New insights in Autoimmune Polyendocrine Syndromes 1 and 2

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“Show me the money!”
Tom Cruise, in *Jerry Maguire* (1996)
TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................ 7

TIIVISTELMÄ .................................................................................................................................... 9

LIST OF ORIGINAL PUBLICATIONS ............................................................................................. 11

ABBREVIATIONS ........................................................................................................................... 12

REVIEW OF THE LITERATURE ...................................................................................................... 14

1. Autoimmune polyendocrine syndromes ................................................................................... 14

2. Autoimmune polyendocrine syndrome type 1 / Autoimmune polyendocrinopathy- 
candidiasis-ectodermal dystrophy ............................................................................................... 15

  2.1. Epidemiology............................................................................................................................ 15

  2.2. Clinical manifestations............................................................................................................ 16

  2.2.1. *Chronic mucocutaneous candidiasis* ................................................................................... 17

  2.2.2. Endocrine manifestations..................................................................................................... 23

  2.2.2.1. Hypoparathyroidism ........................................................................................................ 23

  2.2.2.2. Addison’s disease ............................................................................................................ 24

  2.2.2.3. Gonadal insufficiency ...................................................................................................... 25

  2.2.2.4. Thyroid autoimmunity .................................................................................................... 26

  2.2.2.5. Type 1 diabetes .............................................................................................................. 26

  2.2.2.6. Pituitary failure ............................................................................................................... 28

  2.2.3. Nonendocrine manifestations............................................................................................... 32

  2.2.3.1. Alopecia areata ............................................................................................................... 32

  2.2.3.2. Vitiligo ........................................................................................................................... 33

  2.2.3.3. Nail dystrophy ............................................................................................................... 35

  2.2.3.4. Gastrointestinal manifestations (autoimmune hepatitis excluded) ................................. 35

  2.2.3.5. Hepatitis ........................................................................................................................ 38

  2.2.3.6. Hyposplenia and asplenia ............................................................................................... 39

  2.2.3.7. Kidney manifestations .................................................................................................... 39

  2.2.3.8. Lung manifestations ........................................................................................................ 40

  2.2.3.9. Enamel dysplasia ......................................................................................................... 41

  2.2.3.10. Eye manifestations ....................................................................................................... 41

2.3. Genetics: *AIRE* mutations....................................................................................................... 43

  2.3.1. Autoimmune regulator gene ............................................................................................... 43

  2.3.2. *AIRE* mutations and their implications in the phenotype ............................................... 44

  2.3.3. *AIRE* functions ............................................................................................................... 45

2.4. Autoantibodies in APECED .................................................................................................... 47

  2.4.1. Tissue-specific antibodies ................................................................................................. 47

  2.4.2. Anticytokine antibodies ..................................................................................................... 47

    2.4.2.1. Interferon alpha autoantibodies .................................................................................... 48

    2.4.2.2. Interleukin-17A, -17F and -22 autoantibodies ............................................................. 48

  2.4.3. Cell-immune responses in APECED ................................................................................... 49

3. Quality of life .............................................................................................................................. 51

  3.1. APECED .................................................................................................................................. 51
AIMS OF THE STUDY

SUMMARY OF MATERIAL AND METHODS

1. Patients and patient recruitment
2. Interviews
3. Clinical records
4. AIRE mutations
5. Quality of life (Original studies V and VI)
6. Routine laboratory tests (Original study II)
7. Tissue samples and immunochemistry
   7.1 Gastrointestinal tissues
   7.2 Renal tissues
   7.3 Immunochemistry
8. Autoantibodies and autoreactive T cells
   8.1 Detection of tissue antigens recognised by patient sera and antibodies
      8.1.1 Detection of intestinal antigens by indirect immunofluorescence (IIF)
      8.1.2 Detection of renal antigens by indirect immunostaining of rat kidney
8.2 Detection of circulating antibodies by enzyme linked immunosorbent assay
8.3 Detection of CaSR and NALP5 antibodies
8.4 Other antibodies
8.5 ELISPOT assay against AADC
9. Statistical analysis
10. Ethical considerations

RESULTS AND DISCUSSION

1. Clinical component of APECED and other autoimmune disease in the studied cohorts
   1.1 APECED
   1.2 Addison’s disease and APS-2
2. Clinical manifestations (Original studies I, II and IV)
   2.1 Gastrointestinal symptoms
      2.1.1 Familial history
      2.1.2 Upper gastrointestinal symptoms
      2.1.3 Lower gastrointestinal symptoms
      2.1.4 Other gastrointestinal symptoms
   2.2 Nephrologic and urologic clinical manifestations
3. Tissue immunochemistry and indirect immunofluorescence
   3.1 Gastrointestinal tract
   3.2 Kidney
   3.3 Palpebra (unpublished data)
4. Autoimmune and autoreactive T cells
   4.1 Circulating antibodies against cytokines
   4.2 Circulating antibodies against tissue antigens
   4.3 AADC-specific T cell response
5. Nonendocrine tissue-specific antibodies: simple reflections or active actors in autoimmunity in APECED? .......................................................... 82
  5.1. Origin of the patients and data collection........................................ 82
  5.2. Antibody detection methods.............................................................. 83
  5.3. The role of the targets........................................................................ 84

6. What is the role of autoimmunity in the lower gastrointestinal manifestations of APECED? .......................................................... 85
  6.1. Autoimmune enteropathy during APECED...................................... 85
  6.2. Enterocinocrine cells and the gut microbiota..................................... 86
  6.3. The IL-17/IL-22 pathway and the gut microbiota in APECED.......... 87
  6.4. Paneth cells and the gut microbiota in APECED............................. 88
  6.5. From autoimmunity to disturbance of the gut microbiota (dysbiosis) in APECED.......................................................... 90

7. The quality of life of APECED patients and AD/APS-2 patients (Original studies V and VI) .......................................................... 91

CONCLUDING REMARKS AND FUTURES DIRECTIONS .......................... 96

ACKNOWLEDGEMENTS ............................................................................. 97

REFERENCES .............................................................................................. 99
ABSTRACT

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, OMIM 240300) is a rare autosomal recessive disorder caused by mutations in the autoimmune regulator (AIRE) gene located on chromosome 21 (21q22.3). AIRE deficiency causes a loss in central immune tolerance, leading to the failure to eliminate autoreactive T cells in the thymus and allowing their escape to the periphery. Because of a founder effect, APECED is particularly prevalent in Finland (1/25,000) but is observed worldwide with variable prevalence. APECED patients are susceptible to mucocutaneous candidiasis and multiple endocrine autoimmune diseases such as primary hypoparathyroidism, adrenal insufficiency, primary hypogonadism, type 1 diabetes, hypothyroidism, and hypophysitis. They may also develop additional nonendocrine autoimmune diseases, such as alopecia areata/totalis, vitiligo, gastro-intestinal (GI) diseases, keratitis or tubulointerstitial nephritis (TIN). In addition, the patients typically develop a variety of serum tissue-specific autoantibodies, which are predictive of the development of autoimmune disease and anticytokine antibodies such as those against type I interferons and Th17-related interleukin IL-17 and IL-22.

The aim of this thesis was to study such manifestations of APECED that have not been well characterized before and also, to study health-related quality of life among Finnish APECED and Addison's disease/APS2 patients.

We evaluated the clinical GI features and searched for novel markers of GI dysfunction in a Finnish cohort of 31 APECED patients. The main upper GI symptoms were dysphagia and retrosternal pain (45%) and the lower GI symptoms were constipation (48%), diarrhoea (45%) and malabsorption (16%). Previously, L-amino-acid decarboxylase (AADC) and tryptophan hydroxylase type 1 (TPH-1) antibodies have been demonstrated in APECED. AADC antibodies were found in 51% and TPH-1 antibodies in 39% of all patients. Also, a T cell response to AADC was detected in 43%. One third of the patients had autoimmune enteropathy (AIE)-related 75 kDa antigen (AIE-75, 33%) and villin (29%) autoantibodies, and antibodies against brush borders and Paneth cells (PCs) were detected in 29% and 20%, respectively. Mucosal intestinal IL-17 expression was decreased or negative in 77% of the intestinal samples. Duodenal chromogranin A and serotonin expression was absent or decreased in 50% and 66% of the patients, respectively. Of the clinical symptoms, constipation correlated with negative serotonin staining (p < 0.05) and with AADC antibodies (p = 0.019). Importantly, we found a correlation between autoantibodies against AADC, which are critical for serotonin and DOPA synthesis, and constipation. Constipation was also associated with a lack of serotonin expression in the enteroendocrine cells (EECs). Paneth cells (PCs) were lacking in the duodenum in 20% of our intestinal samples, even though this was not associated with GI symptoms.

In this Finnish APECED patient cohort, 17% (5/30) had moderate-to-severe renal failure, including 10% (3/30) with TIN requiring transplantation, haemodialysis or immunosuppressive treatment. However, the latter did not seem to be efficient in controlling disease progression. All 3 patients with TIN had circulating antibodies against the distal part of the nephron, as did 30% of all cohort cases. The pathogenic relevance of such circulating antibodies is still unclear.

The immunological basis of hypoparathyroidism in APECED was explored by studying circulating calcium-sensing receptor (CaSR) and NALP5 antibodies. Although they were detected in 16 of 44 (36%) and 13 of 44 (30%) patients, respectively, we failed to find any clinically relevant statistical association.

These APECED patients did not present circulating antibodies for other autoimmune diseases such as rheumatoid arthritis, celiac disease, bullous pemphigoid or pemphigus vulgaris. Some patients had antinuclear antibodies at a low-titre without clinical significance.
Secondly, we evaluated the health-related quality of life among Finnish APECED and Addison’s disease/APS2 patients and sought to determine which factors may predict a possible impairment. Using health-related quality of life (HRQoL) questionnaires for APECED (SF-36) and Addison’s disease/APS2 patients (SF-36, 15D), we indeed observed impaired HRQoL. For the APECED patients, general health, emotional well-being and energy/vitality were the most diminished aspects of HRQoL. Among the patients with Addison’s disease/APS2, compared to a large control population, physical or emotional role functioning, energy/vitality and general health were most affected. Discomfort and symptoms, vitality, and sexual activity were the most affected dimensions of the 15D scores. Affiliation with a patients’ association, female gender, the presence of non-APS2 inflammatory comorbidities, lower educational level and a longer disease duration were independent predictors of impaired HRQoL in these patients.

Taken together, the results of this thesis show that APECED patients are genetically prone to develop autoantibodies to a multitude of tissue antigens but are still tolerant to some common autoantigens. The true clinical and biological relevance of these circulating autoantibodies has not yet been elucidated, and it is possible that they are only a reflection of T cell-mediated immunity. They may, however, have a cumulative effect and clinical disease may arise only in patients with a combination of circulating antibodies, as seen in diabetes type 1. This may explain why we failed to find any association between any single type of antibody and a given symptom. For the lower GI tract manifestations, we hypothesise a cumulative effect of the autoimmunity directed against both the enteroendocrine cells and the Paneth cells, leading to a dysfunction in both the secretion of serotonin in the gut and the secretion of antimicrobial defensins. Such a disturbance would have an effect on the gut microbiota. The question of whether the neutralising antibodies against cytokines may have a paradoxical protective effect is open to debate. Lastly, despite having a high number of manifestations, patients with APECED seem to cope with their disease. Patients with Addison’s disease have significantly impaired HRQoL compared to the general population.
Autoimmunipolyendokrinopatia-kandidiaasi-ektodermidystrofia (APECED, OMIM 240300) on harvinainen, autosomissa peittyvä sairaus, joka aiheutuu muttaatioista kromosomissa 21 (21q22.3) sijaitsevasta autoimmune regulator (AIRE) - genistä. AIRE:n toiminnallinen puutos johtaa n.s keskusimmuunitoleranssin menetykseen siten että autoreaktiiviset T-solut eivät tuhoudu kateenkorvassa vaan pääsevät karkaamaan perifeeriseen verenkiertoon. APECED:in esiintyvyys Suomessa on erityisen korkea (1/25,000) johtuen ns. perustaja-mutaatiosta AIRE-geenissä, mutta tautia tavataan myös muualla maailmassa vaihtelevalla esiintyvyydellä. APECED-potilaat ovat alttiita ihon ja limakalvojen krooniselle hiivatulehdukseelle ja heillä voi esiintyä useita autoimmunitauteja, kuten lisäkilpirauhaa, lisämunuaisen kuoren, kilpirauhun sekä sukuelinten vajaatoimintaa, tyypin 1 diabetesta ja aivolisäkkeen tulehdusta. Näiden lisäksi APECED-potilaat kärsivät usein myös muista ei-endokrinisistä autoimmunitaudeista kuten pälvikaljusta (alopesia), valkopälvestä (vitiligo), silmän sarveiskalvon tulehduksesta, muunaisputkien tulehduksesta (tubulointerstitiaalinen nefriitti, TIN,) sekä suoliston sairauksista. Potilailla havaitaan tyypillisesti seerumissa erilaisia kudos- ja elinspesifejä autovasta-aineita sekä vasta-aineita vasta-aineita tulehdusväittäjäaineita eli sytokiineja kohtaan. Viime mainitut kohdistuvat tavallisimmin tyypin I interferonia sekä Th17-immuunivasteeseen liittyviä interferokineneja IL-17 ja IL-22 kohtaan ja ennakkoautoimmuuita puhkeamista.

Tässä väitöskirjassa on tutkittu tarkemmin kahta aiemmin huonosti tunnettua APECED:in esiintyvää oireista: ruoansuolatuskanavan sekä munuaisten ja virtsateiden oireita. Kyselytutkimuksen avulla kartoittimme potilaiden kokemat ruoansulatuskanavanäveräiset oireet ja tutkimme suoliston toimintahäiriöiden tautimekanismia 31 suomalaista APECED-potilasta käsitettävällä aineistossa. Tyypillisä ylemmän ruoansuolatuskanavan oireita olivat nielemishäiriöt ja rintalastan takainen kipu (45%) ja alemman ruoansulatuskanavan osalta ummetus (48%), ripuli (45%) ja imeytymishäiriöt (16%). Jo aiemmin on tiedetty APECED-potailalla esiintyvän vasta-aineita suoliston toiminnan kannalta keskeisten enteroiden (EE) solujen erittämä entsyymejä tryptofaanihydroksylaasia (TPH-1) ja aromaatista L-aminohappodekarboksylaasia (AADC) kohtaan. Potilaat osoittavat jaantamisessa AADC-vasta-aineita esitti 51%:lla sekä TPH-1 vasta-aineita 39%:lla potilaista. Uutena löydöksenä osoitimme T-soluperäisen T-soluperäisen immuneen seerumivasteen AADC:tä kohtaan 43%:lla. Kolmasosa potilaista oli osoitettavissa autovasta-aineita myös autoimmuuni enteropatiaan liittyvää antigenia (AIE-75) sekä ravinnon immyytystä vastaavien enterosyytten mikrovilluksissä esiintyvää villiinä vastaan. Lisäksi löysimme vasta-aineita suolen suksaumaa (29% potilaista) sekä suolistorauhan Panethin soluja (20% potilaista) vastaan. Suolen limakalvon IL-17:ää tuottavien solujen määrä oli alentunut tai puuttui 77%:ssa tapauksista. Pohjakaisuolen kromograini-A-proteiinia tuottavien EE- solujen määrä oli vähentynyt tai puuttui 50%:ssa ja vastaavasti serotoniinia tuottavien solujen määrä 66%:ssa suolistonäytteistä. Potilaaiden oireista ummetus korrelointi negatiivisesti EE- solujen serotoniini-ilmennyisestä (p<0.05) sekä AADC-vasta-aineiden kanssa (p<0.05). Huomionarvoista on, että havaittavat merkittävän yhteyden ummetuksen ja serotoniini- ja dopamiinisynteesille keskeistä AADC-entsyymiä vastaan muodostuneiden vasta-aineiden välillä. Uusi havainto oli myös se, että suolistossa antimikrobisit defensiinejä tuottavat Panethin solut puuttuivat 20%:ssa potilaaiden duodenum-näytteistä, joskaan tämä ei suoraan korreloinut klinisten oireiden kanssa.

Tutkimistamme APECED-potilaista 17% (5/30) kärsi keskivaikeasta tai vaikeasta munuaisen vajaatoiminnasta, ja 10%:lla (3/30) potilaista oli TIN, joka vaati elinsiirteen, hemodialyysit tai immunosuppressivistä hoitoa. Nämä viime mainitut ei kuitenkaan osoittautunut riittäväksi taudinkulun hallinnomisessa. Kaikilla kolmella TIN:istä kärsivällä potilaalla todettiin...


Tässä väitöskirjassa esitettyjen tulokset osoittavat, että APECED-potilailla on runsasti autovasta-aineita monia kudosantigeeneja vastaan mutta samalla potilaat ovat tolerantteja tietylle yleisille autoantigeeneille. Näiden kiertävien autovasta-aineiden lopullinen kliininen sekä biologinen merkitys on vielä tuntematon mutta on mahdollista, että ne ovat vain yksi T-soluvälitteisen immunieettien osa-alue. On mahdollista, että näillä kiertävillä vasta-aineilla on kumulatiivinen vaikutus ja oireiden puhkeamiseen vaaditaan vasta-aineiden yhdistelmää, kuten on esimerkiksi tyypin 1 diabeteksessä. Tämä voisi selittää myös miksi yksittäisten vasta-aineiden ja kliinisten oireiden välillä ei tässä työssä aina voitua osoittaa merkitsevää asosiaatiota.
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original communications, referred to in the text by Roman numerals

I: Kluger N, Jokinen M, Lintulahti A, Krohn K, Ranki A. Gastrointestinal immunity against tryptophan hydroxylase-1, aromatic L-amino-acid decarboxylase, AIE-75, villin and Paneth cells in APECED. Submitted


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AA</td>
<td>Alopecia areata</td>
</tr>
<tr>
<td>AADC</td>
<td>Aromatic L-amino acid decarboxylase</td>
</tr>
<tr>
<td>Abs</td>
<td>(Auto)antibodies</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AD</td>
<td>Addison’s disease</td>
</tr>
<tr>
<td>AIE-75</td>
<td>Autoimmune enteropathy (AIE)-related 75 kDa antigen or harmonin</td>
</tr>
<tr>
<td>AIH</td>
<td>Autoimmune hepatitis</td>
</tr>
<tr>
<td>AIRE</td>
<td>Autoimmune regulator</td>
</tr>
<tr>
<td>AN-Ab</td>
<td>Antinuclear antibodies</td>
</tr>
<tr>
<td>APECED</td>
<td>Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy</td>
</tr>
<tr>
<td>APS</td>
<td>Autoimmune polyendocrine syndrome</td>
</tr>
<tr>
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</tr>
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<tr>
<td>APS4</td>
<td>Autoimmune polyendocrine syndrome type 4</td>
</tr>
<tr>
<td>CaSR</td>
<td>Calcium Ca2+-sensing receptor</td>
</tr>
<tr>
<td>CrA</td>
<td>Chromogranin A</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DLQI</td>
<td>Dermatology Life Quality Index</td>
</tr>
<tr>
<td>EEC</td>
<td>Enteroendocrine cell</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ENA-Abs</td>
<td>Extractable nuclear antigen antibodies</td>
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<tr>
<td>eTAC</td>
<td>Extrathymic AIRE-expressing cells</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamate decarboxylase</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>hD5</td>
<td>Human defensin 5</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
</tr>
<tr>
<td>HP</td>
<td>Hypoparathyroidism</td>
</tr>
<tr>
<td>HRQoL</td>
<td>Health-related quality of life</td>
</tr>
<tr>
<td>IA2</td>
<td>Anti-islet antigen 2</td>
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<td>IAA</td>
<td>Insulin Ab</td>
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<td>Islet cell Ab</td>
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<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IF</td>
<td>Intrinsic factor</td>
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<tr>
<td>IFI</td>
<td>Indirect immunofluorescence</td>
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<tr>
<td>IL-17A</td>
<td>Interleukin-17A</td>
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<tr>
<td>IL-17F</td>
<td>Interleukin-17F</td>
</tr>
<tr>
<td>IL-22</td>
<td>Interleukin-22</td>
</tr>
<tr>
<td>IPEX</td>
<td>Immune dysfunction polyendocrinopathy X-linked</td>
</tr>
<tr>
<td>mTEC</td>
<td>Medullary thymic epithelial cells</td>
</tr>
<tr>
<td>NALP5</td>
<td>NACHT leucine-rich-repeat protein 5</td>
</tr>
<tr>
<td>PA</td>
<td>Pernicious anaemia</td>
</tr>
<tr>
<td>PC</td>
<td>Paneth cell</td>
</tr>
<tr>
<td>PCA</td>
<td>Parietal cell antibodies</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PG</td>
<td>Parathyroid glands</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone or parathormone</td>
</tr>
<tr>
<td>QoL</td>
<td>Quality of life</td>
</tr>
<tr>
<td>RBDI</td>
<td>Raitasalo’s modification of the short form of the Beck Depression Inventory–BDI</td>
</tr>
<tr>
<td>SCC</td>
<td>Side-chain cleavage enzyme</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>TIN</td>
<td>Tubulointerstitial nephritis</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TPH</td>
<td>Tryptophan hydroxylase</td>
</tr>
<tr>
<td>TRA</td>
<td>Tissue-restricted antigens</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid-stimulating hormone</td>
</tr>
<tr>
<td>21-OH</td>
<td>21-hydroxylase</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamin (serotonin)</td>
</tr>
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REVIEW OF THE LITERATURE

1. Autoimmune polyendocrine syndromes

Autoimmune polyendocrine syndromes (APS), sometimes called polyglandular autoimmune syndromes in the literature, are a heterogeneous group of rare disorders characterised by autoimmune activity against at least a minimum of 2 endocrine glands. However, the term of ‘polyendocrinopathy’ is misleading as these patients may also develop nonendocrine autoimmune disorders (Cutolo 2014; Maréchaud 2005). The classification of APS includes 4 types (Neufeld et al 1980, Betterle et al 2003) (Figure 1). However, the 2 major APS are type 1 and type 2, each with a strong genetic component as they occur in multiple generations in type 2 and in siblings in type 1 (Cutolo 2014). The list of polyendocrine syndromes is much broader and includes other diseases that will not be discussed further, such as immune dysfunction polyendocrinopathy X-linked (IPEX) (Eisenbarth et al 2004), thymoma-associated autoimmune disease, POEMS syndrome and Wolfram syndrome (Cutolo 2014).

