Early stages of thyroid autoimmunity: follow-up studies in the Amsterdam AITD cohort
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Citation for published version (APA):
Effraimidis, G. (2012). Early stages of thyroid autoimmunity: follow-up studies in the Amsterdam AITD cohort

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Chapter 8

No causal relationship between Yersinia enterocolitica infection and autoimmune thyroid disease: evidence from a prospective study.

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ABSTRACT

**Objective:** To evaluate prospectively the relationship between Yersinia Enterocolitica (YE) infection and the development of overt autoimmune hypo- or hyperthyroidism (study A) and the de novo occurrence of thyroid antibodies (study B).

**Subjects & Methods:** Prospective cohort study of 790 euthyroid women who were 1st or 2nd degree relatives of AITD patients. Follow-up was five years, with annual assessments.

Study A: Nested case-control study in which YE serological status was measured between cases (subjects who developed overt hypothyroidism (TSH >5.7mU/L and fT4 <9.3 pmol/L) or overt hyperthyroidism (TSH <0.4mU/L and fT4 >20.1 pmol/L) and matched controls.

Study B: 388 euthyroid women without thyroid antibodies at baseline were enrolled. The YE serological status was compared between subjects who developed TPO-Ab and/or Tg-Ab at 4-yr follow up and those who remained negative.

**Results:** Study A. The proportion of subjects positive for YOP-IgG or YOP-IgA did not differ between cases and controls at baseline. One year before the development of overt hypo- or hyperthyroidism, the proportion of subjects with YOP-IgG was not different between cases and controls, but YOP-IgA were less prevalent in cases.

Study B. De novo occurrence of TPO-Ab (or TPO-Ab and/or Tg-Ab) did not differ between subjects in whom at baseline YOP-IgG were positive or negative. Neither persistence nor emergence of YOP-IgG at 4-yr follow up was associated with the occurrence of TPO-Ab or Tg-Ab. Similar results were observed with respect to YOP-IgA.

**Conclusions:** Yersinia enterocolitica infection does not contribute to an increased risk of thyroid autoimmunity.
**INTRODUCTION**

Infectious diseases may provoke several autoimmune diseases, as e.g. observed for Coxsackievirus P2-C and type 1 diabetes, Proteus mirabilis and rheumatoid arthritis [1,2]. In the mid 1970s for the first time an association between Yersinia enterocolitica (YE) infection and autoimmune thyroid disease (AITD) was reported [3,4]. Since then, several groups investigated the potential precipitation of AITD development by YE infection. It has been suggested that molecular mimicry of YE membrane antigens to TSH may induce autoimmunity to the TSH receptor [5].

However, reported studies in the literature on the frequency of YE infection in AITD have shown conflicting results [6-15]. They are all cross-sectional in nature, examining patients who had already developed Graves’ hyperthyroidism or Hashimoto’s hypothyroidism and who had been treated for months or years.

It is obvious that prospective studies are mandatory to support the hypothesis that YE infection might have an aetiologic role in AITD. To this end, we designed a long-term prospective follow up study in euthyroid women at risk for AITD because they were relatives of AITD patients. The aim of the present study was (a) to evaluate the relationship between YE infection and the development of overt autoimmune hypo- or hyperthyroidism and (b) to evaluate the association between YE infection and the de novo occurrence of thyroid antibodies. Serological evidence of YE infection should precede the occurrence of thyroid antibodies or overt AITD if YE infection is involved in the pathogenesis of AITD.

