate an association between previous pregnancy, parity and thyroid antibodies [58]. In contrast, a German study did find an association between parity and AITD [153]. Women with at least one pregnancy had increased odds for AITD (OR 4.6, 95% CI 1.4-15.1) compared to women who had never been pregnant. Similar results were obtained using positive TPO-Ab (OR 1.8, 95% CI 1.0-3.3) as separate dependent variable or using number of births as alternate independent variable.

Pregnancy is characterized by a suppression of Th1-mediated cellular immunity and preservation or enhancement of Th2-mediated humoral immunity [154]. This shift avoids rejection of the fetus by a cell-mediated immune attack by the mother. Accordingly, several Th1-mediated autoimmune disorders usually ameliorate during pregnancy [124]. However, Graves’ disease also ameliorates during pregnancy, and the concentration of thyroid antibodies decreases as well. The switch to a predominantly Th2 pattern of cytokines contributes to the maintenance of transient tolerance to paternal antigens in pregnancy; however the generation of specific regulatory T cells (T\textsubscript{reg}) during gestation is key to this maintenance [155]. T\textsubscript{reg} cells may also be present in the mothers’ circulation and capable of regulating coincidental autoimmune responses through the phenomenon of linked suppression. In turn, this suppression may explain why thyroid antibodies levels decline during pregnancy which leads to remission of Graves’ disease [155]. Recovering from the immune effects of pregnancy, a rebound reaction may activate the Th1-mediated pathway, leading to cellular immunity and destruction of thyroid follicular cells. The likely outcome then will be postpartum hypothyroidism. The rapid fall of T\textsubscript{reg} cells in postpartum may increase thyroid antibodies.

The effect of pregnancy in other autoimmune diseases varies from the spontaneous improvement of rheumatoid arthritis and multiple sclerosis during pregnancy [124,156] to
a possible increased risk for lupus flare [157].

IODINE

In our studies the self-reported iodine excess was not a predictive factor for the occurrence of thyroid antibodies (chapter 3). Iodine excess did not differ between cases (women who developed thyroid antibodies) and their controls at baseline, one year before and at the time of seroconversion.

We abstain from the conclusion that iodine plays no role in the development of AITD because the proportion of women with iodine excess in the Amsterdam AITD cohort is rather low in the order of 6%. The role of dietary iodine in triggering thyroid autoimmunity in genetically susceptible individuals is well demonstrated in animal models [158]. Also in humans, in areas with sufficient iodine intake, the prevalence of TPO-Ab and autoimmune hypothyroidism is higher than in iodine-deficient regions [159], whereas the overall prevalence of thyrotoxicosis is greater in iodine deficient areas [160].

FUTURE RESEARCH

From the various nested case-control studies in the Amsterdam AITD cohort it has become clear that a number of environmental factors are involved in the pathogenesis of AITD. Interestingly the impact of environmental factors may differ between the various stages in the natural history of AITD, and sometimes the same factor has opposite effects on the development of Hashimoto’s and Graves’ disease. For instance, smoking protects against the development of TPO-Ab and overt autoimmune hypothyroidism, whereas it is a clear risk factor for the development of Graves’ disease. The expression of AITD thus to a certain extent depends on the lifestyle of the subject. Why exposure to cigarette smoke has such contrasting effects, is completely obscure, and should be clarified. What also is required is more insight into the aberrations in immunological surveillance in the very early stages of AITD, before TPO-Ab are present in serum. Once the TPO-Ab are detectable in serum, clinical experience tells us that in some instances there will be progression to hypo- or hyperthyroidism, but on the other hand there are many cases in which euthyroidism is maintained and progression to thyroid dysfunction does not occur. The determinants of these two courses in the natural history of AITD are incompletely understood; both genetic and environmental factors could be involved. The same holds true for the spontaneous disappearance of TPO-Ab from serum and for the spontaneous resolvement of subclinical autoimmune hypothyroidism.

Another fruitful area for future research would be gene-environment interactions. Such studies have scarcely been done in thyroid autoimmunity. A notable exception is a Brazilian study on genetic polymorphisms associated with cigarette smoking and the risk of Graves’
Lastly, one could envisage that intervention with selenium in early stages of thyroid autoimmunity might decrease the risk of progression of AITD. A number of studies have shown that TPO-Ab concentrations fall upon selenium supplementation, although the results of several randomized clinical trials on this topic are contradicting each other [162].

References