Briefly, APS type 1 (APS1), or autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), is clinically defined by the presence of 2 of the 3 classical ‘major’ components (also known as ‘Whitaker’s triad’), i.e. autoimmune adrenocortical insufficiency (Addison’s disease, AD), hypoparathyroidism (HP) and chronic mucocutaneous candidiasis (CMC). If a sibling has a definite APECED, having only one of these conditions is sufficient to make the diagnosis (Husebye et al 2009).

APS type 2 (APS2, also called Schmidt’s syndrome) is by far the most common polyendocrine syndrome including AD (prevalence 1/20,000). It is a complex genetic disorder with strong HLA DR3, DR4 and also B8 association. APS2 is usually diagnosed during adulthood (20 to 40 yrs), even though children may be affected. AD is the first manifestation in half of the cases. In 20% of the cases, T1D or thyroid autoimmune disease (autoimmune thyroiditis, primary hypothyroidism, Grave’s disease) can be found simultaneously and, in the remaining 30%, they can precede AD. Patients with APS2 develop autoimmunity sequentially over a period of many years. A high number of endocrine and nonendocrine disorders can be associated with APS2, but HP is by definition not part of APS2 (Maréchaud 2005; Cutolo 2014). The literature also mentions APS3, which involves the same endocrine disorders as in APS2, but without adrenal failure. If the combination of autoimmune endocrine gland
disorders does not meet the above-mentioned criteria, the disease may be categorised as APS4 (Cutolo 2014) (Figure 1).

**Figure 1. Autoimmune Polyendocrine Syndromes: APS1, APS2 and IPEX**

**Autoimmune polyendocrine syndromes**
*(Eisenbarth & Gottlieb, 2004)*

- **APS 1**
  - *AIRE*
    - 21q22.3
    - recessive
  - Addison’s disease
  - Hypoparathyroidism
  - Chronic candidiasis

- **APS 2**
  - *HLA*
    - polygenic
  - Addison’s disease + Autoimmune thyroid disease and/or type 1 diabetes + any autoimmune diseases

- **IPEX**
  - *FOX P3*
    - Xp11.23
    - X-linked
  - Dermatitis
  - Enteropathy
  - Type 1 diabetes
  - Thyroiditis
  - Hemolytic anemia
  - Thrombocytopenia
  - Food allergy

**2. Autoimmune polyendocrine syndrome type 1 / Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy**

APECED (OMIM 240300) is a monogenic autosomal recessive disorder caused by mutations in the *AIRE* (autoimmune regulator) gene, located on chromosome 21 (21q22.3).

**2.1. Epidemiology**

APECED is a rare disease that displays a worldwide distribution, as illustrated by small series of patients collected in the USA (Wang et al 1998), India (Zaidi et al 2009), and Japan, where the prevalence is estimated to be 1:10,000,000 (Sato et al 2002). However, APECED has been mainly reported in Europe. Due to a founder effect, APECED is overrepresented in Finland
with an ‘official’ prevalence of 1/25,000 (Ahonen et al 1990, Perheentupa 2006) and belongs to the ‘Finnish Disease Heritage’ (Norio 2003, Norio 2003b). Nevertheless, it is possible that, due to population migration, the current estimation of APECED patients is lower. However, a higher prevalence was found among Iranian Jews in Israel (1/9000) (Zlotogora et al 1992). Although APECED is very rare in Italy, 3 hot spots have been identified: Sardinia (1/14,400) (Rosatelli et al 1998), Apulia (Betterle et al 2012) and Venetia (Cervato et al 2009). In the northwestern part of France, it was estimated to affect 1/500,000 inhabitants (Proust-Lemoine et al 2010, Proust-Lemoine et al 2012). The prevalence in various Eurasian countries with the main AIRE mutations is summarised in Figure 2.

Figure 2. Prevalence of APECED and the main AIRE mutations in Europe and Israel

2.2. Clinical manifestations

APECED predisposes to mucocutaneous candidiasis, multiple endocrine autoimmune diseases, nonendocrine autoimmune disorders and ectodermic anomalies (Husebye et al 2009, Proust-Lemoine et al 2012). Apart from the 3 ‘major’ components (CMC, HP and AD), there are additional so-called ‘minor’ components that include the aforementioned autoimmunity and
ectodermal anomalies (Figure 3). There is an extreme variability in the number of clinical manifestations, ranging from 1 to 10 (Perheentupa 2006).

APECED manifests mainly during early childhood or adolescence, with a progressive manifestation of various disorders, even though rare cases have been reported during early adulthood between 18 and 28 years of age (Magitta et al 2008). The female-to-male sex ratio varies according to series, from 0.3 to 2.5. The triad is usually complete after the second decade, but it can remain incomplete. The absence of previous family history, the phenotypic variability of APECED, added to a possible lack of knowledge of the disease by the physicians, may lead to mis- or underdiagnosis (Husebye et al 2009). Table 1 summarises the clinical data of approximately 20 published series. The tissue-specific autoantibodies (Abs) are summarised in Table 2.

Figure 3. Tissues affected by APECED (to the exclusion of candidiasis) (modified from Linx2Learn, http://www.webset-lms.com/vtct/Course.aspx?id=5221, with authorization of VCTC)

2.2.1. *Chronic mucocutaneous candidiasis*

CMC may occur in patients with primary immunodeficiencies and various other pathological conditions, such as severe combined immunodeficiency, combined immunodeficiency-like
deterioration of cytokinesis 8 deficiency, signal transducer and activator of transcription (STAT) 3 deficiency causing autosomal-dominant hyper-IgE syndrome (AD-HIES), autosomal recessive (AR) IL-12Rβ1 and IL-12p40 deficiencies, AR tyrosine kinase (TYK) 2 deficiency, and AR caspase recruitment domain nine deficiency. APECED belongs to this list of conditions (Eyerich et al 2010, Soltész et al 2013). Candidiasis is usually the first nonendocrine manifestation of APECED, but can appear much later after the endocrine manifestations (Ahonen et al 1990). On the other hand, it can also be the first manifestation and should therefore raise the suspicion of this diagnosis (Collins et al 2006).

The prevalence and severity of CMC during APECED are variable, according to the series (Table 1), which most likely is the result of different mutations of the AIRE gene (Kisand et al 2011).

In the large Finnish (Ahonen et al 1990) and Italian (Meloni et al 2012) series, CMC appears as early as the first year of life. In Sardinia, it is the first sign of APECED in 86% of the cases, at a median age of 3 years (Meloni et al 2012). In Finland, 26% of the patients have CMC at the age of 1, 55% at the age of 5, and after the second decade, almost all patients have CMC (Ahonen et al 1990). It more rarely develops during adulthood (Meloni et al 2012). The candidal infection affects the nails and the oral, vaginal, and oesophageal mucosa (Husebye et al 2009) (Figure 4).

Oral mucous membranes are almost always affected and the clinical presentation does not differ from regular candidiasis. Lesions are often painful and consumption of acidic or spicy food may be impossible (Collins et al 2006, Husebye et al 2009). Candidiasis oesophagitis affects approximately 15 to 22% of the patients (Perheentupa 2006, Collins et al 2006), causing pain while swallowing, retrosternal pain and dysphagia (Ahonen et al 1990, Perheentupa 2006). These manifestations are not always simultaneously associated with oral candidiasis (Perheentupa 2006). Chronic inflammation may lead to oesophageal stricture (Ahonen et al 1990, Betterle et al 1998, Perheentupa 2006), which needs treatment with balloon dilatation or stenting.

Oral (tongue, gingiva, buccal mucosa) and oesophageal squamous cell carcinomas may develop as a long-term complication of chronic inflammation mediated by CMC. They are currently the most common malignancies associated with APECED. They result from an inadequately treated refractory oral candidiasis, usually due to antifungal resistance. Uittamo et al showed that C. albicans isolated from APECED patients produced potentially mutagenic amounts of acetaldehyde when incubated with glucose (Uittamo et al 2009). Metastasis and
premature death have been reported among patients older than 25 years (Rautamaa et al 2007). Alcohol intake, active smoking, poor oral hygiene, or even the consumption of sweet products, leading to higher acetaldehyde levels (Uittamo et al 2009), as well as immunosuppressive therapies, are risk factors that may promote carcinogenesis for both oesophageal and oral mucosa (Rautemaa et al 2007, Rosa et al 2008). Long-term follow-up is mandatory as recurrences have been observed with the development of multiple carcinomas (Böckle et al 2010, Shepard et al 2012).

Oesophageal cancers during CMC unrelated to APECED have been reported (Rosa et al 2008, Koch et al 2009), supporting the notion that chronic candidiasis plays a role in carcinogenesis rather than APECED itself. Achalasia was reported in a patient with candidal oesphagitis who developed subsequent oesophageal carcinoma. It remains open to debate whether achalasia was an additional factor contributing to the carcinogenicity in this case (McCormack et al 2012).

Management of APECED patients includes ensuring excellent oral hygiene with careful and regular dental follow-up. Dental plaque and sources of yeast in the oral cavity should be actively treated (Rautemaa et al 2007). Patients should be advised to avoid smoking and an excess of alcohol. Candidiasis should be treated aggressively and regular prophylaxis should be given. Azoles, fluconazole especially, are often recommended as first-line treatment but this leads to the selection of resistant strains (Eyerich et al 2010). Therefore, regular fungal sensitivity tests should be performed. Preferably, topical polyenes (amphotericin B) should be chosen as they are less prone to selecting resistant Candida stains (Rautemaa et al 2007). Echinocandins are available as an intravenous infusion, and systemic polyene (amphotericin B) should be restricted to systemic candidiasis (Eyerich et al 2010). Any suspicious lesions, any erosion or ulceration that does not heal within 2 weeks (Shepard et al 2012), or any thickening should be biopsied. Mucosal dysplasia should be surgically removed to prevent rapid progression to carcinoma (Shepard et al 2012). In addition, any difficulties in swallowing or eating or retrosternal pain should prompt oesophageal endoscopy (Rautemaa et al 2007).

Cutaneous candidiasis is rather uncommon (9% of the patients) and affects only the hands and face (Ahonen et al 1990). Destructive candidal onychomycosis and chronic paronychial candidiasis affect the fingernails more often than the toenails. (Collins et al 2006).

Vaginal candidiasis causes a discharge, pruritus and disturbance in the sex life of patients.
The disease is chronic, but often mild with varying intervals of spontaneous regression (Ahonen et al 1990). The incidence of candidiasis diminishes with age. Of note, APECED patients show immunodeficiency only against *Candida* and not against other fungi or bacteria. Systemic dissemination with potential lethal evolution is possible but quite rare and mostly due to additional iatrogenic factors such as immunosuppressive therapies (Betterle et al 1998).

**Figure 4. Big toe nail dystrophy in a Finnish APECED patient possibly related to candidiasis**
Table 1. Percentage frequency of major and minor clinical features in APECED in different populations

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*Prevalence at the age of 30 yo, ** data as given in Betterle et al 2003 *** patients from Dutch (n = 3), Serbian (n = 7) and Slovenian origin (n = 1)
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**Patients recruited among 126 patients with Addison’s disease ***article mentions 36 patients but no data are provided for 2 siblings who died in childhood nd: not determined
2.2.2 Endocrine manifestations

2.2.2.1. Hypoparathyroidism
Primary hypoparathyroidism (HP) is the most common endocrine manifestation of APECED in almost all patient series (Table 1), affecting 75 to 100% of the patients. Lower prevalences are nevertheless found in France (63%) (Proust-Lemoine et al 2010). HP is the first endocrine manifestation in 64% of the Finnish patients (Perhentupa 2006), occurring generally before the age of 10, but it can be diagnosed also during adulthood up to 40 years of age (Gylling et al 2003). It may also remain as the only endocrine manifestations in some patients (Ahonen et al 1990, Zlotogora et al 1992). Interestingly, there is a distinct difference in prevalence between the sexes, as HP occurs in 98% of the women and 71% of the men.

The clinical manifestations of HP include paraesthesia of the face and fingers, muscle cramps, clumsiness, even seizure in the case of frank hypocalcaemia (Husebye et al 2009). Diarrhoea and malabsorption are also symptoms of HP (Ebert 2010). More rarely, serum hypocalcaemia can lead to electrocardiographic anomalies such as prolonged QTc intervals (Meyer et al 2007, Buber et al 2008). Lastly, brain calcifications located in the cerebellum, basal ganglia, thalamus, and the white matter, associated with neurological complaints such as Parkinsonism, have been reported (Abraham et al 2010; Buber et al 2008). Diagnosis is made upon routine laboratory findings: hypocalcaemia, hyperphosphataemia, and possible hypomagnesaemia, in the absence of renal failure and normal or low parathormone (PTH) levels. AIRE mutations do not differ between patients with and without HP, nor does HLA class II haplotype (Gylling et al 2003). However, it was noted among Finnish patients that a lower and later incidence of HP is observed in male patients, probably in relationship with AD as the manifestation in these patients. The authors speculated that corticosteroid substitution for AD might protect against the autoimmune process (Gylling et al 2003, Magitta et al 2008). However, this hypothesis seems unlikely as they receive hydrocortisone only at a substitutive dosage and not at an antiinflammatory dosage.

Several studies have tried to identify potential targets in the parathyroid glands that could explain the occurrence of HP (Table 2). Autoantibodies (Abs) against parathyroid gland (PG) epithelial cells have been found in 19% of the APECED patients, but at low titres and with no difference between patients with and without HP (Gylling et al 2003). Similarly, Abs against hyperplastic PG (37%) and PTH (3%) were ruled out as a possible explanation (Gylling et al 2003). The calcium-sensing receptor (CaSR), a pivotal receptor involved in calcium metabolism homeostasis by sensing serum calcium levels and regulating PTH levels, has been
suspected to be a key target of autoimmunity (Li et al 1996). However, the results have been rather conflicting: Söderbergh et al did not find CaSR Abs in any of their 90 patients (Söderbergh et al 2004), whereas Gylling et al found that 12% of the patients had Abs against CaSR, but with no difference compared with control patients (Gylling et al 2003). Others reported that CaSR Abs were present at a higher incidence among APECED patients, from 35% (Li et al 1996) to 86% (Gavalas et al 2007). It is still unclear whether or not CaSR Abs are a specific or sensitive marker for APECED-associated HP. More recently, Abs against NALP5 (NACHT leucine-rich-repeat protein 5), a protein selectively expressed in the cytoplasm of chief cells in the parathyroid gland, also in testis, breast, and ovaries, has been associated with clinical and biochemical manifestations of HP during APECED. They may have a specificity of 100% and sensitivity of 49% for the diagnosis of HP (Alimohammadi et al 2008, Tomar et al 2012).

Idiopathic HP is quite exceptional in the general population, so APECED should be considered systematically in cases of HP. During the follow-up of patients without HP, plasma calcium and phosphate should be monitored once a year. The mainstay of therapy includes vitamin D derivatives, oral calcium and magnesium supplementation. Regular monitoring of plasma calcium at least every 2 months along with a control of plasma magnesium, phosphate and 24h urinary calcium every 4-6 months is recommended (Husebye et al 2009).

2.2.2.2. Addison’s disease

Except for Iranian Jews, AD affects between 50 to 100% of the APECED patients. It is the result of an autoimmune adrenalitis for which the symptoms appear when 90% of the adrenal cortex has been destroyed (Napier et al 2012). Overall, APECED accounts for 5 to 10% of all cases of AD, but AD occurrence in children should raise the suspicion of possible APECED. Indeed, the age of onset is usually in childhood, before the age of 15 years. It may be the first endocrine manifestation in 30% of the cases before HP, but it is seldom the very first manifestation of APECED (Perheentupa 2006). AD usually manifests with both glucocorticoid and mineralocorticoid deficiency, but one or the other can precede (Napier et al 2012).

The symptoms are usually fatigue, nausea, anorexia, salt craving, hypotension, weight loss, and pigmentation of the whole skin, skin folds and gingiva. Hypotension, hyponatraemia and hyperkalaemia are signs of mineralocorticoid deficiency while hyponatremia, hypoglycaemia, and more rarely, anaemia, lymphocytosis, and eosinophilia disclose glucocorticoid deficiency.
Adrenal crisis, which is usually triggered by a physical stress such as infection or surgery, is potentially lethal in the case of lack of or delayed diagnosis with vomiting, abdominal pain, and hypotension up to hypovolemic shock (Napier et al 2012). Adrenal glucocorticoid deficiency is detected by a Synacthen test. In evolved stages, basal serum cortisol is low and adrenocorticotropic hormone (ACTH) levels are elevated. Mineralocorticoid deficiency is diagnosed by low aldosterone levels and elevated plasma renin activities (Husebye et al 2009). Abs against 21-hydroxylase (21-OH) are found and confirm the autoimmune origin of the disease. Patients with 21-OH Abs may develop AD over time. In APECED, 2 other adrenal targets are considered as markers of AD: side-chain cleavage enzyme (SCC) and 17alpha-hydroxylase (17α-OH) (Table 2). Abs against at least one of the 3 aforementioned antigens is found in 84% of the patients with AD (Söderbergh et al 2004), but the detection of 21-OH and SCC is enough to suspect AD (Söderbergh et al 2004).

Replacement therapy is similar to that for patients with isolated AD, with oral hydrocortisone with or without a fludrocortisone regimen (Husebye et al 2009, Napier et al 2012).

2.2.2.3. Gonadal insufficiency

Primary hypogonadism affects females more often than males, mainly during the second decade (Proust-Lemoine et al 2012). Ovarian insufficiency (or premature ovarian failure) may occur at an early age, in the adolescent or adult years, resulting in the development of primary amenorrhea, lack of puberty or delayed or arrested puberty and premature menopause. By the age of 30 years, 60% of the women have ovarian failure. Oestrogen levels are low while gonadotropins (follicle-stimulating hormone and luteinizing hormone) are elevated (Dragojević-Dikić et al 2010). Oestrogen replacement is started at pubertal onset to maintain normal feminine development. In the case where Abs are detected, pregnancy should not be delayed. Cryoconservation of oocytes and embryo donation are possible (Husebye et al 2009) and available in Finland.

Testicular insufficiency is rarer among males as it affects only from 12 to 35% of the APECED patients (Ahonen et al 1990, Zlotogora et al 1992). However, the causes are diverse: azoospermia, primary testicular atrophy, or LH and FSH deficiency. AD is often associated. However, in some cases, the cause can be unrelated to APECED (mumps, orchitis, etc.) (Perheentupa 2006).

Abs against SCC were found to be significantly associated with hypogonadism (79% of the patients) (Söderbergh et al 2004) and they can be detected before any manifestations. The role
of 17α-OH Abs is less clear (Söderbergh et al 2004). NALP5 Abs, which are already possibly involved in HP in a context of APECED, were also found to be significantly associated with ovarian insufficiency during APECED (Alimohammadi et al 2008). NALP5 is indeed not only expressed in the PG but also in the ovaries. Almost 70% of the patients with hypogonadism disclose significant NALP5 Abs titres. However, these Abs are neither specific nor sensitive, as 30% of the patients without hypogonadism have those (Alimohammadi et al 2008). Of note, there are several other Abs against ovarian targets potentially involved in immune aggression, such as gonadotropin receptor autoantigens, 3β-hydroxysteroid dehydrogenase Abs, gonadotropin receptors Abs, zona pellucida Abs, and anti-oocyte cytoplasm Abs against MATER (‘maternal antigen that embryos require’) (Dragojević-Dikić et al 2010). To our knowledge, none of these have been studied in APECED women with ovarian failure.

2.2.2.4 Thyroid autoimmunity
Thyroid involvement is reported in 0 to 50% of the cases (Table 1). It is infrequently the first manifestation and occurs mainly during adulthood (mean age 26.5 yrs) (Perheentupa 2006). The clinical presentation and pathology are similar to APECED-non-related hypothyroidism. The latter can be brutal, with inflammation, thyroid gland enlargement and high titres of Abs, or slowly progressive with a slightly altered thyroid gland. Abs against thyroglobulin, microsomes and thyroid peroxidase are found in patients with hypothyroidism. The Abs against thyroid peroxidase are the most sensitive and specific and should be used in clinical practice. Some patients with normal thyroid function may disclose circulating Abs (Perniola et al 2008) as an indication of autoimmune thyroiditis. Hyperthyroidism (Graves’s disease) is not a feature of APECED and very few cases have been reported. However, a transient thyrotoxicosis can occur as the first manifestation of thyroiditis before shifting to hypothyroidism (Perheentupa 2006). This is due to the transient leakage of thyroid hormones to the periphery because of the inflammation.
As the occurrence of thyroid dysfunction is unpredictable, patients should be monitored regularly for TSH levels.