**Subjects & Methods**

**Subjects**

The present study was carried out among the 803 subjects from the Amsterdam AITD Cohort. The cohort has previously been described in detail [6]. 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 one 1st or 2nd degree relative with documented autoimmune hypo- or hyperthyroidism. Results of thyroid function tests 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 for five years, or shorter when overt hypo- or hyperthyroidism had occurred (defined as TSH >5.7 mU/L in combination with fT4 <9.3 pmol/L or TSH <0.4 mU/L in combination with fT4 >20.1 pmol/L, respectively). At each annual visit to our institution blood samples were collected to measure TSH, fT4, T3, TPO-Ab, Tg-Ab and TBII. Antibodies against Yersinia enterocolitica antibodies were measured in subjects who developed overt hypo- or hyperthyroidism, in the last blood sample taken before this event happened; Yersinia antibodies were also measured in serum at baseline and at 4 years follow-up. Plasma and serum samples were stored at –20° C until assay. Within this cohort we performed two studies.
Study A. In order to evaluate the relationship between YE infection and the development of overt hypothyroidism or overt hyperthyroidism (called events), we designed a nested case-control study among the 790 subjects euthyroid at study entrance. A subject was considered as a case when she had developed overt hypo- or overt hyperthyroidism during follow up. For each case we selected two controls, matched for age at study entrance and duration of follow up. We compared the serological YE status between cases and controls at baseline and at one year before the occurrence of the event.

Study B. In order to evaluate the relationship between YE infection and de novo occurrence of thyroid antibodies, we selected participants from the cohort in the following manner as depicted in Figure 1. First, we excluded the 56 subjects without measurements of YE antibodies at 4 years follow up. Second, from the remaining 734 euthyroid subjects we excluded those who had any serological sign of autoimmune thyroid disease (i.e. serum concentrations of either TPO-Ab ≥ 100 kU/L, Tg-Ab ≥ 100 kU/L, or TBII ≥ 12 U/L) or subclinical hypo- or hyperthyroidism at study entrance. Third, we excluded 98 subjects who had no follow-up. The remaining 388 subjects were thus included in this analysis. 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 negative for thyroid antibodies.

**Laboratory measurements**

Serum TSH and fT4 were measured using time-resolved fluoroimmunoassay (Delphia, Turku, Finland). Reference values are for TSH 0.4-5.7 mU/L and for fT4 9.3-20.1 pmol/L. Thyroid peroxidase (TPO) antibodies and thyroglobulin (Tg) antibodies were measured by

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**Figure 1.** Flowchart of the recruitment of subjects on the Amsterdam autoimmune thyroid disease (AITD) cohort for study B.
chemiluminescence immunoassays (LUMI-test anti-TPO and LUMI-test anti-Tg respectively, Brahms, Berlin, Germany). Improved versions of both assays became available during follow-up: detection limits of these new assays were for TPO-Ab 30 kU/L and for Tg-Ab 20 kU/L. 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 >100 kU/L. TSH receptor antibodies were determined as TSH binding inhibitory immunoglobulins (TBII) using the TRAK assay (Brahms, Berlin, Germany); detection limits in the 1st and 2nd generation TRAK assays were 5 and 1 IU/L respectively, and values above 12 and 1.5 U/L respectively were considered as positive.

Specific IgG and IgA antibodies against purified plasmid-encoded virulence associated YOPs of YE serotype O9 (LCR) in sera were demonstrated by immunoblotting with a YOP-Ab assay (AID, Strassberg, Germany). In short, antigens (25,34,36,37,39,40,46,48 kDa) are blotted onto nitrocellulose. Sera are diluted 1:51 in PBS-Tween and incubated with the antigen-coated nitrocellulose strips overnight at 22°C. The IgG and IgA antibody-antigen complexes formed are quantified after immunostaining with the AID-Scan-System. Controls are included in each assay run, using human acute sera (culture-positive YE infection) containing antibodies to the YOPs. Test sera are judged positive if at least three bands (IgG) or two bands (IgA) are seen in immunoblotting at a level greater than 10% (IgG) or 5% (IgA) of reference standards. The inter-assay variation of the YOP-Ab assay is <3% according to the manufacturer. The YOP-Ab assay was performed without prior knowledge of thyroid function tests or the presence of TPO-Ab in the serum samples.

**Statistical analysis**

Differences between cases and controls were evaluated by Students’ t test for age, by Mann-Whitney U-test for TSH, fT4, TPO-Ab and Tg-Ab, and by χ2 test or if appropriate Fisher’s exact test for the other parameters. Values are given as mean±SD for age and TSH, but as median and interquartile range for all other parameters. A p-value of <0.05 was considered to indicate significant differences between groups.