2.2.2.5. Type 1 diabetes
T1D is a rare feature of APECED, reported in 2 to 13% in the cohorts. In Finland, the prevalence seems a bit higher, as stressed by 2 publications: 17% among a cohort of 47
Finnish patients (Tuomi et al 1996) and 18% of 68 Finns (Gylling et al 2000). T1D results from the destruction of pancreatic insulin-producing β-cells. It is characterised by insulitis, islet cell Abs, β-cell-specific T lymphocytes and a restricted set of class II major histocompatibility alleles. In addition, environmental factors may be involved (Boitard 2012). In APECED, T1D develops late, usually around 30 to 40 years of age (Perheentupa 2006). Patients with T1D present Abs against islet cell Ab (ICA), anti-islet antigen 2, a tyrosin phosphatase-like transmembrane protein (IA2), glutamate decarboxylase (GAD), and insulin (IAA) (Van den Driessche et al 2009, Boitard 2012). The detection of Abs remains the only diagnostic marker for autoimmunity in T1D in clinical practice (Boitard 2012). The more Abs, the more at risk a normoglycaemic patient is to develop T1D within the next few years (Van den Driessche et al 2009). Having more than 2 circulating Abs is highly predictive of development of T1D while one or 2 is less predictive. However, the detection of these Abs does not predict the onset of the disease in a normoglycaemic individual (Boitard 2012). Overall, numerous pancreatic autoantigens have been identified as being recognised by T cells in T1D patients: as mentioned above, β-cell antigens (insulin, zinc transporter 8), neuroendocrine antigens (GAD-65, GAD-67, IA-2), ubiquitous HSP-60 and -70 or CD38. Evidence of the involvement of autoantigens like chromogranin or IGRP in human T1D is lacking (Boitard 2012).

In a series of 90 Scandinavian APECED patients, Söderbergh et al found a prevalence of positivity of 37% and 7% for GAD-65 and IA-2, respectively (Söderbergh et al 2004). Only IA-2 was associated with T1D, with a specificity of 87% but a low sensitivity of 33%. In previous studies, Gylling et al also showed that IA-2 and/or IAA have low sensitivity (36%) but high specificity (96 or 100%) with a positive predictive value of 67% for T1D. Approximately 75% of the patients with T1D have GAD-65 Abs (Tuomi et al 1996, Gylling et al 2000), but a third of the patients without T1D also present such Abs (Tuomi et al 1996, Gylling et al 2000) (Table 2).

To conclude, the risk of T1D increases with the number of Abs (Gylling et al 2000). The detection of only GAD65 Abs does not invariably lead to clinical diabetes (Tuomi et al 1996). IA-2 Abs do not predict a rapid onset as APECED patients may have circulating Abs for several years before any functional signs of diabetes. No specific HLA haplotypes were found to confer either susceptibility or protection from T1D (Tuomi et al 1996, Gylling et al 2000).
2.2.2.6. Pituitary failure

Autoimmune hypophysitis leading to hypopituitarism is a rare manifestation of APECED, being diagnosed between the ages of 5 and 15 years. The main manifestation is growth hormone deficiency, reported in 5 to 7% of the cases. Only Meloni et al reported a higher prevalence in their series (23% of the cases) (Meloni et al 2012) (Table 1). Other manifestations such as hypogonadotrophic hypogonadism (Ahonen et al 1990), corticotrophic hormone deficiency (Castells et al 1971) and diabetes insipidus have been reported in anecdotal cases (Hung et al 1984; Scherbaum et al 1986). Multiple central deficiencies are also possible (Arvanitakis et al 1973).

O’Dwyer et al found that 58% (39/67) of Finnish APECED patients disclosed Abs against 49 kDa pituitary enolase (O’Dwyer et al 2007). Eighteen patients displayed reactivity against more than one pituitary cytosolic autoantigen (105 kDa pituitary cytosolic protein n = 2, 60 kDa pituitary cytosolic protein n = 8, 40 kDa n = 10, 45 kDa n = 11). As the authors had patient sera obtained over 2 to 25 years, they could show a seroconversion during life and the stability of the Abs after. However, the presence of such Abs was not related to clinical hypopituitarism. The question of whether such Abs are pathogenic, a simple epiphenomenon or the possible consequence of molecular mimicry and cross-reaction with other Abs such as candidal enolase remains open (O’Dwyer et al 2007).

In a series of 86 patients, Bensing et al found that 49% (48/86) of the patients had Abs against TDRD6 (tudor domain-containing protein 6), a protein mainly expressed in the testis but also at low level in various endocrine glands, including the pituitary glands (Bensing et al 2007). Only 6 patients in this series had GH deficiency (7%) (Bensing et al 2007). However, this Ab was not statistically associated with any APECED manifestation. Bellastella et al (2010) showed that there is no relationship between antipituitary Abs in the serum and anterior pituitary dysfunction. Among the patients who had circulating pituitary Abs, only those with positive immunostaining of only a portion of the pituitary cells and at high titres developed clinical hypopituitarism (Bellastella et al 2010). But their series of patients with hypopituitarism in the context of polyendocrine syndromes concerned only patients with either APS-3 or -4 and no APECED.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Tissue affected</th>
<th>Antigenic target</th>
<th>Antigen role</th>
<th>Prevalence in APECED (component)</th>
<th>Prevalence in patients without APECED</th>
<th>Prevalence in controls without APECED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenocortical failure</td>
<td>Adrenal glands</td>
<td>21-hydroxylase (21-OH) side-chain cleavage enzyme (SCC)</td>
<td>Steroidogenic enzymes in the adrenal cortex</td>
<td>66% (Söderbergh et al 2004)</td>
<td>75% (Söderbergh et al 2004)</td>
<td>61% (Söderbergh et al 2004)</td>
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<tr>
<td></td>
<td></td>
<td>17-α hydroxylase</td>
<td></td>
<td>52% (Söderbergh et al 2004)</td>
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<td></td>
<td></td>
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<td></td>
<td>44% (Söderbergh et al 2004)</td>
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<tr>
<td>Hypoparathyroidism</td>
<td>Parathyroid gland</td>
<td>PG</td>
<td>Parathyroid gland</td>
<td>19% (Gylling et al 2003)</td>
<td>19% (Gylling et al 2003)</td>
<td>0% (Gylling et al 2003)</td>
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<tr>
<td></td>
<td></td>
<td>PTH</td>
<td>Parathormone</td>
<td>3% (Gylling et al 2003)</td>
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<td>3% (Gylling et al 2003)</td>
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<tr>
<td></td>
<td></td>
<td>CaSR</td>
<td>Extracellular domain of the calcium-sensing</td>
<td>0% (Söderbergh et al 2004) – 12% (Gylling et al 2003)</td>
<td>0% (Söderbergh et al 2004) – 12% (Gylling et al 2003)</td>
<td>3-4% (Gylling et al 2003)</td>
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<td></td>
<td></td>
<td></td>
<td>receptor on parathyroid cells</td>
<td></td>
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<td></td>
<td></td>
<td>NALP5</td>
<td>NACHT leucine-rich-repeat protein 5</td>
<td>41% (Alimohammadi et al 2008)</td>
<td>49% (Alimohammadi et al 2008)</td>
<td>0% (Alimohammadi et al 2008)</td>
</tr>
<tr>
<td>Hypogonadism</td>
<td>Ovaries and testis</td>
<td>Side-chain cleavage enzyme (SCC)</td>
<td>Steroidogenic enzymes in the gonads</td>
<td>52% (Söderbergh et al 2004)</td>
<td>79% (Söderbergh et al 2004)</td>
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<td></td>
<td></td>
<td>17-α hydroxylase</td>
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<td>44% (Söderbergh et al 2004)</td>
<td>57% (Söderbergh et al 2004)</td>
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<tr>
<td></td>
<td></td>
<td>NALP5</td>
<td>NACHT leucine-rich-repeat protein 5</td>
<td>41% (Alimohammadi et al 2008)</td>
<td>68% (Alimohammadi et al 2008)</td>
<td>0% (Alimohammadi et al 2008)</td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>Endocrine pancreas</td>
<td>GAD-65</td>
<td>Glutamate decarboxylase</td>
<td>37% (Söderbergh et al 2004) - 43% (Gylling et al 2000)</td>
<td>72.7% (Gylling et al 2000)</td>
<td>nd</td>
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<tr>
<td></td>
<td></td>
<td>ICA</td>
<td>Islet cells antibodies</td>
<td>34% (Gylling et al 2000)</td>
<td>54% (Gylling et al 2000)</td>
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<tr>
<td></td>
<td></td>
<td>IA2</td>
<td>Tyrosine phosphatase-like transmembrane protein</td>
<td>7% (Söderbergh et al 2004) - 10% (Gylling 2000 et al)</td>
<td>33% (Söderbergh et al 2004) - 36% (Gylling et al 2000)</td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>Organ/Region</td>
<td>Gene/Protein/Pathway</td>
<td>Reference 1</td>
<td>Reference 2</td>
<td>Reference 3</td>
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<tr>
<td><strong>Hypothyroidism</strong></td>
<td>Thyroid</td>
<td>TPO, Tg, Thyroid peroxidase</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td></td>
<td></td>
<td>Thyroglobulin, Potassium channel regulator</td>
<td>8% (Alimohammadi et al 2009)</td>
<td>88% (Alimohammadi et al 2009)</td>
<td>nd**</td>
<td></td>
</tr>
<tr>
<td><strong>Pulmonary disease</strong></td>
<td>Lung</td>
<td>KCNRG (terminal bronchioles)</td>
<td>8% (Alimohammadi et al 2009)</td>
<td>88% (Alimohammadi et al 2009)</td>
<td>nd**</td>
<td></td>
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<tr>
<td><strong>Atrophic gastritis</strong></td>
<td>Gastric mucosa</td>
<td>Parietal cell, Intrinsic factor</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td></td>
<td></td>
<td>Sodium-potassium channel molecule of the parietal cells</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td></td>
<td></td>
<td>Fixation of B12 vitamin</td>
<td>47% (Ekwall et al 1998)</td>
<td>89% (Ekwall et al 1998)</td>
<td>0% (Ekwall et al 1998, Dal Pra 2004)</td>
<td></td>
</tr>
<tr>
<td><strong>Gastrointestinal dysfunction</strong></td>
<td>Gastrointestinal tract</td>
<td>TPH-1, Histidine decarboxylase</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td></td>
<td></td>
<td>Tryptophan hydroxylase</td>
<td>47% (Ekwall et al 1998)</td>
<td>92% (Scarpa et al 2013)</td>
<td>58% (Sköldberg et al 2003)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Pyridoxal phosphate-dependent enzyme</td>
<td>37% (Sköldberg et al 2003)</td>
<td>92% (Söderbergh et al 2004)</td>
<td>51% (Hasebye et al 1997, Söderbergh et al 2004)</td>
<td></td>
</tr>
<tr>
<td><strong>Autoimmune hepatitis</strong></td>
<td>Liver</td>
<td>Aromatic L-amino acid decarboxylase (AADC)</td>
<td>51% (Hasebye et al 1997, Söderbergh et al 2004)</td>
<td>92% (Söderbergh et al 2004)</td>
<td>92% (Söderbergh et al 2004)</td>
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<tr>
<td></td>
<td></td>
<td>TPH</td>
<td>45% (Söderbergh et al 2004)</td>
<td>92% (Söderbergh et al 2004)</td>
<td>nd</td>
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<tr>
<td></td>
<td></td>
<td>Tryptophan hydroxylase</td>
<td>47% (Ekwall et al 1998)</td>
<td>92% (Söderbergh et al 2004)</td>
<td>nd</td>
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<td></td>
<td></td>
<td>7.9% (Alimohammadi et al 2009)</td>
<td>92% (Söderbergh et al 2004)</td>
<td>92% (Söderbergh et al 2004)</td>
<td>nd</td>
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<tr>
<td></td>
<td></td>
<td>ANA</td>
<td>12.5% (Obermayer-Straub et al 2001)</td>
<td>5.9-9% (Obermayer-Straub et al 2001)</td>
<td>0% (Obermayer-Straub et al 2001)</td>
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<tr>
<td></td>
<td></td>
<td>SMA</td>
<td>nd</td>
<td>0%</td>
<td>6-10%</td>
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<tr>
<td></td>
<td></td>
<td>SLA/LP</td>
<td>nd</td>
<td>0%</td>
<td>0%</td>
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<tr>
<td></td>
<td></td>
<td>Antinuclear antibodies</td>
<td>nd</td>
<td>12.5% (Obermayer-Straub et al 2001)</td>
<td>5.9-9% (Obermayer-Straub et al 2001)</td>
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<tr>
<td></td>
<td></td>
<td>Smooth muscle antibodies</td>
<td>nd</td>
<td>0%</td>
<td>6-10%</td>
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<td></td>
<td></td>
<td>Soluble liver Antigen/liver pancreas antigen</td>
<td>nd</td>
<td>0%</td>
<td>0%</td>
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<tr>
<td></td>
<td></td>
<td>Liver kidney microsome 1</td>
<td>8% (Obermayer-Straub et al 2001)</td>
<td>50% (Obermayer-Straub et al 2001)</td>
<td>50% (Obermayer-Straub et al 2001)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Cytochromes expressed mainly in the liver</td>
<td>nd</td>
<td>6% (Obermayer-Straub et al 2001)</td>
<td>35% (Söderbergh et al 2004)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>CYP1A1, CYP1A2</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<td></td>
<td>CYP2A6</td>
<td>CYP2B6</td>
<td>Histidine decarboxylase</td>
<td>Pyridoxal phosphate-dependent enzyme</td>
<td>al 2001)-8% (Söderberg et al 2004)</td>
<td>15.6% (Obermayer-Straub et al 2001)</td>
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<tr>
<td><strong>Vitiligo</strong></td>
<td>Skin (melanocytes)</td>
<td>Aromatic L-amino acid Decarboxylase (AADC)</td>
<td>Tyrosine hydroxylase (melanocytes)</td>
<td>SOX9 (cross-reaction with SOX10 autoantibodies)</td>
<td>SOX10 (melanocytes)</td>
<td>Serotonin and dopamine</td>
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<tr>
<td><strong>Alopecia areata</strong></td>
<td>Hair follicle</td>
<td>Tyrosine hydroxylase (keratinocyte)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Hypopituitarism</strong></td>
<td>Pituitary gland</td>
<td>49 kDa enolase</td>
<td>40 kDa</td>
<td>45 kDa</td>
<td>60 kDa</td>
<td>105 kDa</td>
</tr>
</tbody>
</table>
2.2.3. Nonendocrine manifestations

2.2.3.1. Alopecia areata

Alopecia areata (AA) is an autoimmune disease characterised as asymptomatic, well-defined patches of non-scarring hair loss with no epidermal changes. It affects both males and females and occurs in 0.1 to 0.2% of the general population (Alkhalifah et al 2010). AA mainly affects the scalp in 90% of the cases, but can occur on virtually any hair-bearing area. The alopecia is defined as *patchy, totalis* (loss of 100% of scalp hair) or *universalis* (loss of all scalp and body hair) (Alkhalifah et al 2010). Nail involvement is observed in up to 66% of the patients, mainly nail pitting, but other anomalies have been described. The nail abnormalities can occur before, during or after the hair loss and can be associated with more extensive hair loss. In the active phase, the histopathology of the AA area shows a peribulbar lymphocytic infiltrate on anagen follicles with oedema, apoptosis, necrosis, and foreign-body giant cells around the affected follicles (Alkhalifah et al 2010). AA prevalence ranges from 13 to 53% and increases with age, but the prognosis is no different from that of non-APECED AA. Patients experience single or multiple recurrences of AA during their lifetimes (Collins et al 2006). AA is very rarely one of the first manifestations of APECED (Perheentupa 2006). Although AA is not lethal, it may impair the quality of life (QoL) of patients by affecting their body image.

AA appears to be a multifactorial disorder related to autoimmune targeting against the hair follicle. Mouse models indicate that CD4+ and CD8+ T cell subsets are involved in the hair loss promotion (Alkhalifah et al 2010). Other factors include genetic factors like DQB1*03 and DRB1*1104 (Alkhalifah et al 2010), as well as environmental factors like stress. Halonen et al found a significant association with DRB1*04-DQB1*302 in APECED patients (Halonen et al 2002). Lastly, intestinal malabsorption during APECED leads to underlying hypoalbuminaemia and multiple vitamin deficiencies that can contribute to hair loss (Puzenat et al 2010).

Abs against the hair follicle matrix, cuticle and cortex keratinocytes have been observed to be significantly associated with total AA in APECED patients, while patients with patchy AA did not have such Abs (Hedstrand et al 1999). The same team then found a significant correlation between Abs against tyrosine hydroxylase (TH) and the occurrence of AA using the sera of 94 patients (including 29 patients with AA). Forty-four per cent of the patients presented such Abs, but the prevalence was higher in the group with AA (62%) compared with those without (35%, p = 0.02) (Hedstrand et al 2000). TH is involved in the conversion of tyrosine to L-dopa, which can be found in human hair follicles and cultured keratinocytes.
The authors also observed a mildly reduced activity of the enzyme with the patients’ serum, even though normal controls also showed this inhibition. The authors failed to find any specific TH staining on human scalp using either TH Abs or APECED patients’ serum. They hypothesised a possible autoimmune attack on the nerve-endings close to the hair follicles. A more recent study failed to show any association between TH Abs and AA (Bratland et al 2013). The role of such Abs in the pathophysiology of AA is speculative.

2.2.3.2. Vitiligo
Vitiligo is the most common depigmenting disorder, with a prevalence of approximately 0.5% in the world population. The hallmark is a loss of epidermal melanocytes. The initial cause of nonsegmental vitiligo is still debated but appears to involve immunologic factors, oxidative stress, or a sympathetic neurogenic disturbance (Taïeb et al 2009). In vitiligo-associated multiple autoimmune disease patients, the variants in the gene encoding NACHT leucine-rich-repeat protein 1, or NALP1, were recently identified (Jin et al 2007). In APECED, the prevalence of vitiligo ranges from 8 to 33 % of the patients (Figure 5). It may show a patchy distribution, affect hair follicle areas (poliosis) and be responsible for the halo-naevi (Sutton) phenomenon (Collins et al 2006). Vitiligo is very rarely the first manifestation of APECED (Perheentupa 2006). It can be associated with AA and, similar to AA, it may impair the QoL of APECED patients.

Anti-melanocyte Abs have been reported in APECED patients (Howanitz et al 1981). Abs against aromatic L-amino acid decarboxylase (AADC) have been found with vitiligo in patients with APECED. The clinical relevance of these Abs is uncertain. Abs against TH have been found to be more prevalent among patients with non-APECED nonsegmental vitiligo (Kemp et al 2011), especially when the vitiligo is active. Among series of APECED patients, no association with vitiligo has been demonstrated (Hedstrand et al 2000, Bratland et al 2013). Kemp et al suggested that, given that TH is an intracellular enzyme, TH Abs probably arise by exposure of the antigen following damage to melanocytes by either oxidative stress or an autoimmune attack on the melanocytes. It is unlikely that the Abs have any effect on the function of the enzyme. They may serve as markers for the melanocyte disruption and/or a cellular response to TH (Kemp et al 2011).

Transcription factors SOX9 and SOX10 have been shown to be antigen targets of Abs in APECED (Hedstrand et al 2001). They were found in 47% and 63% of the patients with vitiligo, respectively. SOX9 and 10 are involved in the embryonic development of various
organs. SOX9 would be a target by cross-reactivity while SOX10, which is expressed in melanocytes, would be the main autoantigen.

Lastly, Husebye et al reported that 80% of the APECED patients with vitiligo had Abs against AADC compared with those without vitiligo (43%, p = 0.021) (Husebye et al 1997). However, AADC is mainly expressed in the central nervous system and the liver, but not in the skin. The authors speculated that this could be due to a possible cross-reaction with tyrosinase. Nevertheless, they failed to report whether the patients having TH Abs also had AADC Abs. In a small Italian study, Dal Pra et al (2004) found results similar to those of Husebye et al (1997). However, they mentioned that 6 out of 8 (75%) patients with vitiligo and/or chronic hepatitis had Abs against AADC. Among the 6 patients for which data are available, it appears that 3 had vitiligo and 3 others autoimmune hepatitis. The control group in this study consisted of only 8 patients with vitiligo. Söderbergh et al found AADC Abs in 12 patients with Addison’s disease without APECED and only one of these patients had vitiligo (8%) (Söderbergh et al 2000).

**Figure 5. Vitiligo universalis in a woman with APECED**
2.2.3.3. Nail dystrophy

Nail dystrophy is a bothersome symptom that is often reported in patient series. However, the aetiopathogenesis is far from clear. In a series of 18 Irish patients examined by a dermatologist, none had nail pitting or distinctive nail dystrophy (Collins et al 2006). All nail changes were related to candidal infection (Figure 4). Nail infection is the most common cause of nail dystrophy. According to Perheentupa, 22 to 50% of the Finnish APECED patients may have had nail pitting without positive candidiasis on fungal culture (Ahonen et al 1990, Perheentupa 2006). ‘Nail dystrophy’ encompasses a large number of nail symptoms and it is not clear whether the authors had ever considered other possible causes for nail pitting, such as alopecia areata (LeBoeuf et al 2007, Puzenat et al 2010).

2.2.3.4. Gastrointestinal manifestations (autoimmune hepatitis excluded)

Chronic atrophic gastritis, with pernicious anaemia or not, affects 7 to 33% of the patients (Table 1). Chronic atrophic gastritis is an autoimmune disease affecting the gastric parietal cells and intrinsic factor (IF). It eventually leads to gastric atrophy, which in turn is associated with vitamin B12 deficiency and pernicious anaemia (PA, Biermer disease) (Toh et al 1997). These patients have Abs against the sodium-potassium channel molecule of the parietal cells in the corpus and fundus of the stomach (parietal cell Abs, PCA). The process starts with superficial gastritis, characterised by lymphocytic infiltration below the epithelium, leading to a diffuse gastritis with lymphocytic infiltration extending to specific glands and to glandular destruction (atrophic gastritis). As hydrochloric acid and pepsinogen-producing cells disappear, so does the secretion of gastric IF. PA is thus the end stage of gastric immunologic destruction, caused not only by the lack of IF, but also by Abs recognising the vitamin B12-binding protein and preventing the subsequent translocation of vitamin B12 from the ileum to circulation (Toh et al 1997). The circulating Abs reacting with parietal cells and IF may be detected before any clinical symptoms (Perheentupa 2006, Betterle et al 1998). PA usually develops during early adulthood (Betterle et al 1998), although sometimes even during childhood (Oliva-Hemker et al 2006); this differs from solitary PA, which is usually diagnosed in the sixth decade (Toh et al 1997).

The clinical symptoms are those of a chronic lack of vitamin B12. They may include atrophic glossitis with a smooth red beef tongue, diarrhoea, malabsorption, peripheral neuropathy, degeneration of the spinal cord, and personality defects. The red cell count is low along with macrocytosis, leukopenia, thrombocytopenia, and pancytopenia. Over the long term, PA may lead to gastric carcinoid tumours and adenocarcinoma, and therefore regular screening by
gastric fibroscopy should be instituted. Treatment includes only intramuscular injections of vitamin B12 every 1 to 3 months and regular fibroscopy for gastric carcinoma screening (Kluger et al 2013).

In contrast to the main organ-specific autoimmune symptoms of APECED, the GI symptoms and their underlying pathogenesis are poorly understood. The prevalence of GI symptoms (excluding hepatitis) is roughly estimated at up to 25%. Cohorts and review articles usually describe GI manifestations as ‘diarrhoea’, ‘constipation’ and ‘malabsorption’ (Kluger et al 2013). On the other hand, isolated case reports and small series depict severe intestinal involvement in children leading to malabsorption, multiple nutritional deficiencies, growth impairment (Ward et al 1999) and possible death (Perheentupa 2006). Studies of GI function with intestinal biopsies are rare. GI symptoms may be the first manifestation of APECED and may be have various causes and, therefore, effective treatment will vary accordingly.

Chronic diarrhoea is the first manifestation of APECED in 5% of the patients and severe constipation in 2% (Perheentupa 2006), while malabsorption varies from 9 to 26%, depending on the series (Kluger et al 2013). Approximately half of the Sardinian patients disclose periodic intestinal dysfunction (Meloni et al 2012). The precise identification of the cause of intestinal symptoms in association with APECED, i.e. abdominal pain, bloating, and recurrent, watery or fatty (steatorrhoea) diarrhoea or constipation, constitutes a real challenge for the physician as the cause is far from obvious. Several disorders may co-occur or may follow each other over the lifetime. Therefore, each new episode may either be related to a previously identified cause or to a new one that has not yet been diagnosed. Reports on APECED patient cohorts often lack precision on this aspect and various criteria have been chosen to define GI manifestations. Authors may also evaluate only one aspect of the GI symptoms. For instance, Ahonen et al focused only on malabsorption defined as floating stools and increased faecal fat excretion (Ahonen et al 1990); therefore, other causes of diarrhoea in APECED patients may have been overlooked. Often, the precise cause of malabsorption has not been analysed (Ahonen et al 1990, Zaidi et al 2009) or may be multifactorial (Proust-Lemoine et al 2010). The main cause of lower GI tract dysfunction includes hypocalcaemia whether it is induced by HP or malabsorption of D vitamin, intestinal infection or autoimmune enteropathy with circulating Abs against tryptophan hydroxylase 1 (TPH-1) (Kluger et al 2013). Moreover, oral calcium intake is responsible for constipation. In the late 90s, Ekwall et al identified TPH as an intestinal autoantigen in APECED patients (Ekwall et al 1998). TPH-1 is expressed in serotonin-producing cells in the central nervous
system and intestine. In their series of 80 patients, they were able to relate ‘GI symptoms’ to the presence of circulating TPH-1 Abs and also to the total absence of enterochromaffin cells in the mucosa of the small bowel. TPH-1 Abs were detected in 90% of APECED patients with GI symptoms (Ekwall et al 1998, Scarpa et al 2013) and sometimes preceded the clinical symptoms. Other studies confirmed this association (Posovszky et al 2012, Scarpa et al 2013). Nevertheless, up to 34 to 50% of the patients with such Abs do not have any GI symptoms (Ekwall et al 1998, Scarpa et al 2013). Several studies have shown a link between the absence of EECs and the occurrence of GI manifestations (Ekwall et al 1998, Posovsky et al 2012, Scarpa et al 2013) (Figure 6).

In addition, Sköldberg et al (Sköldberg et al 2003) identified Abs against histidine decarboxylase expressed by EEC-like cells in the gastric mucosa. On the other hand, celiac disease remains exceedingly rare among APECED patients (Betterle et al 1998), while to the best of our knowledge inflammatory bowel disorders have not been reported. An exhaustive list of GI diseases associated with APECED is summarised in Table 3.

**Figure 6. The dopamine and serotonin pathway**

![Dopamine and Serotonin Pathway Diagram](image)

- **TH**: Tyrosine Hydroxylase
- **AAAD**: Amino Acid Decarboxylase
- **TPH**: Tryptophan Hydroxylase
- **AADC**: Amino Acid Decarboxylase

AADC, L-Amino-Acid Decarboxylase
TH: Tyrosine Hydroxylase
TPH: Tryptophan Hydroxylase
Table 3. A review of gastrointestinal manifestations in APECED (Kluger et al 2013)

<table>
<thead>
<tr>
<th>Organs</th>
<th>Conditions</th>
</tr>
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| **Oesophagus** | Candida oesophagitis  
|              | Oesophageal carcinoma                                                     |
| **Stomach**  | Pernicious anaemia (Biermer’s anaemia)  
|              | Autoimmune gastric atrophy  
|              | Gastric adenocarcinoma  
|              | Gastric carcinoid tumour                                                  |
| **Bowel**    | Primary hypoparathyroid-induced hypocalcaemia  
|              | Secondary hypocalcaemia due to malabsorption of vitamin D  
|              | Intestinal infection: bacterial overgrowth, Candida infection, Giardiasis lamblia, Clostridium difficile  
|              | Exocrine pancreatic insufficiency (related to): hypocalcaemia, diabetes, celiac disease, cystic fibrosis, Intestinal megaloblastosis due to vitamin B12 deficiency, autoimmune pancreatitis (?)  
|              | Autoimmune enteropathy  
|              | Lactase nonpersistence / deficiency  
|              | Intestinal lymphangiectasia  
|              | Celiac disease  
|              | Functional disease                                                       |
| **Autoimmune hepatitis** |  
| **Cholelithiasis** | (?): no cases reported in APECED thus far, but considered according to the pancreatitis induced in the knock out Aire-/- mouse model (Ramsey et al 2002)  

2.2.3.5. Hepatitis

Autoimmune hepatitis (AIH) affects 8 to 27% of APECED patients (Betterle et al 1998, Obermayer-Straub et al 2001). Its spectrum ranges from asymptomatic and/or self-limited cytolysis that may fluctuate over time to fulminant hepatitis with liver failure and potential lethal outcome (Obermay-Straub et al 2001). Interestingly, the autoimmune targets are different from those of the usual AIH type 1 and type 2. Usually, in type 1, patients disclose antinuclear Abs and/or smooth muscle Abs and soluble liver antigen/liver pancreas antigen (SLA/LP), while liver-kidney microsomal Abs (LKM-1 and LKM3) and liver cytosolic antigen (LC1) are the biological signs of AIH type 2. APECED patients have a different variety of Abs mainly against cytochrome P4501A2 (CYP1A2), cytochrome P4502A6 (CYP2A6) and aromatic L-amino acid decarboxylase (AADC) (Husebye et al 1997, Dal Pra et al 2004). According to Obermayer et al, anti-CYP1A2 is likely to be specific for APECED-associated AIH; however, half of the patients with hepatitis do not have this Ab (Obermayer-Straub et al 2001). CYP2A6 Abs would be more suitable for a diagnosis of APECED rather
than AIH. In any case, the serological follow-up of an APECED patient with hepatitis clearly showed an evolution in the Ab types and titres over time, with the appearance of new Abs against cytochrome P450 (Obermayer-Straub et al 2001). Thus, anti-CYP1A2 Abs is likely to parallel the severity of hepatitis. Other Abs of interest have been found to be associated with hepatitis, but with sometimes conflicting results. For instance, Söderbergh et al found an association with TPH Abs (tryptophan hydroxylase) but failed to find a statistical association with AADC Abs, despite being present in 92% of the patients with hepatitis (Söderbergh et al 2004). TPH is not expressed in the liver (Söderbergh et al 2004). In the study by Bratland et al on 48 Scandinavian patients, no link between hepatitis (20% of the patients) and TPH1 or 2 was found (Bratland et al 2013).

2.2.3.6. Hyposplenia and asplenia

The spontaneous regression of the spleen leading to functional asplenia is considered to be an infrequent feature of APECED (Parker et al 1990, Pollak et al 2009). It occurs in 9% of the affected 5- to 15-year-olds and in 19% of those over 17 years old (Perheentupa 2006), but its prevalence is otherwise poorly documented. Abdominal ultrasound and CT scans can show the progressive regression in size of the spleen to complete atrophy over the course of patient follow-up (Starzyk et al 2001). Blood anomalies such as thrombocytosis or Howell-Jolly bodies on peripheral blood smears raise the suspicion of asplenia. Unmanaged asplenia predisposes to potentially life-threatening fulminant S. pneumoniae or H.influenza sepsis. Management includes patient education, regular self-administered oral antibiotic prophylaxis and updated vaccination against S. pneumoniae and H.influenza (Husebye et al 2009).

The mechanisms of spleen reduction in APECED are not known. Aburano et al showed that the sequestration function remained while the reticuloendothelial system was impaired in an APECED patient (Aburano et al 1997). Immune destruction of the spleen has been suggested but has not been confirmed (Friedman et al 1991).

2.2.3.7. Kidney manifestations

Renal impairment in APECED is rarely mentioned in large series of patients. Only a few severe paediatric cases of renal failure leading to haemodialysis and transplantation with sometimes acute and chronic rejection have been reported (Hannigan et al 1996, Ulinski et al 2006, Al-owain et al 2010). Betterle et al mentioned the death of an 18-year-old patient from renal failure among their 41 Italian patients (1/41, 2.4%) without further detail (Betterle et al
Renal diseases associated with APECED include specific autoimmune tubulointerstitial nephritis (TIN) that can manifest by renal tubular acidosis, nephrocalcinosis, and chronic renal failure requiring kidney transplantation. Perheentupa estimated that up to 9% (8/92) of patients develop TIN (Perheentupa 2006). The suspected pathogenic mechanism is immunity against tubular cells. Nephrocalcinosis may also be related to hypoparathyroidism. In addition, T1D, hypertension and drug-induced nephrotoxicity, such as from cyclosporine to control the severe autoimmune manifestations of APECED, can be additional causes of renal failure.

2.2.3.8. Lung manifestations

Respiratory-related symptoms are quite uncommon in APECED. In a large series of 110 European patients, Alimohammadi et al found only 7 with respiratory symptoms (6.4%) (Alimohammadi et al 2009). The symptoms included recurrent lower respiratory infections, obstructive respiratory asthma-like symptoms, chronic cough, dyspnoea, thoracic pain, and airways hyperresponsiveness (Alimohammadi et al 2009). The disease is progressive with worsening of the symptoms and chronic respiratory failure that in rare cases can be lethal (De Luca et al 2008, Alimohammadi et al 2009). Some patients may develop lymphocytic bronchiolitis with interstitial pneumonitis (Popler et al 2012). Only one case of rapidly lethal primary pulmonary hypertension with no underlying pneumopathy was reported in a 30-year-old APECED woman in Poland (Korniscewski et al 2003). However, a possible fortuitous association cannot be ruled out.

Recently, the efficacy of anti-CD20 monoclonal Abs in a 9-year old patient with organising pneumonia and lymphocytic bronchiolitis was reported (Popler et al 2012). Abs against a potassium channel regulator named KCNRG has been shown to be present in 88% of the patients with self-reported respiratory symptoms, while less than 1% of the APECED patients with no respiratory complaints had this Ab (Alimohammadi et al 2009). KCNRG is mainly expressed in the lung in the epithelial cells of terminal bronchioles. A pathogenic link between the Abs and the symptoms is still open and KCNRG Abs may merely reflect the autoimmune aggression of the terminal bronchioles. Besides, respiratory symptoms may be related to other causes such as unrelated asthma, tobacco-related chronic bronchitis, lung infection or cystic fibrosis. It could be useful in the evaluation of an APECED patient with respiratory symptoms to distinguish a specific autoimmune pneumopathy from other
conditions and to suggest the use of immunosuppressive therapies (Alimohammadi et al 2009; Popler et al 2012).

### 2.2.3.9. Enamel dysplasia

Defective dental enamel formation of the permanent teeth is frequent and affects from 25 to 82% of the patients (Mylärniemi et al 1978; Perheentupa 2006). The pattern of enamel involvement differs from patient to patient, ranging from horizontal hypoplastic patches/pitted enamel surface of variable width alternating with zones of well-formed enamel, to complete hypoplastic enamel. Usually all permanent teeth are hypoplastic but sometimes only premolars and second molars are affected. Such hypoplasia exposes to further damage and caries. Dental care is necessary and should be performed promptly. The occurrence of enamel hypoplasia is independent of hypocalcaemia and hypoparathyroidism (Mylärniemi et al 1978). Some authors have suggested the possibility of immune aggression against ameloblasts or against one of their proteins secreted in the extracellular matrix as potential targets, but this hypothesis has never been explored (Pavlic et al 2009). Interestingly, Bratland et al found also a statistical association between TH Abs and enamel dysplasia in a Norwegian subgroup of patients with APECED, but these results need to be confirmed by larger series (Bratland et al 2013).

### 2.2.3.10. Eye manifestations

Considered as one of the ‘ectodermal’ manifestations of APECED (Husebye et al 2009), ocular manifestations are also one of the most disabling as they can lead to severe visual impairment and even complete blindness. Virtually any of the ocular structures can be affected, from the eyelashes, the sclera and the cornea to the retina and even the optic nerve. Ocular symptoms usually appear at a mean age of 5 years but with extreme variations from early childhood to adulthood (Perheentupa 2006). They are the first manifestation of APECED in only 4% of the cases (Perheentupa 2006).

The main manifestation is keratitis (or keratoconjunctivitis), whose prevalence is about 25% (Gass 1962, Wagman et al 1987, Merenmies et al 2000, Perheentupa 2006). Higher prevalences of 36% (Chang et al 2006) and even up to 52% have been reported in older reviews (Gass 1962). The symptoms are those of any keratoconjunctivitis, including photophobia, blepharospasm and lacrimation, conjunctival redness, sensation of sand in the eyes and reduced lacrimation. Chronic evolution leads to subepithelial scarring, perforation
(Yeh et al 2009), nodulus, leukoma and deep vascularisation. Keratitis is not considered to be of fungal origin (Merenmies et al 2000).

An autoimmune mechanism has been suspected to be responsible for the immune aggression of the eye. Local treatment relies on vitamin A and immunosuppressive treatments (corticosteroid (Merenmies et al 2000), tacrolimus or cyclosporine (Chang et al 2006)). Corneal transplant may be necessary to restore useful sight (Tarkkanen et al 2001). Low corticosteroid maintenance is necessary although it does not always prevent recurrence (Merenmies et al 2000).

The mechanism of the keratitis is poorly understood. An autoimmune attack of the corneal/conjunctival epithelium has been postulated. Yeh et al recently showed that Aire-deficient mice are prone to develop an infiltration of the conjunctival epithelium by CD4, CD8 and CD11b+ and the Meibomian glands under dessicating stress with an increase in CD4, CD8, CD11b+ and CD45 cells. In addition, conjunctival goblet cell density was lower in Aire -/- mice (Yeh et al 2009). Goblet cells are mucin-producing cells that are critical to tear film and ocular surface homeostasis, loss or reduction, associated with a severe ocular surface phenotype. The Meibomian glands are involved in the production of the hydrophobic lipid layer of the tear film, and disruption of the Meibomian function can also contribute to chronic ocular surface disease. Recently, Laakso et al reported that several Finnish patients developed flares of keratoconjunctivitis after a cutaneous Mantoux reaction was performed, stressing that keratoconjunctivitis is of autoimmune origin and has nothing to do with an ectodermal ‘dystrophy’ (Laakso et al 2014). DeVoss et al observed Abs against proteins present in and around the ducts upon indirect immunofluorescence and identified an 18kDa protein, odorant-binding protein 1a (OBP1a), a putative pheromone transporter belonging to the lipocalin family, as a target of the Abs present in the tear fluid and the lacrimal glands (DeVoss et al 2010). Recently, another hypothesis was raised with a possible deficiency among the limbic stem cells (Shah et al 2007).

Other ocular manifestations of APECED are summarised in Table 4. Briefly, reduced tear production as assessed by Schirmer’s tests may develop even without any keratitis (Chang et al 2006). The association with Sjögren’s syndrome has been reported in a few patients (Betterle et al 1998). The development of cataract (18%) (Chang et al 2006) has been related to hypocalcaemia (Rajendram et al 2003). The retina and the optic nerve can be affected in APECED (Orlova et al 2010). Autoimmune retinopathies are a rare group of heterogeneous ocular diseases characterised by rapidly progressive, painless, unilateral or bilateral visual loss.
A high number of retinal Abs has been identified as potential biomarkers but there are no validated standardised methodological approaches (Braithwaite et al 2014). In APECED, Abs against the retina and the optic nerve have been detected (Wood et al 1991). Anti-retinal Abs were found in some patients with retinopathy (enolase, GAPDH, and other undefined targets identified by western blots) necessitating oral immunosuppressive therapies. There is a high number of other Abs against recoverin, enolase, rod transfucin, and numerous other targets against photoreceptors, Müller cells, cones and rods that are possible targets. However, none has ever been explored in APECED, and the direct pathogenicity of such Abs is debatable because of the blood-retina barrier (Braithwaite et al 2014).

Table 4. Overview of the ocular manifestations of APECED

<table>
<thead>
<tr>
<th>Specific manifestations</th>
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<tbody>
<tr>
<td>Keratoconjunctivitis</td>
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<tr>
<td>Dry eyes</td>
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<tr>
<td>Iridocyclitis</td>
</tr>
<tr>
<td>Retinitis pigmentosa (Orlova et al 2010)</td>
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<tr>
<td>Optic atrophy</td>
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<tr>
<th>Manifestations related to other autoimmune conditions during APECED</th>
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<tbody>
<tr>
<td>Cortical lenticular opacities/cataract secondary to hypoparathyroidism</td>
</tr>
<tr>
<td>Diabetic retinopathy</td>
</tr>
<tr>
<td>Hypotrichosis</td>
</tr>
<tr>
<td>Loss of eyelashes and/eyebrows due to alopecia areata</td>
</tr>
<tr>
<td>Sjögren’s syndrome</td>
</tr>
<tr>
<td>Meibomian gland dysfunction (?)</td>
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<table>
<thead>
<tr>
<th>Other</th>
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<tbody>
<tr>
<td>Myopia</td>
</tr>
<tr>
<td>Retinal detachment</td>
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<tr>
<td>Hypertrichosis</td>
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</table>

2.3. Genetics: AIRE Mutations

2.3.1. Autoimmune regulator gene

APECED is caused by loss-of-function mutations in the Autoimmune Regulator gene (AIRE), identified in 1997 in the q22 region of chromosome 22 (Nagamine et al 1997; Finnish-German APECED Consortium, 1997). The gene is approximately 13 kb in length and the coding sequence is composed of 14 exons coding for a 58 kDa protein of 545 amino acids (Nagamine et al 1997; Finnish-German APECED Consortium, 1997). AIRE presents domains that are characteristic of transcription regulators and chromatin-binding proteins. It is similar
to SP100-family proteins that are involved in DNA binding, modulation of transcription and repression mechanisms (Peterson et al 2008).

AIRE contains

- an N-terminal six-helix structure caspase-recruitment domain (CARD) involved in the **oligomerisation of AIRE**
- a SAND domain that is a **putative DNA-binding domain**
- two plant homeodomains (PHD1 and PHD2) fingers involved in **protein-protein interaction**. PHD1 has been shown to mediate the binding of AIRE to non methylated histone H3
- a prolin-rich region (PRR) involved in **transcription**
- The N-terminus also contains a nuclear localisation signal (NLS) for nuclear import of the protein, while the C-terminus is important for transcriptional activation (Arstila et al 2013) (Figure 7).

Figure 7. The structure of AIRE protein (adapted from Arstila et al 2013)

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2.3.2. AIRE mutations and their implications in the phenotype

To date, over 60 mutations (nonsense, deletion or missense) have been localised in the AIRE gene of APECED patients (Fierabracci 2011, Arstila et al 2013). It has been rather difficult to draw definitive conclusions on genotype-phenotype correlation, since the number of
mutations is high but the number of carriers for each mutation is low. In Finland, 83% of the patients carry the so-called Finn major mutation R257X (c.769C>T) in exon 6, indicating a founder effect in this geographically isolated population (Heino et al 2001) (Figure 3). The mutation is not, however, restricted to the Finns as it is the most common mutation worldwide. The other frequent mutation 967-979del13bp, a 13-base pair deletion (c.967-979del) in exon 8, is more frequent among North American and British patients (Heino et al 2001). Except for one family in Italy (Cetani et al 2001), there is no dominant mutation/dominant fashion transmission reported thus far, meaning the patient must be a homozygous or heterozygous composite for the AIRE mutation to develop APECED.