**Results**

**YE infection and development of overt hypo- or hyperthyroidism (Study A).**

During the 5 years follow up period 38 cases of overt autoimmune hypothyroidism and 13 cases of overt autoimmune hyperthyroidism occurred after a mean follow-up of 2 years, as reported previously [6].

The proportion of subjects positive for YOP-IgG was not different between cases and controls, neither at baseline nor at one year before the event (Table 1). The proportion of subjects with Yersinia YOP-IgA antibodies was not different between cases and controls at study entrance. However, the frequency of YOP-IgA antibodies at one year before the event was lower in cases than in controls (p=0.02). No significant differences in YOP IgG and IgA frequencies were observed when hypothyroid and hyperthyroid cases with their respective controls were analyzed separately.
Table 1. Comparison of characteristics and YE serological status between patients who developed overt hypo- or hyper-thyroidism and their corresponding controls matched for age and follow up, in a nested case-control study of 153 women with 1st or 2nd degree relatives with proven AITD.

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
<th>OR (95% CI)</th>
<th>P-value</th>
<th>Cases</th>
<th>Controls</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N =</td>
<td>51</td>
<td>102</td>
<td></td>
<td></td>
<td>51</td>
<td>102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Age in years</td>
<td>39</td>
<td>39 ± 12</td>
<td>0.96</td>
<td></td>
<td>41</td>
<td>41 ± 13</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>- TSH mU/l</td>
<td>2.9 (1.6-5.4)</td>
<td>1.6 (1.1-2.3)</td>
<td>&lt;0.001</td>
<td></td>
<td>3.4 (1.7-5.6)</td>
<td>1.6 (1.2-2.2)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>- fT4 pmol/l</td>
<td>11.1 (10.0-13.2)</td>
<td>13.0 (11.7-15.0)</td>
<td>&lt;0.001</td>
<td></td>
<td>11.5 (10.0-13.0)</td>
<td>13.1 (11.8-14.3)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>- TPO Antibody positive</td>
<td>78 %</td>
<td>20 %</td>
<td>&lt;0.001</td>
<td>86 %</td>
<td>25 %</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Tg Antibody positive</td>
<td>23 %</td>
<td>10 %</td>
<td>0.001</td>
<td>37 %</td>
<td>19 %</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- YOP IgG Antibody positive</td>
<td>43 %</td>
<td>47 %</td>
<td>0.85(0.43,1.68)</td>
<td>0.65</td>
<td>31 %</td>
<td>33 %</td>
<td>0.91(0.44,1.88)</td>
<td>0.81</td>
</tr>
<tr>
<td>- YOP IgA Antibody positive</td>
<td>23 %</td>
<td>31 %</td>
<td>0.67(0.31,1.45)</td>
<td>0.31</td>
<td>14 %</td>
<td>30 %</td>
<td>0.36(0.15,0.90)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Data are given as means ± SD or medians with interquartile range or proportions, OR: odds ratio, CI: confidence intervals, P-value of cases versus controls. Time of event: visit when diagnosis was made or confirmed.

TSH: thyroid stimulating hormone, fT4: free T4, TPO: thyroid peroxidase, Tg: thyroglobulin, YOP: Yersinia enterocolitica outer membrane proteins.
**Chapter 8**

**YE infection and development of TPO-Ab and/or Tg-Ab (Study B).**

At study entrance the mean age of the 388 participants was 34.3±11.2 years and the mean serum TSH concentration was 1.67±0.73 mU/L. At 4 years follow up, 36 subjects (9.3%) had developed only TPO-Ab while 51 subjects (13.1%) had developed TPO-Ab and/or Tg-Ab. At the time of seroconversion the TPO-Ab concentration had a median value of 160 kU/L (range 100-2833 kU/L) and the Tg-Ab concentration had a median value of 130 kU/L (range 100-195).