Despite the growing number of reported APECED-causing mutations, the phenotypic variations among APECED patients cannot be explained by allelic variation in the AIRE gene. The typical example comes from the phenotypic variation in APECED among Finnish patients, despite most of them being homozygous R257X. Variation in phenotype between siblings indicates that other genetic or environmental factors determine the clinical severity of the disease (Heino et al 2001, Arstila et al 2013). Two clinical genotype-phenotypes are nevertheless recognised: i) patients who have the R257X mutation do develop CMC (Halonen et al 2002) and ii) and the Iranian Jews carrying the Y85C mutations have a milder form of APECED. Compared with the Finns, the Iranian Jews do not present keratopathy or CMC (Zlotogora et al 1992, Heino et al 2001). Other genes, such as the HLA class II alleles (Halonen et al 2002), the gender of the patients and additional environmental factors may influence the clinical phenotype of APECED.

### 2.3.3. AIRE functions

AIRE has been proposed to function as a nonconventional transcription factor with a certain number of functions: DNA-binding, assembly into a large DNA-binding complex, induction of elongation by recruiting the positive transcription elongation factor b (pTEFb), direct binding to histone H3, and recruitment of genes to the nuclear matrix. Given the complexity of AIRE functioning, many key questions remain unanswered (Fiebaracci 2011).

AIRE is expressed mainly in the thymus, but also in lymph nodes and foetal liver (Kluger et al 2012). The AIRE protein functions as a transcription factor (Peterson et al 2008, Gardner et al 2009, Fierabracci 2011). AIRE is mainly expressed in the medullary thymic epithelial cells (mTECs) (Peterson et al 2008, Arstila et al 2013) and cells of the monocyte/dendritic cell lineage (Kogawa et al 2002). mTECs through the expression of MHC classes I and II express
a wide array of tissue-restricted antigens (TRAs) derived from different organs in the body. TRAs include self-proteins with patterns of expression restricted to a single or small handful of organs. Thymic expression of TRA serves as an important source of self-antigens to allow the negative selection of autoreactive T cells. Collectively, mTEC and thymic monocyte/dendritic cells play a crucial role in establishing self-tolerance by eliminating autoreactive T cells (negative selection) and/or by producing immunoregulatory FOXP3+ T cells, which prevent CD4+ T cell-mediated organ-specific autoimmune diseases. Collectively, several studies in mouse and man have shown that AIRE regulates thymic expression of several genes of ectopic peripheral proteins including many TRAs. Thus, AIRE dysfunction leads to a decrease in the expression of TRAs in the thymus, and consequently, autoreactive T cell clones escape into the periphery (Derbinski et al 2005, Moraes-Vasconcelos et al 2008, Gardner et al 2009, Fierabracci 2011). Besides, extrathymic AIRE expressing cells (eTACs) in the secondary lymph nodes of mice are capable of expressing self-antigens and may delete naive autoreactive T cells, reinforcing the immune tolerance by preventing the maturation of autoreactive T cells that could escape thymic negative selection (Gardner et al 2008) (Figure 8).

Figure 8. AIRE in human immune tolerance in the thymus and the periphery (modified from Proust-Lemoine et al 2012)
2.4. Autoantibodies in APECED

2.4.1. Tissue-specific antibodies

Patients with APECED may disclose a multitude of tissue-directed Abs (Arstila et al 2013, Table 2). The Abs are mostly directed against enzymes involved in hormone synthesis. Interestingly, because those targets are mainly intracellular, the real impact on each disease component is not clear. The precise mechanism of the production of autoantibodies in APECED has not yet been elucidated. Lindh et al reported in a mouse model that Aire$^{−/−}$ mice displayed a T cell-independent response against some antigens with autoantibodies. This response was dependent on marginal zone B cells in the spleen (Lindh et al 2008). Such an exaggerated response was linked to increased levels of a cytokine, the B cell-activating factor (BAFF) produced by dendritic cells. Interestingly, BAFF was also found to be elevated in APECED patients (Lindh et al 2008). Therefore, a cell-intrinsic effect of AIRE deficiency in peripheral DCs would lead to higher serum BAFF and enhanced activation of B cells, especially in the spleen, making them more prone to produce autoantibodies, independently of the autoreactive T cells (Lindh et al 2008). In addition, Campbell et al observed in a collagen-induced arthritis model that Aire knock-out mice had increased CD4 T cell help to B cells for cross-reactive Abs production (Campbell et al 2009). The CD4 T-cells were indeed more efficient in this model at stimulating wild-type B cells to produce specific Abs against collagen II (Campbell et al 2009).

Another hypothesis is that the circulating antibodies may simply reflect the autoimmunity directed against a specific organ without being specifically involved in the pathogeny. The tissue destruction preceding the failure of the endocrine organs may have a role. Tissue destruction, caused by trauma, viral infection or a T cell-mediated autoimmune attack, would probably lead to the release of potential tissue-specific autoantigens and, thus, to Ab formation against these proteins in a patient with AIRE deficiency.

This aspect is important to take into account as it may explain why various publications report the ‘statistical’ significance between certain Abs and symptoms or diseases, while the real pathologic relevance remains rather unclear.

2.4.2. Anticytokine antibodies

Anticytokine Abs are a newly recognised cause of autoimmune and infectious diseases (Browne et al 2010). Neutralising high-titre Abs can cause a wide variety of potentially life-
threatening illnesses (Browne et al 2010). Thus, Abs against interferon gamma, erythropoietin, G-CSF or GM-CSF have been described (Browne et al 2010). APECED is definitely the prototype of a disease with circulating anticytokine Abs.

2.4.2.1. Interferon alpha autoantibodies

The presence of anti-IFN type I Abs in APECED was first reported by Meager et al in 2006 (Meager et al 2006), and then confirmed by others (Meloni et al 2008). All the patients of their series (76/76) had neutralising IgG Abs against IFN-α subtypes compared with none in the controls. These Abs can be detected early in life (even before the age of 1 year), antedating or coinciding with CMC infection or other autoimmune features (Wolff et al 2013), and they remain positive for decades (Meager et al 2006). Overall, 90% of APECED patients have Abs against IFN-α and 100% against IFN-ω (Kisand et al 2011). The precise role and the impact of such Abs on the disease pathogenesis are not known. There is no relationship between the clinical phenotype or the AIRE mutation (Meloni et al 2008, Wolff et al 2013). Abs against IFN-α cause highly significant down-regulation of interferon-stimulated gene expression in the T cells in APECED patients' blood (Kisand K et al 2008). Despite this high activity, the Abs do not predispose patients to an increased risk of viral infections. It is possible that other IFNs such as type II or III or IFN-β compensate for such a deficiency. It may also be that the biological tests are ‘too’ sensitive and overestimate the in vivo biological activity in addition to APECED (Kisand et al 2011). To date, the anti-IFN Abs have a diagnostic value as, apart from APECED, they are found only in thymoma-associated myasthenia gravis (Kisand et al 2011). Abs against IFN-β, λ1 and other interleukins such as IL-10 and -12 were found at low non-neutralising levels in APECED patients (Meager et al 2006).

2.4.2.2. Interleukin-17A, -17F and -22 autoantibodies

Neutralising Abs against IL-17 and IL-22 are also present at diagnosis or earlier and persist for decades at high titres in APECED patients. As for Abs against IFN-α, these Abs can precede the occurrence of any clinical symptoms in siblings with AIRE mutations (Wolff et al 2013). A strong association with CMC has been shown (Kisand et al 2010, Puel et al 2010). Kisand et al found anti-IL-17A, -IL-17F and IL-22 in 41%, 74% and 90% of their 162 APECED patients, respectively (Kisand et al 2010). Puel et al identified anti-IL-17 Abs in 33 APECED patients, including 29 with CMC, whilst controls had no Abs. The Abs were able to
inhibit the production of IL-6 from IL-17-responsive fibroblasts (Puel et al 2010). In only a few cases, patients with such Abs did not have any CMC. Conversely to IFN-α, the production of IL-17F and -22 is especially impaired in patients with such Abs (Kisand et al 2011). Nevertheless, there are numerous causes of CMC other than APECED, and therefore IL-17 and IL-22 Abs are not absolutely necessary for the development of CMC. Besides, there are notable cases of APECED patients with CMC and without any Abs against IL-17F or IL-22 (Wolff et al 2013). Laakso et al recently showed a defect in expression of IL-22 in the skin of APECED patients in a steady-state resident population and in the cells participating in a tuberculin-induced recall response (cf below, Laakso et al 2014). An IL-22 defect is likely to be a contributing factor in CMC in APECED patients (Laakso et al 2014).

2.4.3. Cell-immune responses in APECED

It is generally believed that the destruction of the endocrine organs in APECED is caused by the autoreactive CD8+ cytotoxic T cells, although definitive evidence for this mechanism is still lacking (Betterle et al 2003, Moraes-Vasconcelos et al 2008). This hypothesis is reinforced by microscopic examination of samples, sometimes obtained postmortem. Typically, the parathyroid, adrenal glands or ovaries show atrophy and lymphocytic infiltration that suggest a cell-mediated attack leading to atrophy and dysfunction (Betterle et al 2003). This is also highlighted, indirectly, by the analysis of the Aire−/− mice, which also develop lymphocytic infiltration in some inner organs along with atrophy (Ramsey al 2002). Immunological studies have stressed an increased frequency of CD8+ CD45RA+ CCR7-effector T cells in the blood (Laakso et al 2011). On the other hand, immune regulation is also defective in APECED as illustrated by the decreased number of T regulatory cells (T reg, CD4+ CD25+ Foxp3+), especially the CD45RO+-activated subset (Ryan et al 2005, Laakso et al 2010). Whether the T reg deficiency is related to thymic AIRE deficiency per se or is secondary to the severe autoimmune disease in the patients is unknown. Laakso et al recently showed a defect in the expression of IL-22 in the skin of APECED patients (Laakso et al 2014). The Th22 population was the main affected population, even though IL-17 production was also seen to be impaired. It is not known to what extent the circulating Abs against IL-22 and the T reg activation deficiency are involved in the IL-22 defect (Laakso et al 2014). It was recently suggested that the pathogenic T cells are primed before their export from the AIRE-deficient thymus (Kisand et al 2014).
Overall, APECED is now seen as a disease with both increased T cell effector and decreased regulator activity (Arstila et al 2013). However, the defective negative selection of autoreactive clones in AIRE-deficient thymus cannot, by itself, explain fully the autoimmune symptoms of APECED.

The various subclasses of anomalies of the peripheral immunocompetent cells reported in various series are summarised in Table 5 (Wolff et al 2010). However, it is important to remember that such results are often variable, mostly because of variation among the study populations in terms of disease duration or activity at the time of the immunophenotyping (Wolff et al 2010).

Table 5. Immunocompetent cells and immunoglobulin anomalies observed in APECED

<table>
<thead>
<tr>
<th>T cells</th>
<th>CD3+; CD4+; CD8+ normal rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Memory T cells</td>
<td>(CD4+CD45RA-CD45RO+) diminished</td>
</tr>
<tr>
<td>Th17 subset</td>
<td>CD4+CCR6+CXCR3+: diminished</td>
</tr>
<tr>
<td>T regulatory cells</td>
<td>T reg (CD4+CD25+FoxP3+) cells diminished</td>
</tr>
<tr>
<td></td>
<td>T reg subset (CD3+CD4+CD25+CD127-) diminished</td>
</tr>
<tr>
<td>B Lymphocytes</td>
<td>B-cell CD19+ : normal</td>
</tr>
<tr>
<td>NK Cells</td>
<td>NKT cells (CD3+CD56+) normal or decreased</td>
</tr>
<tr>
<td></td>
<td>NK cells (CD3-CD56+) normal</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CD14+/live cells normal</td>
</tr>
<tr>
<td></td>
<td>CD14+CD16+/CD14+ cells diminished</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>Normal</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>Increased serum IgM, IgG, IgE; deficiency in IgA</td>
</tr>
</tbody>
</table>
3. Quality of life

3.1. APECED

It has been well-established that health-related quality of life is impaired in chronic diseases. In the field of endocrinology, numerous studies have shown that T1D, hypothyroidism and Addison’s disease have an impact on the HQoL of patients before any medical intervention. Patients with APECED find themselves in a very peculiar situation, due to the unique accumulation of mainly endocrine diseases and the early onset in childhood. Early in life, oral CMC impairs food intake and the pleasure of eating (López-Jornet et al 2009; Liu et al 2012). Later on in adulthood, the sex life of the women with genital candidiasis is affected. Some of the endocrine diseases, namely AD, HP and HT, expose to potentially lethal complications (adrenal crisis, hypocalcaemia and coma in cases of untreated hypothyroidism). This underlines the importance of educating patients and local healthcare providers to detect early symptoms and manage any complications. Autoimmune hepatitis, bronchiolitis or TIN, albeit rare, may rapidly become lethal if not controlled by immunosuppressive therapy. Asplenia exposes to fulminant sepsis and, lastly, immunosuppressive treatment increases the risk of sepsis as well as other side effects. The risk of infertility is a burden for young women. Preventive measures, such as early pregnancy in young adults or cryoconservation of oocytes, cause additional stress. The patients are also exposed to the risk of developing oral squamous cell carcinoma, while atrophic gastritis exposes to gastric carcinoma. Hormonal imbalance of thyroid function or adrenal glands typically may interfere with mood and general well-being as well. Diabetes type 1 alone exposes to a high number of complications such as the macro-and microangiopathies. All these diseases also imply the need for regular follow-up, blood test monitoring, multidisciplinary management and oral intake of numerous hormones and supplements. Keratoconjunctivitis may lead to severe visual impairment, and skin manifestations such as vitiligo or alopecia areata can be additional causes of stress, negatively affecting the body image of the patient. Therefore, exploring the impaired quality of life in APECED patients seems amply justified (Figure 9).
AD accounts for approximately 80% of all cases of adrenal insufficiency. In recent years, many studies have shown that both QoL and life expectancy are reduced in AD (Arlt et al 2003, Betterle et al 2011, Schalin-Jäntti 2011). Too small as well as too large doses of hormone replacement (cortisol, aldosterone) therapy affect the patient's well-being. In recent years, many studies have demonstrated that the doses previously recommended for cortisol replacement have been much too large (Arlt et al 2003). In addition, timing of the dosage may not be correct. Non-optimal replacement therapy may thus have a strong impact on QoL, both on personal and professional levels (Arlt et al 2003). The adverse effect of chronic adrenal insufficiency on QoL is comparable to that of congestive heart failure (Løvås et al 2002). Fifty percent of the patients with primary adrenal insufficiency considered themselves unfit to work and 30% needed household help (Arlt et al 2003). Patients with adrenal insufficiency show a significantly impaired HRQoL, irrespective of age, sex, concomitant disease and primary or secondary origin of the disease (Hahner et al 2007). The QoL may also depend on the condition that underlies the chronic AI (Reisch et al 2011). It is not known whether Finnish AD patients suffer from impaired QoL and to what extent, and whether replacement
doses, timing and surveillance at healthcare centres versus specialist centres influence these factors.

Patients with AD have a life-long risk for potential acute emergencies during stressing conditions, ranging from infections to surgical procedures and labour. Such conditions may precipitate an acute adrenal crisis that can be lethal in the absence of correct management (Schalin-Jäntti 2011). It is generally believed that AD patients may therefore have to visit emergency healthcare facilities and frequently warrant hospital care. Whether this is the case for Finnish AD patients is not known.

Fertility may be affected by AD, although it is primarily due to primary ovarian failure. Indeed, AD and primary ovarian autoimmune failure do share common autoantibodies against 21-hydroxylase (Hoek et al 1997, Husebye et al 2009 b). Gonadal failure has been identified in approximately 17% of the patients with APS type 1 and in 3.6% of patients with type 2 cases (Kowal et al 2006).

Depression and psychological/psychiatric disorders have been reported in patients with AD (Virtanen et al 1998, Thomsen et al 2006). Depression may even be the presenting sign (Virtanen et al 1998). Patients with AD may disclose increased levels of anxiety and fear and overreaction to stimuli, but decreased performance efficiency and need for social contact. Such psychological characteristics may affect the doctor-patient relationship, leading potentially to poor compliance and less efficacious therapy.

Lastly, the persistent risk of developing new autoimmune disorders (such as thyroid dysfunction, type I diabetes, celiac disease or vitiligo), the risk of possibly life-threatening adrenal crisis that may occur at any time and may be misdiagnosed, and the necessity of continuous hormone substitution can be sources of continuous distress.

Overall, all these factors may have a deep impact on the QoL of patients presenting with AD.
AIMS OF THE STUDY

APECED is a monogenic disease manifesting as chronic candidiasis and a high number of autoimmune disorders. Our objective was to better delineate the clinical manifestations of two ‘minor’ components of APECED that have been sparsely studied in the literature: the gastrointestinal and nephrologic manifestations. We also reviewed the implication of Abs against NALP5 and CaSR in hypoparathyroidism as well as other more common Abs during APECED. Lastly, we investigated the impact of APECED on patient HRQoL and tried to determine which factors are most involved.

Specifically, the aims were:

To study in detail the gastrointestinal and nephro-urologic manifestations among Finnish APECED patients.

To investigate the occurrence and association of various Abs and the clinical manifestations, mainly the gastrointestinal and renal manifestations.

To study the significance of parathyroid-directed Abs in APECED.

To study the occurrence of common non-tissue-specific Abs in APECED.

To evaluate the health-related quality of life among Finnish APECED and Finnish patients with Addison’s disease/APS2 and to explore which factors may be specifically involved in the QoL impairment.
SUMMARY OF MATERIAL AND METHODS

The material and methods are described in greater detail in the original publications.

1. Patients and patient recruitment

In 2010-2011, we had track of 51 patients living with APECED, scattered all over the country (Rönn 2014, unpublished observations). Most were members of a patients’ association founded in 1995 (the Finnish registered APECED and Addison’s association: Apeced ja Addison Ry). The association provides peer support and information to patients in the form of meetings, information leaflets and a web page (http://www.apeced.org). The association also has a permanent support person for eventual crises, trained by the Finnish Red Cross. The Finnish patient group has a history of willingly participating in research on their disease. Thus, the APECED patients were recruited through the association circuit between 2011 and 2013. The number of patients for each study varied according to their availability, varying from 26 (Original study V) to 44 patients (Original study III). This explains the discrepancies between published cohorts as regards gender, age, clinical manifestations and other outcomes. The cohorts had a mean age ranging from 33 (Original study III) to 41 years (Original study V) with female predominance (Original studies I to V). In addition, Rönn conducted a national survey between 2010 and 2011 regarding the self-assessment of diseases and clinical symptoms among Finnish patients with APECED. A database was established for this and used as a reference for the studies of this thesis as well (Rönn 2014).

For AD and APS2 patients (Original study VI), we included 107 patients who had been diagnosed and treated at the Helsinki University Central Hospital (Meilahti, Jorvi and Peijas hospitals) from 1997 onwards and from the national patients’ organisation. Patient charts with the ICD code for Addison’s disease (E27.1, E27.1 or E 27.4) and the questionnaires from subjects recruited via the patients’ organisation were screened by an endocrinologist and only subjects fulfilling the diagnostic criteria for AD were included in the study. APS-2 was defined as patients with AD associated with at least one of the following additional autoimmune conditions: hypo- or hyperthyroidism, T1D, ovarian failure, celiac disease, pernicious anaemia/atrophic gastritis, vitiligo or alopecia areata. The characteristics of the patients for each study are summarised in Table 6.
Table 6. Patient characteristics in studies I to VI

<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Female/Male</th>
<th>Median age in years (range)</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>31</td>
<td>21/10</td>
<td>40 (5-65)</td>
<td>APECED</td>
</tr>
<tr>
<td>II</td>
<td>30</td>
<td>20/10</td>
<td>45 (7-68)</td>
<td>APECED</td>
</tr>
<tr>
<td>III</td>
<td>44</td>
<td>26/18</td>
<td>33* (8-67)</td>
<td>APECED</td>
</tr>
<tr>
<td>IV</td>
<td>30</td>
<td>20/10</td>
<td>42 (7-65)</td>
<td>APECED</td>
</tr>
<tr>
<td>V</td>
<td>26</td>
<td>19/7</td>
<td>44 (17-65)</td>
<td>APECED</td>
</tr>
<tr>
<td>VI</td>
<td>107</td>
<td>84/23</td>
<td>51 (22-76)</td>
<td>AD and APS2</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>28/14</td>
<td>48 (22-73)</td>
<td>AD</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>55/9</td>
<td>52 (23-76)</td>
<td>APS2</td>
</tr>
</tbody>
</table>

*For this study only the mean age of the cohort is available

2. Interviews

For 3 studies (Original studies II, V and VI), we used self-reported questionnaires.

For Original study II, the questionnaire explored the past or present history of urinary tract infections (pyelonephritis, cystitis, number of episodes), kidney or urinary tract stones, TIN, renal biopsies, hypertension and uveitis. In addition, male patients were specifically asked if they had previously been diagnosed with prostate disease (acute/chronic prostatitis, adenoma or cancer). In order to ensure good comprehension and to avoid misunderstandings, the questions used both medical terms and an explanatory definition of their meanings. The questionnaire was mailed with an explanatory letter describing the intended research and prepaid return envelopes. No second attempt was made to contact non-responders.

For Original study V, 3 self-reported questionnaires, for which a Finnish translation has been validated, were used: the SF-36 (or RAND-36), Raitasalo’s modification of the short form of the Beck Depression Inventory–BDI (RBDI) and the Dermatology Life Quality Index (DLQI). The questionnaires were mailed along with an explanatory letter, an informed consent form and a prepaid envelope for return of the questionnaire. In the case of no response, patients were contacted a second time by phone.

Because of the high number of possible GI symptoms, we chose for Original study I a direct interview or a telephone interview by a single physician (Martta Jokinen), using a structured questionnaire developed by Nicolas Kluger and Annamari Ranki. For children, one of the parents was asked to answer the questions. The questionnaire included demographic data, familial and personal GI conditions (including e.g. lactose intolerance and celiac disease) and symptoms. The upper GI tract symptoms included sensation of dry mouth, pain with swallowing (odynophagia), difficulties in swallowing solid and liquid food (dysphagia),
vomiting, retrosternal pain and discomfort. The lower GI tract symptoms included chronic/repeated episodes of constipation and/or diarrhoea, stool consistency, anal incontinence and use of laxatives for constipation and pancrelipases for pancreatic exocrine failure. Past history or active chronic mucosal candidiasis and organ-specific autoimmune manifestations were also collected.

For Original study VI, apart from the HRQoL questionnaires, we recorded current hormone replacement therapy and dosage (hydrocortisone, HC or prednisone, fludrocortisone, FC, dehydroepiandrosterone DHEA), social and demographic information, current weight and height, blood pressure, other chronic comorbidities such as hypertension, coronary heart disease, type 2 diabetes, thyroid dysfunction, and other autoimmune diseases, affiliation with the patients’ association, and current level of disease follow-up in the healthcare system (university hospital/specialist in endocrinology, general hospital/specialist in internal medicine or local healthcare centre/general practitioner). The questionnaires were mailed along with an explanatory letter, an informed consent form, and a prepaid envelope for return of the questionnaires.

3. Clinical records

We recently created a clinical database of APECED patients, including demographic data (gender, age, age at APECED diagnosis, age at first onset of symptoms), clinical symptoms, autoimmune endocrine and nonendocrine diseases, and occurrence of oral carcinoma or other malignancies (Rönn 2014, unpublished observations), which allowed us to compare these findings with the renal and urinary findings (Original study II). In the case of discrepancies or unclear data, we collected information directly from the patients’ medical files, as authorised by the patients themselves by written consent. In a few cases, the responsible physician was directly contacted by one of us to cross-check the clinical data and the patients’ narratives.

4. AIRE mutations

All the patients with APECED had confirmed mutations of AIRE genes. Among the 44 patients included in Original study III, the exon 6 AIRE mutation c.769C_T was found in all of them (100%), and 36 (82%) were homozygous for the allele. The mutation analyses were done in the laboratories of the late Professor Leena Peltonen-Palotie, National Institute of Health, and Professor Kai Krohn, University of Tampere. Other AIRE genotypes are summarised in Table 7.
Table 7. AIRE mutations in 44 APECED patients described in Original study III

<table>
<thead>
<tr>
<th>AIRE mutation</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.769C_T/ c.769C_T</td>
<td>36 (82%)</td>
</tr>
<tr>
<td>c.769C_T/c.967–979del13bp</td>
<td>3 (6.8%)</td>
</tr>
<tr>
<td>c.769C_T/c.923G_A (C311Y, in exon 8)</td>
<td>2 (4.5%)</td>
</tr>
<tr>
<td>c.769C_T/c.1638A_T (X546C, in exon 14)</td>
<td>1 (2.3%)</td>
</tr>
<tr>
<td>c.769C_T/11631164insA (M388fsX422, in exon 10)</td>
<td>1 (2.3%)</td>
</tr>
<tr>
<td>c.769C_T/undetermined allele</td>
<td>1 (2.3%)</td>
</tr>
</tbody>
</table>

5. Quality of life (Original studies V and VI)

To assess the impairment of the HRQoL of APECED patients (Original study V), 3 questionnaires were used. The SF-36 is one of the most widely used HRQoL survey instruments in the world today (Hays et al 1993). It is a self-rated, profile-based HRQoL measure and originates from the Medical Outcomes Study 36-item short-form health survey. It comprises 36 items that assess 8 health concepts: physical functioning (10 items), physical role limitations (4 items), emotional role limitations (3 items), social functioning (2 items), bodily pain (2 items), general mental health/emotional well-being (5 items), energy/vitality (4 items), and general health perceptions (5 items). We used the Finnish version of the SF-36 as it was shown to have good reliability and construct validity in the general Finnish population (Aalto et al 1999). The SF-36 is scored in 2 steps. Each item is first scored on a scale from 0 to 100 (a higher score defining a higher HRQL), and an average value is calculated for each of the 8 dimensions. If more than 50% of the items in a dimension are missing, the average value cannot be calculated (Hays et al 1993).

The RBDI mood questionnaire (Beck et al 1972; Beck et al 1974) is a self-reported questionnaire that has been used in Finland for the past 30 years for depression. This Finnish-modified version of Beck’s 13-item depression scale has 13 questions for depression (Raitasalo 2007). Depression items are scored from 0 to 3 and the total score ranges from 0 to 39 points; 5 to 7 points indicates mild depression, 8 to 15 points moderate depression, and over 16 points severe depression.

The DLQI® is the first dermatology-specific quality of life instrument and the most frequently used instrument in studies of randomised controlled trials in dermatology (Finlay et al 1994). It is a simple validated 10-question questionnaire which has been used for 33 skin
conditions and is available in Finnish [20]. Patients respond on the basis of the symptoms they may have presented in the previous week to permit accurate recall. Each question has 4 possible answers for a maximum of 3 points and a total maximum score of 30. Higher scores indicate more severely affected QoL. The patients were given 3 DLQI questionnaires to assess the differential effects of alopecia, vitiligo and CMC. A score of 0 to 1 indicates no effect on the patient's life, 2 to 5 a small effect, 6 to 10 a moderate effect, 11 to 20 a very large effect, and 21 to 30 an extremely large effect. Questionnaires were not anonymous for the purpose of enabling comparison with the data in the recently created clinical database (see chapter 3).

For the study of the QoL of patients with AD (Original study VI), we used 2 scores: the SF-36 and the 15D. The 15D is a generic, comprehensive, 15-dimensional, standardised, self-administered measure of HRQoL that can be used both as a profile and as single index instrument (Sintonen 2001). It is a well-validated test consisting of 15 dimensions: mobility, vision, hearing, breathing, sleeping, eating, speech, elimination, usual activities, mental function, discomfort and symptoms, depression, distress, vitality and sexual activity. Each dimension is divided into 5 ordinal levels, by which more or less of the attribute can be distinguished. The patient or person chooses the level best describing his or her current health status for each dimension. The evaluation system of the 15D is based on an application of the multi-attribute utility theory. A set of utility or preference weights, elicited from the general public through a 3-stage evaluation procedure, is used to generate the within-dimension level values and the overall utility score, that is, the 15D score (single index number) over all the dimensions on a 0–1 scale. The maximum score is 1 (no problems on any dimension) and the minimum score is 0 (equal to being dead). The minimal clinically important difference of the 15D score is 0.03. The advantage of the 15D is that the results of the patients were compared with HRQoL data of an age- and sex-matched general Finnish population (n = 5671), available from the National Health 2000 Health Examination Survey.

6. Routine laboratory tests (Original study II)

Laboratory analysis included recent (less than a year old) plasma creatinine, urea nitrogen, plasma cystatin C, urinary proteins, low-molecular-weight protein and glucose values, a urinalysis, a urine bacteriology and the value for prostate-specific antigen for men. The
glomerular filtration rate (GFR) was assessed using the Cockcroft–Gault formula for adults or the bedside Schwartz equation for children.

7. Tissue samples and immunohistochemistry

7.1. Gastrointestinal tissues (Original study I)
Archival formalin-fixed, paraffin-embedded biopsies from the upper (duodenum) and lower GI tract (ileum, colon or rectum) were available from 12 patients (20 tissue samples altogether), obtained during fibroscopy/colonoscopy for various symptoms (retrosternal pain/dysphagia/oesophagitis, gastritis, constipation, diarrhoea, swollen abdomen).

7.2. Renal tissues (Original study II)
Three patients had undergone kidney biopsies. Archival formalin-fixed, paraffin-embedded biopsies could not be traced in one patient and a second sample was too small to allow any valuable reinterpretation. Therefore, only one representative biopsy sample was available for the study and the review of the histologic and immunofluorescence results.

7.3. Immunohistochemistry (Original study I)
Immunohistochemistry was performed as follows. After antigen retrieval (heating in citrate buffer at pH 6 for 10 min), the tissue sections were incubated overnight at +4°C with monoclonal Abs against human defensin 5 (hD5, dilution of 4µL in 1 mL, clone 8C8; Thermo Scientific, Rockford, IL, USA), CgA (dilution 1:400, Clone DAK-A3; DakoCytomation, Glostrup, Denmark), 5-HT (dilution 1:200, NB120-16007, Novus Biologicals, Cambridge, UK), villin (1:200, Clone 1D2 C3; DakoCytomation, Glostrup, Denmark), and interleukin-17 (IL-17, dilution 1:50, AF-317-NA, R&D Systems, Minneapolis, MN, USA). The bound Abs were detected with the ImmPRESS Universal Ab (anti-mouse Ig/anti-rabbit Ig, peroxidase) Polymer Detection Kit and AEC SK 4200 (Vector Laboratories Inc., Burlingame, CA, USA) for CgA, 5-HT, hD5 and villin Abs and the VECTASTAIN Elite ABC Kit (Goat IgG) PK-6105 and AEC SK 4200 (Vector Laboratories Inc., Burlingame, CA, U.S.A) for IL-17 Abs. Normal healthy duodenum, ileum and colon sections from elective surgery patients were used as reference samples. CgA, 5-HT, and IL-17 positive cells were calculated in 3 different areas per slide, selected randomly and bounded by 50 crypts in well-oriented duodenal, ileal or colonic samples. If the sample contained fewer than 50 crypts, the number
was normalised to 50 crypts. Villin expression was analysed according to the patterns of reactivity in the enterocytes: brush borders (apex), cytoplasmic and/or basal expression.

8. Autoimmune and autoreactive T cells

8.1. Detection of tissue antigens recognised by patient sera and antibodies

8.1.1. Detection of autoantibodies against intestinal structures by indirect immunofluorescence (IIF, Original study I)

Formalin-fixed, paraffin-embedded biopsies of normal human duodenum and ileum were used as target tissue. After antigen retrieval (as above) and blocking with 1% BSA (bovine serum albumin), the slides were incubated for 1 hr at room temperature with each patient serum (1:10 in phosphate-buffered saline, PBS) or with serum from healthy blood donors (n=15, Finnish Red Cross blood service), washed in PBS, followed by FITC-conjugated anti-human IgG (F0203; DakoCytomation, Glostrup, Denmark) at 1:40 for 30 min at room temperature. The nuclei were visualised with DAPI (4',6-diamidino-2-phenylindole, Vectashield®, Vector Laboratories Inc., Burlingame, CA, USA), after which the slides were analysed with an Olympus BX50 microscope.

8.1.2. Detection of autoantibodies against renal structures by indirect immunostaining of rat kidney (Original study II)

Indirect immunostaining of formalin-fixed, paraffin-embedded rat kidney (original study II) sections was performed to detect tissue-specific cross-reactive Abs. Abs are typically cross-reactive, and rodent tissues can be used for their detection (Fetissov et al 2009). Tissue sections were deparaffinised, and the endogenous peroxidase activity was quenched with 1:10 hydrogen peroxide. Antigen retrieval was performed by heating slides in citrate buffer at pH 6 for 10 min. Blocking was performed with 1% BSA. Slides were incubated overnight at +4°C with individual patient sera (1:1000 in PBS). As a detection system, the VECTASTAIN Elite ABC Kit (Goat IgG) PK-6105 and AEC SK 4200 (Vector Laboratories Inc., Burlingame, CA, USA) were used. As control tissue, rat spleen sections, immunostained simultaneously on the same slide, were used. Sera from healthy individuals served as control sera.
8.2 Detection of circulating antibodies by enzyme-linked immunosorbent assay (Original studies I and II)

Patient sera were collected between 2010 and 2012 and stored at −70°C. We analysed Abs against IFN-α (α1beta, α2beta, α4, α5), −γ, −ω, IL-17A, IL-17F, IL-22, and IL-23, and against various intestinal autoantigens of interest: TPH-1, AADC, gastrin, AIE-75 and villin, with a validated in-house ELISA assay. Plates were coated with recombinant human INF−α,−γ, −ω, IL-17A, IL-17F, IL-22, and IL-23 (ImmunoTools GmbH, Friesoythe, Germany), TPH-1 (Sino Biologicals, Beijing, China) and villin (Novus Biologicals, Cambridge, UK) with 0.75 µg of protein/ml in PBS, pH 7.0, blocked with 2% HSA. AIE-75 (Novus Biologicals, Cambridge, UK) and AADC (Nordic BioSite, Helsinki, Finland) were used at 1 mg/ml concentration and gastrin (Novus Biologicals, Cambridge, UK) at 30 µg/ml in PBS with pH 7.0. The precoated plates were then incubated with patient serum samples (1:250 dilution) for 2 hr at 22°C, washed, and developed with anti-human IgG horseradish peroxidase conjugate (Sigma-Aldrich), 1-Step Ultra TMB-ELISA (ThermoScientific, Rockford, IL, USA) and Stop Solution (2M, H2SO4). Absorbance was read at 450 nm. Sera from 53 to 62 healthy age- and sex-matched (except for children) blood donors, provided by the Finnish Red Cross blood service, served as controls. The cut-off value was defined as mean + 3SD.

8.3 Detection of CaSR and NALP5 antibodies (Original study III)

The following experiments were performed by Kemp et al in the University of Sheffield, United Kingdom. Immunoprecipitation (IP) assays for detecting CaSR Abs were carried out using CaSR-FLAG protein expressed in human embryonic kidney 293 cells (HEK293, ECACC, Public Health England, Salisbury, UK): Proteins immunoprecipitated from the HEK293 cell extract containing expressed CaSR-FLAG protein were separated by SDS-PAGE in 7.5% (w/v) polyacrylamide gels and transferred to Trans-Blot® Transfer Membrane (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). The CaSR-FLAG protein was detected using Anti-FLAG® M2-Peroxidase Conjugate (Sigma-Aldrich) and an ECL™ Western Blotting Analysis System. The results were compared by immunoprecipitation of CaSR-FLAG anti-CaSR Ab, APECED patient sera, control sera and no serum or anti-CaSR Ab (beads alone).

A CaSR antibody index for each serum was calculated as: densitometry value of tested serum/mean densitometry value of a population of healthy control sera. Each serum was
tested in at least 2 experiments and the mean CaSR antibody index was calculated. The upper limit of normal for the CaSR immunoprecipitation assay was calculated using the mean CaSR antibody index + 3SD of a population of healthy individuals. Any serum with a CaSR antibody index above the upper limit of normal was designated as positive for CaSR antibodies.

NALP5 Abs were detected in radioligand binding assays (RBAs) using $^{35}$S]-labelled NALP5. The latter was produced \textit{in vitro} in a TnT® T7-Coupled Reticulocyte Lysate System (Promega, Southampton, UK) and then used in radioligand binding assays (RBA) with patient and control sera, and anti-NALP5 Ab. Following RBAs, immunoprecipitation of [35S]-labelled NALP5 protein was analysed by SDS-PAGE in 8% (w/v) polyacrylamide gels followed by autoradiography. Antibody levels were expressed as an NALP5 antibody index. This was calculated for each serum tested as: cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by the population of healthy control sera. Each serum was tested in at least 2 experiments and the mean NALP5 antibody index was calculated. The upper limit of normal for the NALP5 RBA was calculated using the mean NALP5 antibody index + 3SD of a population of healthy controls. Any serum sample with a NALP5 antibody index above the upper limit of normal was designated as NALP5 antibody-positive.

\subsection*{8.4 Other antibodies (Original study IV)}

We investigated the occurrence of antinuclear Abs (AN-Abs) and Abs against extractable nuclear antigens (ENA-Abs), citrullinated peptide, and transglutaminase in 24 patients and against bullous pemphigoid antigen 180 and desmogleins 1 (Dsg1) and Dsg3 in 30 patients of a Finnish cohort of APECED patients. These immunological assays were performed at the accredited Helsinki University Central Hospital laboratory, HUSLAB. Sera from 8 healthy blood donors were used as controls for each autoantigen, although the reference values of HUSLAB (http://www.huslab.fi), the largest university hospital laboratory in Finland, are based on the values in a large normal population as indicated in the accreditation documents of the laboratory (www.finas.fi).

\subsection*{8.5 ELISPOT assay against AADC (Original study I)}

The lymphocyte reactivity against AADC antigen was assessed in 14 patients. Precoated plates with anti-IFN$\gamma$ mAb (ELISpotPro for Human IFN-gamma, Mabtech, Nacka Strand,
Swedish) were used to detect antigen-specific T cells secreting IFNγ in response. After blocking of the plates as instructed, 1 µg/ml of recombinant human AADC protein (PAT-80291-1, Nordic BioSite, Helsinki, Finland) was added, followed by the patient cells. Frozen and thawed PBMCs of the 14 patients were allowed to rest for 24 hr after thawing and seeded in 300,000 cells per well in 100 µl total volume of serum-free culture medium (RPMI Medium 1640, Gibco, Grand Island, NY, USA). Polyclonal T cell stimulation with CD3-2 mAb (Mabtech) served as a positive control. After 48 hr of culture, the cells were discarded and IFNγ secretion visualised according to the manufacturer’s instructions with BCPI/NBT-plus (Mabtech). The coloured spots were counted using an Immunospot Analyser and the means of triplicate wells were calculated. The cut-off for a positive response was set at 2SD above the average basal reactivity (i.e. reactivity w/o antigen), as previously described in similar experiments.

9 Statistical analysis
Statistical analyses were carried out using several software packages: SPSS statistics 18.0 (SPSS Inc., an IBM company); NCSS 2007 (QoL) and GraphPad Instat 3 software (GraphPad Software, La Jolla, Ca). Patient characteristics and quantitative data in general were expressed as mean (range) values and SD for continuous variables and frequencies and proportions for categorical variables. Comparisons of categorical variables were performed using the \( \chi^2 \) test. Patient characteristics were compared using Fisher’s exact test for categorical variables and the Mann-Whitney U-Test for continuous ones. Spearman’s rank correlation coefficient was applied for continuous variables. The Pearson correlation coefficient was a measure of the correlation between variables. The statistical significance threshold was set at \( P < 0.05 \).

10 Ethical considerations (Original studies I-VI)
The studies were approved by the Medicine Ethical Review Board of Helsinki and Uusimaa Joint Authority (dnro 8/13/03/01/2009 for studies I to VI and dnro 276/13/03/01/2012 for study VI) and the principles of the Helsinki Declaration were followed in collecting the samples. Written informed consent was obtained from the patients or, for patients under 18 years, from the parents of the subjects.
RESULTS AND DISCUSSION

1. Clinical component of APECED and other autoimmune diseases in the studied cohorts

1.1. APECED (Original studies I to V)

The number of patients ranged from 26 (Original study V) to 44 (Original study III), depending on the study (Table 6).

Among the 34 APECED respondents, 12 were male (35%) and 22 were female (65%). All were unrelated except for 2 young girls who were sisters. The mean age was 36.9 yrs ± SD 15.0 (range 7-65). APECED had manifested in early infancy or childhood (4.0 ± 3.4 yrs) and had evolved for 32.9 ± 13.9 yrs. The main clinical manifestations of the interviewed APECED cohort (Rönn 2014, unpublished observations) are summarised in Table 8.

Sixty-five percent (22/34) had a complete ‘triad’ (CMC+AD+HP), while 29% (10/34) displayed only 2 components as follows: 15% (5/34) CMC+HP, 15% (5/34) CMC+AD, and 2.9% (1/34) AD+HP (Rönn 2014, unpublished observations). Only one patient (2.9%) had only CMC as a symptom (Figure 10).