The rate of TPO-Ab conversion was not different between subjects with positive or negative YOP IgG status at baseline (6.3% vs 10.7% p=0.16) (Table 2) as was the rate of TPO-Ab and/or Tg-Ab conversion (8.7% vs 15.3% p=0.06). The difference in TPO-Ab conversion rate between YOP IgG positive and YOP IgG negative subjects at baseline was -4.4% (95 CI: -10.6%, 1.7%); the difference for TPO-Ab and/or Tg-Ab conversion rate was -6.7% (95 CI: -13.8%, 0.5%).

In subjects positive for YOP IgG at study entrance, the rate of TPO-Ab conversion (and of TPO-Ab and/or Tg-Ab conversion) was not different between participants in whom YOP IgG persisted and in whom YOP IgG disappeared. Likewise, in the group who tested negative for YOP IgG at baseline, no difference in the rate of TPO-Ab conversion (and of TPO-Ab and/or Tg-Ab conversion) was observed in subjects who remained YOP IgG negative and those who became YOP IgG positive.

Similar results were observed for YOP IgA status (Table 3). De novo occurrence of TPO-Ab (or TPO-Ab and/or Tg-Ab) was not related to the YOP IgA status at baseline. Neither persistence nor emergence of YOP IgA at 4 years follow up was associated with the TPO-Ab or Tg-Ab seroconversion.

**Discussion**

In the present prospective study we evaluated any association between seroreactivity against YE and both early stages (when thyroid antibodies emerge but thyroid function is still normal) and late stages (when overt thyroid dysfunction develops) of the natural course of AITD.

In our nested case-control study, the prevalence of YOP-IgG was similar in cases and controls, both at baseline and at one year before the development of overt hypo- or hyperthyroidism. Whereas the prevalence of YOP-IgA was also not different between cases and controls at baseline, YOP-IgA was less prevalent at follow-up just before the development of overt thyroid dysfunction. If YE would play a causative role in this development, one would expect a higher no lower frequency of YOP-IgA. Our results thus indicate that YE infection is not associated with 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 [7-10] whereas other didn’t find any relationship [11,12]. Brix et al. reported in a classical case-control study that the prevalence of YOP IgG-Ab and YOP IgA-Ab in Graves’ disease was higher than in controls (51% vs 35% and 49 vs 34%, respectively) [13]. Similar results were found in twin pairs discordant for Graves’ disease. In contrast, we didn’t observe differences in YOP IgG
and IgA frequency when we limited our analysis to the 11 Graves’ hyperthyroid cases.

One of the earliest detectable events in the natural history of AITD is the occurrence of thyroid antibodies in serum. The hypothesis that Yersinia infection is an aetiological factor for AITD presupposes that YE infection precedes the occurrence of thyroid antibodies. In our study, neither chronic Yersinia infection (as reflected by YOP IgG status) nor the more recent stages of Yersinia infection (as reflected by the presence of YOP IgA) had any association with thyroid antibody status.

Our study is the first to evaluate the relationship between YE infection and thyroid antibodies in a prospective manner. All studies in the past were cross-sectional studies and concerned patients who had been already treated for Hashimotos’s thyroiditis or Graves’ disease. Three recent cross-sectional studies on this topic reached essentially similar conclusions. Strieder et al reported that the presence of antibodies against YE was unrelated to the presence of TPO-Ab in euthyroid female subjects who were relatives of patients with AITD [14]. A Danish twin study also failed to find an association between thyroid antibodies and YOP antibodies in a case-control study in which controls were recruited either from an external-twin population or from the co-twins [15]. A recent study from China [16] reported that thyroid microsomal antibodies and Tg-Ab were not correlated with the antibodies to YE.

### Table 2. Yersinia enterocolitica YOP IgG status at baseline and at 4-yr follow up in relation to de novo development of thyroid antibodies.