Figure 10. Prevalence of the main components of APECED in 34 Finnish patients (Rönn 2014, unpublished observations)
Table 8. Prevalence of the main components of Finnish APECED patients according to Rönn (n = 34) (Rönn 2014, unpublished observations)

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>34</td>
<td>12 (35%)</td>
<td>22 (65%)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>37 (7 – 65)</td>
<td>38 ± 13 (16 – 65)</td>
<td>36 ± 16 (7 – 57)</td>
</tr>
<tr>
<td>Age at the beginning of APECED (yrs)</td>
<td>4 ± 3 (1 – 18)</td>
<td>4,4 ± 5 (1 – 18)</td>
<td>4 ± 3 (1 – 10)</td>
</tr>
<tr>
<td>Duration of APECED (yrs)</td>
<td>33 ± 14 (5 – 53)</td>
<td>33 ± 10 (14 – 47)</td>
<td>33 ± 16 (5 – 53)</td>
</tr>
<tr>
<td>BMI</td>
<td>23 ± 5 (16 - 37)</td>
<td>24 ± 5 (16 - 34)</td>
<td>22,7 ± 4 (18 - 37)</td>
</tr>
<tr>
<td>Addison’s disease</td>
<td>82% (28/34)</td>
<td>92% (11/12)</td>
<td>77% (17/22)</td>
</tr>
<tr>
<td>Hypoparathyroidism</td>
<td>82% (28/34)</td>
<td>58% (7/12)</td>
<td>95% (21/22)</td>
</tr>
<tr>
<td>Mucosal candidiasis</td>
<td>97% (33/34)</td>
<td>100% (12/12)</td>
<td>95% (21/22)</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>29% (10/34)</td>
<td>17% (2/12)</td>
<td>36% (8/22)</td>
</tr>
<tr>
<td>IDDM</td>
<td>15% (5/34)</td>
<td>25% (3/12)</td>
<td>9% (2/22)</td>
</tr>
<tr>
<td>Eye manifestations</td>
<td>76% (26/34)</td>
<td>75% (9/12)</td>
<td>77% (17/22)</td>
</tr>
<tr>
<td>Vitiligo</td>
<td>24% (8/34)</td>
<td>42% (5/12)</td>
<td>14% (3/22)</td>
</tr>
<tr>
<td>Alopecia areata</td>
<td>50% (17/34)</td>
<td>83% (10/12)</td>
<td>32% (7/22)</td>
</tr>
<tr>
<td>Pernicious anaemia</td>
<td>9%</td>
<td>0/12</td>
<td>14% (3/22)</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>12% (4/34)</td>
<td>25% (3/12)</td>
<td>5% (1/22)</td>
</tr>
<tr>
<td>Asplenia</td>
<td>26% (9/34)</td>
<td>17% (2/12)</td>
<td>32% (7/22)</td>
</tr>
<tr>
<td>Ovarian insufficiency</td>
<td></td>
<td></td>
<td>64% (14/22)</td>
</tr>
<tr>
<td>Testicular insufficiency</td>
<td></td>
<td>17% (2/12)</td>
<td></td>
</tr>
<tr>
<td>Hypogonadism</td>
<td>47% (16/34)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth hormone deficiency</td>
<td>12% (6/34)</td>
<td>25% (3/12)</td>
<td>5% (1/22)</td>
</tr>
<tr>
<td>Malabsorption</td>
<td>9% (3/34)</td>
<td>25% (3/12)</td>
<td>0/22</td>
</tr>
<tr>
<td>GI-dysfunction</td>
<td>56% (19/34)</td>
<td>50% (6/12)</td>
<td>59% (13/22)</td>
</tr>
<tr>
<td>Tubulointerstitial nephritis</td>
<td>9% (3/34)</td>
<td>0/12</td>
<td>14% (3/22)</td>
</tr>
<tr>
<td>Oral cancer</td>
<td>3% (1/34)</td>
<td>0/12</td>
<td>5% (1/22)</td>
</tr>
</tbody>
</table>

Yrs: years

More than half of the patients, 56% (19/34), had 6 or more components while 44% (15/34) had 5 or fewer. The patients had overall 3 to 10 components (Figure 11). On average, a patient had a mean number of 6.3 components of APECED (Rönn 2014, unpublished observations).
None of the patients had a history of autoimmune connective tissue disease, rheumatoid arthritis, celiac disease, or autoimmune cutaneous bullous disorders (Original study IV). It is rather interesting to note that despite a lack in the negative selection of autoreactive clones, APECED patients develop only a certain number of autoimmune diseases. For instance, ‘common’ autoimmune diseases have not been reported, such as multiple sclerosis, bullous disorders, autoimmune thrombocytopenia or neutropenia, or Goodpasture syndrome. Cases of celiac disease, haemolytic anaemia, and Sjögren’s syndrome are rather anecdotal (Betterle et al 1998, Betterle et al 2003, Perheentupa 2006), raising the question of whether these associations are fortuitous or not.

None of our patients had systemic lupus or rheumatoid arthritis (Original study IV). There are several explanations for such discrepancies: i) the rarity of APECED makes it difficult to detect unusual autoimmune manifestations, ii) premature deaths of patients make it difficult to detect a disease like bullous pemphigoid that affects the elderly, iii) autoreactive T cell clones for ubiquitous molecules may be deleted at an early stage of development, thereby preceding AIRE-regulated events in the thymic medulla, iv) other factors may be involved, such as HLA alleles, and v) paradoxically, by their neutralising effect, anticytokine (anti-IFN, IL-17 or IL-22) Abs may have a protective action against the development of some autoimmune diseases.

It is interesting to note that thymoma, an uncommon tumour derived from the thymic epithelium, which is also characterised by a lack of or diminished expression of AIRE in the thymic tissue (Scarpino et al 2007, Liu et al 2014), is associated with a high frequency of autoimmune diseases (Holbro et al 2012). However, these autoimmune manifestations are quite different from those seen in APECED, mainly myasthenia gravis. For instance, cases of pemphigus-associated thymoma have been reported (Shelly et al 2011), but not in APECED thus far (Original study IV).
1.2. Addison’s disease and APS-2 (Original study VI)

Among the 107 respondents of study VI, 84 were women (78.5%) and 23 were men (21.5%) with a mean age of 49.8 yrs. Sixty percent (64/107) fulfilled the criteria of APS-2. The number of autoimmune components in APS-2 patients ranged from 2 to 5 (Figure 12). There was no difference between the AD and APS-2 groups regarding age, age at diagnosis, duration of the disease or hormonal treatments. However, a significant female predominance was observed in the APS-2 group.
2. Clinical manifestations (Original studies I, II and IV)

2.1. Gastrointestinal symptoms (Original study I)

2.1.1. Familial history
Regarding past family history of GI conditions, 3 unrelated patients acknowledged GI cancers among their relatives: gastric carcinoma (n = 1) and colonic carcinoma (n = 1) in first-degree relatives and gastric carcinoma (n = 1) in a second-degree relative. None of these relatives was known to have a diagnosis or symptoms evocative of APECED. Lactose intolerance was present in the families of 2/3 of the patients (63%, 19/30) and celiac disease in 4 families (13%, 4/30) (Original study I).
2.1.2. Upper gastrointestinal symptoms

GI symptoms were overall very frequent in our cohort (Figure 13). The most commonly reported upper GI tract symptoms included occasional dysphagia to solid food in 45% (14/31) of the APECED patients. Dysphagia was associated with concomitant oropharyngeal Candida infections only in 3 patients (Figure 14). In addition, 3 patients had a stricture/stenosis of the oesophagus diagnosed by fibroscopy. Episodes of retrosternal chest and abdominal pain or discomfort were present in 45% (14/31). Most of the patients reported symptoms on a monthly basis. Also, 3 children complained of epigastric/lower stomach pain episodes. The parents did not link the pain episodes to any specific activity or other symptoms.

Occasional odynophagia, described as difficulties or pain when swallowing, was present in 35% of the cases (11/31). These symptoms were associated with oral and pharyngeal candidiasis in only 3 patients (27%, 3/11). Subjective symptoms of xerostomia, i.e. a repeated or daily feeling of dry mouth, were present in 29% of the study population (9/31). Two patients stressed that the symptoms fluctuated according to an active Candida infection. Although no accessory salivary gland biopsies were made, primary Sjögren’s syndrome did not explain these symptoms since none of the patients displayed autoantibodies against nuclear or extractable nuclear antigens, including anti-SSa/Ro and anti-SSb/La (Original study IV). Pyrosis, defined as chest burn and the sensation of gastric acid regurgitation, was present in 26% of the cases (8/31). One patient reported regular vomiting, about once a month, and one child on a regular basis, albeit rarely. Lastly, 8 patients (26%, 8/31) complained of both swallowing difficulties and dysphagia (Original study I).

A rather low percentage of patients linked the symptoms to candidiasis. In Finland, mucous candidiasis affects almost all APECED patients (Ahonen et al 1990). According to the literature, 15 to 22% of the APECED patients present with Candida oesophagitis (Perheentupa 2006, Collins et al 2006). Thus, the upper GI tract symptoms may reflect oral and oesophageal candidiasis, but due to the retrospective nature of our study, a recollection bias may explain the discrepancy. Self-treatment by antifungal therapies on the appearance of first symptoms and the improvement of candidiasis with time can also explain our results. Eight patients had oesophagitis confirmed by fibroscopy, but only 4 of them were found to have candidiasis by PAS staining (12%). These results are consistent with the literature (Perheentupa 2006).
2.1.3. Lower gastrointestinal symptoms

Half (48%) of the patients reported constipation (Figure 14). Three patients mentioned that they had been symptom-free since childhood or adolescence. Forty percent of them (6/15) regularly used laxatives and 2 used natural remedies or extradietary fibres. One woman regularly performed manual anal stimulation due to severe constipation. Less than half (45%) had unexplained periods of diarrhoea, unrelated to any viral or bacterial gastroenteritis and lasting longer than a couple of days. In 1/3 of them, diarrhoea has been alleviated since childhood. Importantly, 3 out of 4 children had a history of diarrhoea and in 2 cases, diarrhoea had been the main symptom leading to APECED diagnosis. Three patients had experienced diarrhoea only in childhood while one adult male patient reported diarrhoea lasting for decades and relieved only after an increased dosage of oral corticosteroids, given as supplementation for adrenal insufficiency. Five of the patients with diarrhoea had steatorrhoea compatible with exocrine pancreas deficiency and 2 of them took daily pancrelipase. Typically, in 26% of the patients constipation and diarrhoea alternated. One third (32%) did not present any past symptoms of constipation or diarrhoea (Original study I). Lower GI symptoms in APECED may have 3 main causes: 1) hypoparathyroidism, 2) infection and 3) autoimmune enteropathy. Because of the retrospective nature of our study,
we could not assess whether episodes of diarrhoea might have been related to an episode of hypocalcaemia or constipation to a period of hypercalcaemia or the intake of oral calcium. Similarly, we cannot rule that some episodes may have been related to an undiagnosed intestinal infection (Figure 15). Antifungal medication for oral candidiasis may also heal intestinal candidiasis. One patient had chronic giardiasis found on biopsies over 10 years and this may explain the symptoms (Figure 16). We failed to find any significant association between any other autoimmune features of APECED and the presence or absence of chronic diarrhoea, constipation or the alternation of the two symptoms (Original study I).

Figure 14. Lower gastrointestinal manifestations in Finnish APECED patients (n = 31) (Original study I)
2.1.4. Other gastrointestinal symptoms

Less than a third of the patients (29%, 9/31) complained of subjective symptoms associated with consuming dairy products. However, only 3 of them tested positive for lactose deficiency. A low-lactose or lactose-free diet did not lead to symptom remission, irrespective of the patients having been tested or not. One patient is still on a strict lactose-free diet but continues to have chronic diarrhoea (Original study I).

None of our patients had symptoms suggestive of gluten intolerance (Original study IV). Two patients had B12 vitamin deficiency, possibly in the context of pernicious anaemia.
2.2. Nephrologic and urologic clinical manifestations (Original study II)

The main manifestations are summarized in Figure 17. Ten percent of our patients (3/30) had tubulointerstitial nephritis (TIN), confirmed with histology and without any identified cause other than APECED. The outcome was different for each patient: one underwent kidney transplantation, one is under dialysis and the youngest is still under follow-up. The latter, aged 12 yrs, received immunosuppressive treatment including anti-CD20 chimeric Abs (Rituximab) with no efficacy on the creatinine levels. Renal histology disclosed the features of TIN with an interstitial lymphocytic infiltrate, fibrosis and tubular atrophy. The infiltrating lymphocytes were mainly T cells (CD4+ rather than CD8+) and rarely B cells (CD20+). Immunofluorescence analysis showed deposits of IgG, C1q, C3 and kappa and lambda chains on the tubular basement membrane (Original study II).

Figure 17. Main nephro-urologic manifestations in 30 Finnish APECED patients (Original study II)
3. Tissue immunohistochemistry and indirect immunofluorescence

3.1. Gastrointestinal tract (Original study I)
As reported previously, we found a decrease or a complete lack of enteroendocrine cells (EECs) in the GI tract in 50 to 77% of the intestinal samples based on immunostaining for chromogranin A and serotonin, respectively (Table 9, Figure 18). The EECs are present in most of the digestive tract. They are not regrouped in clusters, but are instead distributed singly throughout the GI tract and resemble the neurosecretory cells of the central nervous system. They produce gastrointestinal hormones (secretin, gastrin, and cholecystokinin), somatostatin, and neurotransmitters. We found that the lack of serotonin expression was significantly associated with the complaint of constipation (p = 0.035) (original publication I). We also observed the lack of expression of human beta defensin 5 (hD5), secreted by PCs in the GI crypts in 20% of our samples, possibly indicating the lack of PCs here (Original study I).

Table 9. CgA- and 5-TH positive cells in the intestinal tract of APECED patients who underwent endoscopic investigations

<table>
<thead>
<tr>
<th></th>
<th>Chromogranin A-positive/crypt</th>
<th>Serotonin-positive cells/crypt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total patients</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Biopsies</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Duodenal positivity</td>
<td>50% (5/10)</td>
<td>22% (2/9)</td>
</tr>
<tr>
<td>Ileal positivity</td>
<td>0% (0/1)</td>
<td>0% (0/1)</td>
</tr>
<tr>
<td>Colon and rectal positivity</td>
<td>0% (0/3)</td>
<td>0% (0/3) and 0% (0/2)</td>
</tr>
</tbody>
</table>
Lastly, we performed staining of villin, a 92.5kDa actin-binding cytoskeletal protein which associates with the apex and axial bundle structures of intestinal microvilli to mediate cytoskeletal organisation in the intestine. Several teams recently showed that villin is a potential target of Abs in IPEX patients (Kobayashi et al 2011, Lampasona et al 2013). As IPEX exposes to a paediatric form of autoimmune enteropathy, we investigated the possibility that villin is a possible target of autoimmunity in APECED. Compared with normal intestinal tissue (strong staining in the brush borders and cytoplasm, it disclosed fainter staining but also a wide range of reactivity patterns (apical expression, basal). However, we could not determine a specific pattern or the relevance of this abnormal expression in the gut (Original study I).

IL-17 is a key cytokine in the control of the gut microbiota (Blaschitz et al 2010, Rubino et al 2012). As APECED patients display circulating antibodies against IL-17, we hypothesised that IL-17 expression would be impaired in the intestinal samples of our patients. Thirteen samples, mainly for the duodenum, from 9 patients were stained for IL-17 expression. We observed that 77% of our samples showed a decrease or lack of expression of IL-17 (Figure 19). However, we could not confirm the relationship between symptoms and lack of IL-17 in the samples (Original study I).
Figure 19. IL-17 staining in a normal colon (left) and absence of staining in an APECED patient (right)

Indirect immunofluorescence (IIF) of normal duodenum and ileum with the sera of 29 patients revealed strong staining directed against cellular parts of PCs (20%, 6/29) and enterocyte brush borders (24%, 7/29) in the APECED patients (Figure 20). The sera of 15 healthy blood donors were negative for staining. The implication of such Abs against PCs is not yet clear as we failed to find a direct correlation between the lack of hBD5 expression and GI symptoms (Original study I). Interestingly though, one of our patients with strong IIF positivity against duodenal and ileal PCs had no duodenal hBD5 positivity.

Figure 20. Strong staining in the crypt of the duodenum with positive staining of the Paneth cells
3.2. Kidney (Original study II)

When we stained rat kidney sections with the sera of our patients, we observed in 30% of the sera (9/30) restricted positive immunostaining in the distal part of the nephron, consisting of the distal tubules, Henle’s loop and collecting tubules. The intensity varied among the patients, but no staining was seen in other renal structures, including the proximal tubular epithelium. All 3 patients with TIN disclosed this pattern (Figure 21). The sera of 15 healthy blood donors were all negative. There was consistent negative staining of rat spleen sections, which were used as a control tissue, for both patient and healthy blood donor sera (Original study II).

Figure 21. Orange staining of the distal part of the tubules with the serum of a young APECED patient with TIN (x 4)

3.3. Palpebra (unpublished data)

The question remains open as to whether the meibomian glands are affected. Indeed, a meibomian gland dysfunction causes symptoms that mimic keratitis, with eye itching, eye burning, puffy eyes, dryness, watery tears, crusty lashes, and ocular redness. We noted in sera from 28 patients that half of them (14/28, 50%) specifically stained the meibomian glands of palpebral rat section (Kluger N et al, data unpublished, Figure 22). None of the controls stained any structure of the palpebral rat sections.
4. Autoantibodies and autoreactive T cells

4.1. Circulating antibodies against cytokines (Original studies I and II)

Twenty-eight sera were assessed for circulating Abs against cytokines (Figure 23). Briefly, all the patients had Abs against IFN-α and IL-22 and 90% against IFN-ω. When correlating this antibody reactivity with the clinical symptoms, we found a statistical association with the presence of constipation and IL-17F Abs. We failed to find any association with TIN.
4.2. Circulating antibodies against tissue antigens (Original studies I, III and IV)

As emphasised in our review of the literature, APECED patients develop circulating Abs against a multitude of target antigens. Anti-TPH1 has been shown to be associated with GI manifestations (Ekwall et al 1998, Scarpa et al 2013). Anti-AADC is found in APECED (Söderbergh et al 2004) but has not been studied specifically in GI manifestations of APECED. Because harmonin (AIE-75) and villin are targeted by antibodies in autoimmune enteropathies (Patey-Mariaud de Serre et al 2009), especially villin in IPEX (Kobayashi et al 2011, Lampasona et al 2013), we investigated whether these Abs are found in APECED. We also investigated Abs against gastrin, a neurohormone that stimulates the secretion of gastric acid by the parietal cells of the stomach and helps in gastric motility. It is released by G cells in the pyloric antrum of the stomach, but also duodenum, and the pancreas (Figure 24).

In our series, 51 and 39% of the patients had anti-AADC and anti-TPH1 Abs (Original study I). The results are quite close to the prevalence found by Söderberg et al (Söderbergh et al 2004) and Husebye et al (1997). As a novel finding, we found a statistical association between anti-AADC positivity and constipation and anti-TPH1 and GID. We also observed a cumulative effect as patients with both Abs had more constipation and GID than those without these Abs (Original study I).

In addition, we observed that 1/3 of our patients had Abs against harmonin (AIE-75), an antigen of interest involved in autoimmune enteropathy (Patey-Mariaud de Serre et al 2009), and also villin, which has recently been named as a possible target in IPEX patients (Kobayashi et al 2011, Lampasona et al 2013). However, there was no statistical link between these Abs and the clinical symptoms.

The question of whether these Abs (AADC, TPH-1, AIE-75, villin) are directly involved in the physiopathogeny of GID remains unanswered. We cannot rule out that these Abs appear secondarily as a consequence of the immune aggression or simply reflect the thymic inability to negatively select autoreactive clones (Original study I).

On the other hand, we found no transglutaminase (for celiac disease, 0/24) or anti-desmoglein 1 (pemphigus foliaceus, 0/30) antibodies in our cohort of patients. Two patients disclosed positivity for anti-BP180 (bullous pemphigoid) with no clinical symptoms. Anti-desmoglein 3 was also positive at a low level in 2 patients without clinical symptoms. Lastly, 25% of our patients had antinuclear Abs at 1:80, a level not considered clinically significant (6/24) and none had anti-citrullinated peptide antibodies (Original study IV).
Thirty-six percent of the patients (16/44) and none in the healthy control group had CaSR Abs (p < 0.0001), while NALP5 Abs were found in 30% of the patients and in none in the control group (13/44, p < 0.0001). However, we found no statistical association between these Abs and the clinical manifestations, including HP, nor was there any gender prevalence among those with Abs. Only an association between a shorter duration of evolution of HP (less than 10 y) and CaSR positivity was found (Original study III).

4.3. AADC-specific T cell response (Original study I)

To better understand the background of the AADC-specific Abs, we studied 14 patients. Forty-three percent (6/14) of our patients and no healthy controls had a positive T cell response against AADC (Figure 25). However, no association was found between the T cell response, the presence or absence of AADC Abs, constipation and GID in this small patient sample (Table 10).

Table 10. T cell response, clinical symptoms and AADC-antibodies in 14 APECED patients

<table>
<thead>
<tr>
<th></th>
<th>ELISPOT + (6)</th>
<th>ELISPOT - (8)</th>
<th>p &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>GID +</td>
<td>50% (3)</td>
<td>62% (5)</td>
<td>NS</td>
</tr>
<tr>
<td>GID -</td>
<td>50% (3)</td>
<td>37.5% (3)</td>
<td>NS</td>
</tr>
<tr>
<td>Constipation +</td>
<td>50% (3)</td>
<td>37.5% (3)</td>
<td>NS</td>
</tr>
<tr>
<td>Constipation -</td>
<td>50% (3)</td>
<td>62% (5)</td>
<td></td>
</tr>
<tr>
<td>AADC-Abs +</td>
<td>66% (4)</td>
<td>62% (5)</td>
<td>NS</td>
</tr>
<tr>
<td>AADC-Abs -</td>
<td>33% (2)</td>
<td>37.5% (3)</td>
<td></td>
</tr>
</tbody>
</table>

NS: Not significant
5. Nonendocrine tissue-specific antibodies: simple reflections or active actors in autoimmunity in APECED?

In our studies, we observed that APECED patients had circulating Abs against various intestinal targets such as TPH-1, AADC, AIE-75, villin and Paneth cells (Original study I) and against renal tubular proximal cells (Original study II), and we confirmed the immunity against parathyroid antigens (Original study III). However, we failed to show a statistically significant association between circulating Abs and the symptoms of clinical failure of the involved organ, sometimes even with results that conflicted with the findings of previous studies published in the literature. There are several explanations for these discrepancies.

5.1. Origin of the patients and data collection

Our cohort includes only Finnish patients. In a high number of published series on Ab assays, the population has often been heterogeneous. Even when all patients are from Northern Europe, discrepancies are noted. For instance, a considerable variation in the prevalence of TPH-1 Abs in Swedish (100% of positivity), Finnish (37%) and Norwegian (22%) patients (Ekwall et al 1998) has been reported. Also, although an association between TPH-1 and
gastrointestinal dysfunction was found in Scandinavian patients, it was not found in an Italian series (Dal Pra et al 2004).

The size of the cohort may play a role in determining the prevalence of circulating Abs, and the variability in the assay methods is discussed below (6.2). Some manifestations are, by definition, so frequent in APECED, like HP, that the number of APECED patients without such a manifestation is too low for a significant result in a comparative study (Original study III).

An important aspect – too often neglected in our opinion – regarding GI manifestations is the lack of specificity about the reported symptoms. The studies are unclear regarding patient GI symptoms. For example, considerable confusion surrounds the definition of GI ‘dysfunction’: for some it is ‘steatorrhoea, watery diarrhoea, or constipation’ (Ekwall et al 1998) and for others it is only ‘malabsorption’ (Dal Pra et al 2004, Bratland et al 2013).

For this reason, we preferred to separate (chronic) ‘diarrhoea’ from (chronic) ‘constipation’ or the alternation of the two. Besides, malabsorption has a very specific definition, mainly based on confirmed steatorrhoea (Kluger et al 2013). As stressed in the discussion, both hypocalcaemia and infections can cause diarrhoea. Thus, confounding factors may explain the discrepancies among studies. Lastly, as stressed by all the studies, patients with no symptoms may also have Abs. Due to the retrospective nature of our studies, based mainly on self-reported symptoms, we cannot totally rule out a potential bias of recollection or misinterpretation of some of the symptoms.

5.2. Antibody detection methods

An important factor that may explain the discrepancies among studies is the method used. We used mainly ELISA assays with a cut-off of 3SD above the mean based on healthy blood donor results. However, other tests like the radioenzymatic assay using full-length or modified radiolabelled antigens have been used in other publications (Ekwall et al 1998, Dal Pra et al 2004), so the outcomes are not completely comparable. Interestingly, Dal Pra et al (2004) observed that Abs against AADC and TPH1 interacted with different epitopes and that different sera had different inhibiting effects, suggesting the heterogeneity of Ab binding sites. The potential in vivo implications are not clear but they may explain the discrepancies between studies about a given symptom and the presence or absence of an Ab of interest.
5.3. The role of the targets

Self-antigens are classified as the following: 1) self-antigens expressed constitutively in all cell types, 2) self-antigens of restricted tissue expression but present in the circulation at various levels, 3) self-antigens of restricted tissue expression that are undetectable in the circulation, and iv) sequestered antigens (Sospedra at al 2008). If the target of the Ab is expressed on the cell surface, the Abs may have a direct functional action through complement fixation or Ab-dependent cytotoxicity. Abs may also have antagonist or agonist activity when binding to the target. If the tissue antigen is intracellular (like intracellular enzymes), a direct effect of Abs is seen as rather improbable (Artstila et al 2013) and in this case the Ab would just be a stigma of the autoimmunity against the organs. With time, as the targeted organ is destroyed, the lack of immune stimulation may result in the disappearance of the Abs.

Lastly, the detected Abs could simply be an epiphenomenon, a reflection of the defect in deleting auto-reactive clones in the thymus and the periphery, leading to the production of a multitude of Abs with limited biological activity (Figure 26).
Figure 26. The genesis of anti-tissue antibodies: pathogenic or simple witnesses of autoimmunity in APECED. Abs are produced after exposure to antigens that are released after cytotoxic aggression by T CD8 cells and a lack of T reg regulation. Abs are detected by various assays and may be a useful tool to diagnose a disease if their sensitivity and specificity are high enough (at best 100%). Hypotheses of their pathogenicity include complement-dependent cytoxocity (1) and/or immune complex deposits (2). Abs may cross-react with other antigens (3), without certainty that such cross-reaction would be pathogenic.

6. What is the role of autoimmunity in the lower gastrointestinal manifestations of APECED?

6.1. Autoimmune enteropathy in APECED

As previously pointed out in the literature review section (Kluger et al 2013), there are three main groups of causes that can lead to lower GI symptoms: chronic infection, metabolic disorders, mainly hypocalcaemia, and autoimmune enteropathy. There are also other rarer causes, not discussed herein. In addition, various causes may be simultaneously combined or may explain different episodes in the patient. Hypocalcaemia and infection(s) should be
systematically ruled out in cases of chronic diarrhoea. Autoimmune enteropathy remains a diagnosis of exclusion. Arguments that support an autoimmune enteropathy include: i) the lack of EECs, ii) the existence of Abs against cellular targets on IFI, iii) circulating Abs against neurotransmitters in the gut, iv) the presence of a lymphocytic infiltration in the gut as reported in the literature in some patients (Kluger et al 2013), and v) the need for immunosuppressive treatment of such symptoms, even though its efficacy is variable (Ward et al 1999, Kluger et al 2013).

The clinical relevance of the brush border staining and the circulating Abs against AIE-75 and villin, which are established targets in IPEX, another cause of autoimmune enteropathy, is of unclear significance in APECED.

It is clear that additional (and possibly numerous) other factors play a role in the pathogenesis of the GI symptoms, including individual genetic and epigenetic factors, local cytokine production, the enteric nervous system, and the enteric microbiota. We hypothesise that immunity has a direct impact on the intestinal microbiome, which may explain the GI manifestations in APECED patients.

6.2. Enteroendocrine cells and the gut microbiota

The local resident intestinal microbiota (gut flora) plays an essential role in mediating host physiology, metabolism and immune response. For instance, this microbiota is essential for regulating proper nutrient and vitamin intake, preventing colonisation by bacterial pathogens and promoting the development of secondary lymphoid structures like Peyer’s patches and intestinal lymphoid follicles (Rubino et al 2012).

The EECs are involved in intestinal growth, blood flow, motility, secretion of pancreatic enzymes and bile. These cells, located throughout the intestinal tract, are accessible to enteric microbiota on the lumen side but are also in contact with afferent and efferent nerve terminals on the lamina propria side, which provides them contact with the central nervous system (Rhee et al 2009). They secrete numerous peptides in response to physiological or pathological stimuli, including serotonin (5-HT). 5-HT can be secreted on both sides of the intestinal epithelium by the EECs, into the intestinal lumen or into the basolateral sides. Pathogenic bacteria in the GI tract can increase 5-HT secretion in the lamina propria (Rhee et al 2009). Similarly, the enteric microbiota can modulate intestinal motility and gut transit. Disruption of the balance between enteric microbiota populations might induce gut motility and secretion alterations that would in turn influence the balance of enteric microbiota (Rhee
et al 2009). Therefore, the lack of EECs can explain severe GI manifestations. However, there is not a strict correlation and the disappearance of EECs is not permanent, as these cells typically have a high turnover. We observed a significant association between the lack of 5-HT expression in the intestinal tissue and constipation. In addition, we report here a novel association between constipation and AADC Abs, AADC being an enzyme involved in the serotonin pathway. Therefore, a cellular immune aggression directed against EECs combined with Abs against the 5-TH pathway may impair the secretion of 5-HT in the gut. In fact, 5-HT levels were shown to be lower in patients with TPH-1 Abs (Scarpa et al. 2013). Different targets on the same pathway may have cumulative effects. A dysfunction of the 5-HT pathway in the gut may disturb the crosstalk between the microbiota, the gut and the brain, leading to dysbiosis (Frank et al 2011, Salzman et al 2013).

6.3. The IL-17/IL-22 pathway and the gut microbiota in APECED

It is currently unknown whether IL-17- and IL-22-neutralising Abs have an influence on gut physiology and the GI manifestations of APECED. The microbiota is required for the generation of Th17 cells in the lamina propria. Segmented filamentous bacteria (SFB, members of the *Clostridium* genus) induce a Th17 response in the small intestine (Rubino et al 2012). Moreover, SFB also upregulate basal gut IFN$\gamma$+ Th1 and FOXP3+ regulatory T cell responses (Rubino et al 2012). Th17 cells and the subset of cytokines they produce, such as IL-17A, IL-17F and IL-22, are involved in the organisation of the mucosal defence against pathogens (Blaschitz et al 2010, Rubino et al 2012). Interestingly, $\gamma$δ T cells, NK, NKT and dendritic cells are also involved in the production of IL-17 and IL-22 (Blaschitz et al 2010). Thus, IL-17 and -22 prevent infection dissemination and upregulate chemokines (CXCL-8, CCL20) and G-CSF, thereby recruiting and activating neutrophil cellular responses at inflammation sites and antimicrobial responses (iNos, lipocalin-2, defensins); they also reinforce tight junctions between enterocytes and enhance cell proliferation (Blaschitz et al 2010, Rubino et al 2012). We could not find any link between the lack of IL-17 expression in the mucosa and the presence of clinical lower GI symptoms. However, the presence of IL-17 Abs, which are known to have neutralising activity, and the diminished expression of IL-17 in the small intestine of some of our patients are strong indications of an impact on the gut flora. For instance, chronic intestinal giardiasis has been reported in APECED patients and was observed in one of our patients (Kluger et al 2013). It was recently shown that IL-17 production is important in the
clearance of a *Giardia muris* infection with early induction of peroxisome proliferator-activated receptor alpha (Dreesenet al 2014). Thus, the IL-17 pathway may favour not only CMC in APECED but also the occurrence of intestinal infections (bacteria, candidiasis or parasitic infections).

**6.4. Paneth cells and the gut microbiota in APECED**

Paneth cells are located at the base of the small intestinal crypts of Lieberkühn, interspersed with epithelial stem cells at this location. They have an intensive secretory activity for a wide variety of peptides, the most abundant being the antimicrobial peptides (Salzman et al 2013), especially a-defensins, but also lectins, lysozyme and secretory phospholipase A2, which contribute to mucosal host defence. They regulate the composition of the intestinal bacterial microbiome (Salzman et al 2010) and they protect from pathogenic microbes (Salzman et al 2013). Mouse models overexpressing hD5 show a significant loss of segmented filamentous bacteria (SFB), resulting in reduced numbers of Th17 cells in the lamina propria. PC defensins may thus regulate the small intestinal microbiome (Salzman et al 2010). They are also involved in maintaining the growth and survival of crypt stem cells (Salzman et al 2013). There are various genetic and environmental factors that can induce PC dysfunction (Salzman et al 2013), but direct immunity as a cause of PC depletion has not been reported, to our knowledge.

The role of the circulating Abs against PCs remains to be defined in APECED, but a pathophysiological role is possible. The precise target is not known; although the immunostaining we observed was located in the granules, it could be directed to one of the antimicrobial peptides. As some of our patients had clearly specific Abs against granules and we observed the lack of hD5 expression in 20% of our cases, defensins could be a possible target of autoimmunity in APECED.

However, as stressed by Salzman et al, it is not always clear whether modification of the microbiota is a cause or the consequence of PC dysfunction (Salzman et al 2013) (Figure 27).
Figure 27. The role of innate IL-17 and IL-22 responses to enteric bacterial infections and the endocrine cell-mediated signalling in the enteric microbiota-host interaction (adapted from Rubino et al 2012 & Rhee et al 2009). Dendritic cells (DC) are activated by pathogens and secrete several cytokines that stimulate different subsets of T cells (Th17 cells, γδ T cells, NK and NKT cells) to secrete IL-17 and IL-22. IL-17 promotes amplification of the host response by recruiting neutrophils, while IL-22 promotes barrier functions by enhancing production of antimicrobial peptides from Paneth cells. The Th17 response prevents bacterial dissemination from the gut and promotes colonisation of the mucosa by pathogens resistant to some of the antimicrobials. Furthermore, EECs can secrete 5-HT on either side of the intestinal epithelium either under the influence of the microbiota or under the action of afferent neurons in response to the content of the digestive tract.
6.5. From autoimmunity to disturbance of the gut microbiota (dysbiosis) in APECED

Based on all these data, we hypothesised that constipation is most likely related to EEC deficiency/dysfunction of the serotonin pathway in the gut through autoimmune aggression. The Abs against AADC (and TPH1) could promote symptoms of constipation by blocking the serotonin metabolism. Conversely, isolated chronic diarrhoea may have additional causes (hypocalcaemia, candidiasis, infections, malabsorption) (Kluger et al 2013) or the calcium supplementation itself is responsible for constipation. Of course, other Abs and pathways may play additional roles, such as histidine decarboxylase as reported previously.

Furthermore, the Abs against PCs combined with those against the IL-17/IL-22 pathway may modify the local microbiota. They may facilitate the growth of infectious agents such as *Candida albicans* or *Giardia lamblia* and lead to symptoms, adding on possible other causes such as hypocalcaemia or pancreatic exocrine failure.

According to the models of dysbiosis (*the modification of microbiota to an unfavourably skewed one*) as highlighted by Frank et al (Frank et al 2011), we think that the GI manifestations in APECED fit best with the following pathway: the dysbiosis arises because of the pathological condition itself (autoimmunity) and the change in composition of microbiota may further contribute to disease severity and/or duration (Figure 28). Therefore, future prospective studies on the intestinal microbiota of patients with newly diagnosed APECED are warranted.
Figure 28. Significance of dysbiosis. Dysbiosis is a primary factor causing disease pathogenesis (a), dysbiosis has no aetiological consequences on the disease but is the result of aetiological agents (b), dysbiosis is the result of the disease itself (c), and the change in composition in microbiota might contribute to disease severity, duration or frequency (d). In the case of GI manifestations in APECED, we speculate that the path is (c)/(d) (adapted from Frank et al (Frank et al 2011)).

7. The quality of life of APECED patients and AD/APS-2 patients (Original studies V and VI)

Twenty-six patients with APECED took part in our study on HRQoL (Original publication V). Briefly, we found that general health perceptions and energy/fatigue/vitality were the most affected items in the SF-36 questionnaire (Figure 29). Male patients also seemed more affected than women, even though the difference was not significant. We could not find any correlation between the outcomes of the SF-36 items and the number of clinical components that the patient had. We also used a specific depression-related questionnaire (RBDI) to detect whether these patients had depressive symptoms. One third (30%) of the patients had mild to severe depressive symptoms, but only 7.7% had moderate to severe depressive symptoms. Six patients had mild symptoms, while 2 patients, under antidepressive treatment, still had
moderate to severe depressive symptoms. Two additional patients were under antidepressive therapy with good efficacy as they both scored 0. Fatigue, well-being, and general health were negatively correlated with the RBDI score. In other terms, depressive symptoms were associated with increased fatigue and decreased well-being and general health perceptions. Lastly, we investigated whether visible skin manifestations such as vitiligo, candidiasis or AA had an impact on QoL using a specific questionnaire related to skin disease, the DLQI. Overall, the impact of skin disease was rather low. Not surprisingly, women with AA universalis reported the highest scores of impairment, even though these scores indicated only a ‘moderate’ effect on QoL. Before our study, only Løvås et al had assessed the HRQoL of APECED patients but in a smaller series (Løvås et al 2002), and our results are close to their observations. We found some interesting results in our studies despite the small number of patients, the obvious design limitations with the small sample size, the lack of an age-, sex- and dispersed geography-matched Finnish healthy control group, and the absence of data regarding other potentially confounding factors like socioeconomic features. One of these was that HRQoL was not as impaired as one might have anticipated. The accumulation of disease components did not have an impact on HRQoL. The male patients appeared to be more fragile, although the difference was not significant. We also concluded that the older the patients were, the better they coped, as illustrated by a negative correlation between a low total RBDI score, elevated age and long duration of this disease. Such results may be explained mainly by the fact that we interviewed a cohort of patients who have lived a long time with the disease (e.g. 35 yrs) and have learned to cope with it. Besides, disabling symptoms like CMC improve with time and patients are used to adapting self-medication at the first sign of symptoms. The beneficial effect of the patients’ association is also possible, as most of the patients were members. The low number of patients may help ensure the quality of peer support. Nevertheless, our results cannot be applied to an individual level, and clearly some patients suffered more from the disease at certain points in their lives due to the accumulation of components or because of specific disabilities (Figure 30). We also observed that young patients seemed to be more sensitive to an impairment in QoL.
Figure 29. SF-36 quality of life outcomes for the Finnish APECED patients

![SF-36 Quality of life of APECED patients (n = 26)](image)

Figure 30. The overall burden of APECED

- **Life threatening situations**
  1. Due to the disease
     - Adrenal crisis
     - Hypocalcemia
     - Asplenia (sepsis)
     - Autoimmune hepatitis
     - Autoimmune bronchiolitis
  2. Immunosuppressive therapies
  3. Cancer
     - Oral cancer
     - Oesophageal cancer
     - Gastric cancer

- **Multiple daily and lifetime oral hormonal supplementation**
- **Oral and mucosal chronic candidiasis**
- **Infertility**
- **Eye involvement**
  - Vision impairment
  - Blindness
- **Skin manifestations**
  - Vitiligo
  - Alopecia
  - Nails dystrophy
  - Enamel dystrophy
- **Depression**
  - Reactive or organic
  - Addison’s disease
  - Hyperthyroidism
  - Diabetes type 1
  - Pituitary deficiency

- **Fear of developing additional components during life**
One year later, we performed a similar study of HRQoL among Finnish patients with AD and/or APS2 (Original study VI). For this study, we used 2 HRQoL scales: the 15D (Figure 31) and the SF-36 (Figure 32). This 15D score study is much stronger as we compared the data with an age- and gender-standardised population. There was a significant impairment of the 15D score in the patient group compared with the controls (0.853 vs 0.918, p < 0.001). The impairment in HRQoL was related to patients’ association membership, female gender, lower education, other autoimmune or inflammatory disorders that were not part of APS-2, and longer disease duration. Interestingly though, having APS-2 (as opposed to only AD) did not lead to any significant difference, nor did the number of autoimmune components of APS-2, again indicating that this criterion was not decisive in our Finnish cohort. Another very positive aspect was that the Finnish AD patients were treated according to the current hydrocortisone substitution recommendations (a hydrocortisone dose of 20 mg/d). As we did not perform a comparative study with a normal population using the SF-36 in either study, we cannot draw direct comparisons between these two. There are numerous factors that might have interfered, such as the age of the cohort and also the delay in disease progression, as typically AD/APS2 is diagnosed during adulthood.

We also wondered about the possible impact of belonging to the patients’ association, since we observed in the second study that belonging to the patient’s association was associated with lower HRQoL. We speculate a possible deleterious impact of membership on the QoL for patients with only AD/APS2 because they belong to an association guided by patients with APS1. Despite a similarity in the name, APS1 and APS2 are quite different diseases. Mixing patients with different diseases under the same association may have an impact on HRQoL. The patients’ expectations may be different in terms of information to patients, topics dealt with in patient meetings and overall patient objectives.
Figure 31. 15D score outcomes for the Finnish AD patients and the control group

Health-related quality of life in patients with Addison’s disease compared to that of age-matched general population

Figure 32. SF-36 quality of life outcomes for the Finnish AD and APS-2 patients

SF-36 Quality of life in Addison's disease (n = 43) and APS-2 (n = 64) patients

Physical functioning
Physical role limitations
Emotional role limitations
Energy/Vitality
Emotional well being
Social Functioning
Bodily pain
General health perception
CONCLUDING REMARKS AND FUTURE DIRECTIONS

Despite the clear monogenic background, the pathogenesis of human APECED remains unresolved. The reasons why patients present such a diversity of manifestations is unclear. Our study showed that gastrointestinal manifestations were frequent when patients were specifically asked to report them. Patients develop Abs against various antigens (TPH-1, AADC, AIE-75, etc.) in the gut, mainly neuro-endocrine targets. The pathogenic relevance of such Abs is nevertheless not that clear. The GI manifestations seem to be multifactorial, including disturbances in calcium metabolism, possible intestinal infections, and humoral and cellular autoimmunity directed against the gut tissue. We hypothesised that all these factors may affect the local gut microbiota of patients, leading to a vicious circle. Further studies should focus on analysing the gut microbiota to assess whether or not it is disturbed in APECED patients and related to their GI symptoms. Moreover, studies on Abs in APECED in general should screen multiple Abs and try to link them to a symptom rather than just to one autoantibody. The targets of immunity in the kidney seem to be located towards the renal tubules, yet the antigens remain to be identified. Our study confirmed that distal tubular cells are likely to be the target of autoimmunity during APECED. Specific immunoscreening with serum samples from APECED patients of a human renal cDNA library should help to detect possible molecular renal targets.

We failed to show that CaSR and NALP5 antibodies were specific or sensitive markers for HP in our cohort. The precise role of the autoimmune response against CaSR and NALP5 in the pathogenesis of APECED is still unknown. A parathyroid-specific antigen associated with HP to allow serologic diagnosis of the disease is still missing.

The role of cytokine Abs and how they influence autoimmune manifestations remain to be elucidated. APECED patients seem to be protected from some of the ‘classic’ autoimmune diseases such as systemic lupus or polyarthritis, raising questions about whether these Abs are protective.

HRQoL is impaired for some of the patients with APECED, but not as much as one would have expected when looking at the accumulation of symptoms. The role of peer support via a patients’ association may well be involved, as would better patient management in Finland than in the past. Interestingly, the question of whether patients with AD and APS-2 should still be affiliated with the association of APECED patients is open for debate, as patients with AD/APS-2 have a different profile than that of APECED patients and, paradoxically, this may impact their HRQoL.
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