<table>
<thead>
<tr>
<th>YOP IgG status</th>
<th>TPO-Ab converters</th>
<th>TPO-Ab and/or Tg-Ab converters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>baseline positive</td>
<td>8/127</td>
<td>6.3%</td>
</tr>
<tr>
<td>baseline negative</td>
<td>28/261</td>
<td>10.7%</td>
</tr>
<tr>
<td>baseline pos - 4yr pos</td>
<td>6/91</td>
<td>6.6%</td>
</tr>
<tr>
<td>baseline neg - 4yr neg</td>
<td>2/19</td>
<td>10.7%</td>
</tr>
<tr>
<td>baseline neg - 4yr pos</td>
<td>26/242</td>
<td>10.5%</td>
</tr>
</tbody>
</table>

TPO: thyroid peroxidase, Tg: thyroglobulin, Ab: antibodies, YOP: Yersinia enterocolitica outer membrane proteins.

Δ: difference in conversion rate, CI: confidence interval

### Table 3. Yersinia enterocolitica YOP IgA status at baseline and at 4-yr follow up in relation to de novo development of thyroid antibodies.

<table>
<thead>
<tr>
<th>YOP IgA status</th>
<th>TPO-Ab converters</th>
<th>TPO-Ab and/or Tg-Ab converters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>baseline positive</td>
<td>3/64</td>
<td>4.7%</td>
</tr>
<tr>
<td>baseline negative</td>
<td>33/324</td>
<td>10.2%</td>
</tr>
<tr>
<td>baseline pos - 4yr pos</td>
<td>1/35</td>
<td>2.9%</td>
</tr>
<tr>
<td>baseline neg - 4yr neg</td>
<td>2/29</td>
<td>6.9%</td>
</tr>
<tr>
<td>baseline neg - 4yr pos</td>
<td>5/33</td>
<td>15.2%</td>
</tr>
<tr>
<td>baseline neg - 4yr neg</td>
<td>28/291</td>
<td>9.6%</td>
</tr>
</tbody>
</table>

TPO: thyroid peroxidase, Tg: thyroglobulin, Ab: antibodies, YOP: Yersinia enterocolitica outer membrane proteins.

Δ: difference in conversion rate, CI: confidence interval

and IgA frequency when we limited our analysis to the 11 Graves’ hyperthyroid cases.

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A higher prevalence of YOP IgG and IgA in female relatives of patients with AITD than in controls derived from the general population has been reported previously [14]. The higher rate of persistent YE infection in AITD relatives might be due to susceptibility genes for AITD contributing to the risk for YE infection. The Danish twin study indicated that the genetic contribution in the association with YE is modest, and that it is more likely that environmental exposures to confer to the reported association between and YE and AITD [13].

Our study has several limitations. Sample size was dependent on the size of the Amsterdam AITD cohort: all cohort participants eligible for the present studies were included. Nevertheless, the confidence intervals of the odds ratios (study A) and of the differences in the thyroid antibodies conversion rates (study B) clearly do not support the hypothesis that YE infection is causally related to AITD. We measured YOP-Abs not at the time of the occurrence of overt hyper- or hypothyroidism, but in the year prior to the event. One could argue that we have missed acute YE infection in these cases. This limitation does not apply to study B, in which YE serology was assessed precisely when for the first time thyroid antibodies were detected in serum. Another limitation is that outcome of YE infection may differ in a population with different genetic background. Indeed, some rat strains infected with YE are able to clear the infection and YOP-Ab disappear, whereas other rat strain develop chronic YE infection with persistent YOP-Ab [17]. These limitations are in our view well balanced by the strengths of our study. Its prospective nature provides more solid evidence than obtained for cross-sectional studies. Also the nested case-control study design allowed for equal exposure times to environmental insults in cases and controls.

In conclusion, the present prospective study demonstrates that YE infection is not a risk factor neither for the occurrence of thyroid antibodies nor for the development of overt autoimmune hypo- or hyperthyroidism. Thus, Yersinia infection is not a causal factor contributing to the pathogenesis of autoimmune thyroid disease.

REFERENCES