Autoimmune polyendocrine syndrome type I

Novel diagnostic assays and immune regulation

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Scientific Environment

The research contained in this thesis was performed from 2007 to 2011 at the Section for Endocrinology, Institute of Medicine, University of Bergen, under the leadership of Professor Eystein S. Husebye and Dr. Anette S. B. Wolff.

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Summary

Autoimmune polyendocrine syndrome type I (APS-I) is a rare, monogenetic recessively inherited disease caused by mutations in the autoimmune regulator (*AIRE*) gene. The patients display different endocrine and ectodermal manifestations, where the majority develop at least two of the three main components of autoimmune adrenocortical failure (Addison's disease), hypoparathyroidism and chronic mucocutaneous candidiasis (CMC). In addition, patients frequently develop autoantibodies against molecular targets in their affected organs and against cytokines of the immune system.

In two studies, robust immunoassays detecting APS-I patients with high sensitivity and specificity were developed. Autoantibodies against *in vitro* transcribed and translated interferon omega (IFN-ω) and interleukin (IL) 17F and IL-22 were measured, the latter shown to correlate to CMC. Antibodies against IFN-ω were confirmed as a universal marker for APS-I, suggesting this assay as an ideal screening tool preceding mutational analyses in suspected APS-I cases. We have shown that single nucleotide polymorphisms and variation in copy number of *AIRE* have no association with Addison's disease. However, large deletions in one allele of *AIRE* together with a concomitant disease-causing mutation were discovered in two APS-I patients, underpinning the importance of copy number analysis. A wide range of immune cell subsets were studied, identifying disturbances in the regulatory T cell compartment together with less $CCR6^{+}CXCR3^{+}$ T cells and a reduction in $CD16^{+}$ monocytes in patients with APS-I, possibly resulting in susceptibility for developing autoimmune manifestations.

The characterisation of genetic variations in *AIRE* and of abnormalities in the immune cell subsets contribute to our understanding of the function of AIRE, while establishing immunoassays ease the identification of APS-I patients.

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Abbreviations

17-OH	steroid cytochrome P450 17-alpha-hydroxylase
21-OH	steroid cytochrome P450 21-hydroxylase
AAD	autoimmune Addison's disease
AADC	aromatic L-amino acid decarboxylase
AChR	acetyl-choline receptor
AHR	aryl hydrocarbon receptor
AIRE	autoimmune regulator
AP-1	activator protein-1
APC	antigen presenting cell
APECED	autoimmune polyendocinopathy-candidiasis-ectodermal dystrophy
APS	autoimmune polyendocrine syndrome
AVINA	antiviral interferon neutralising assay
BCR	B cell receptor
Bp	base pair
CARD	caspase recruitment domain
CaSR	calcium sensing receptor
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CMC	chronic mucocutaneous candidiasis
CNV	copy-number variation
Cpm	counts per minute
CTLA4	cytotoxic T-lymphocyte antigen 4
CV	coefficient of variation
CXCL	chemokine (C-X-C motif) ligand
CXCR	CXC Chemokine receptor
CYP1A2	cytochrome P450 1A2
DC	dendritic cell
DP	double positive
DTT	dithiothreitol

ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
eTAC	extrathymic Aire-expressing cell
FGF	fibroblast growth factor
FN	false negative
FOXP3	forkhead box P3
FP	false positive
GAD65	glutamic acid decarboxylase 65
H3K4me0	unmethylated lysine 4 of histone 3
HLA	human leukocyte antigen
HSR	homogeneously staining domain
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNKT	invariant natural killer T cell
IPEX	X-linked immunodysregulation, polyendocrinopathy enteropathy
JNK	c-Jun N-terminal kinase
LD	linkage disequilibrium
LTi	Lymphoid tissue inducer cells
MAPK	mitogen-activated protein kinase
MG	myasthenia gravis
MHC	major histocompability complex
MS	multiple sclerosis
mTEC	medullary thymic epithelial cells
NALP5	NACHT leucine-rich-repeat protein 5
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NKT	natural killer T-cell
OMIM	online Mendelian inheritance in men
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PHD	plant homeodomain zinc finger
PLZF	promyelocytic leukemia zink finger
PRRs	pattern recognition receptor

PTPN22	tyrosine-protein phosphatase non-receptor type 22
PTA	peripheral tissue antigen
RA	rheumatoid arthritis
Rb-1	retinoblastoma-1
RIA	radioimmunoassay
RLBA	radioligandbinding assay
ROAS	register for organ-specific autoimmune diseases
ROR	retinoic-acid-receptor-related orphan receptor
RORγt	RAR-related orphan receptor C gamma
SAND	named after Sp100, AIRE-1, NucP41/75, DEAF-1
SCC	cholesterol side chain cleavage enzyme
SD	standard deviation
SLE	systemic lupus erythematous
SNP	single nucleotide polymorphism
SP	single positive
STAT	signal transducer and activator of transcription
T-bet	T-box transcription factor
Tc	cytotoxic T cell
TCR	T cell receptor
Th	T helper cell
TH	tyrosine hydroxylase
TLR	toll-like receptor
TGF	transforming growth factor
TN	true negative
TNF	tumor necrosis factor
TF	true positive
TPH	tryptophan hydroxylase
Treg	regulatory T cell

List of Publications

This thesis is based on the following papers, and will be referred to in the text by Roman numerals:

Paper I

AS Bøe Wolff*, B Oftedal*, S Johansson, O Bruland, K Løvås, A Meager, C Pedersen, ES Husebye, PM Knappskog (2008)

AIRE variations in Addison's disease and autoimmune polyendocrine syndromes (APS): Partial gene deletion contribute to APS I

Genes and Immunity 9, 130-136

*These authors contributed equally to this work

Paper II

BE Oftedal, AS Bøe Wolff, E Bratland, O Kämpe, J Perheentupa, AG Myhre, A Meager, R Purushothaman, S Ten, ES Husebye (2008)

Radioimmunoassay for autoantibodies against interferon omega; its use in the diagnosis of autoimmune polyendocrine syndrome type I

Clinical Immunology 129, 163-169

Paper III

AS Bøe Wolff, BEV Oftedal, K Kisand, E Ersvær, K Lima, ES Husebye (2010)

Flow Cytometry Study of Blood Cell Subtypes Reflects Autoimmune and Inflammatory Processes in Autoimmune Polyendocrine Syndrome Type I

Scandinavian Journal of Immunology 71, 459-467

Paper IV

BEV Oftedal, O Kämpe, A Meager, KM Ahlgren, A Lobell, ES Husebye, AS Bøe Wolff (2011)

Measuring Autoantibodies against IL-17F and IL-22 in Autoimmune Polyendocrine Syndromes Type I by Radioligand Binding Assay Using Fusion Proteins

Scandinavian Journal of Immunology 74, 327-333

Introduction

A functioning immune system is a necessity for survival as it provides protection from a wide array of bacteria, viruses and fungi that we encounter in our daily lives. If the physical barriers of the skin and mucosal membranes are breached, invading pathogens are cleared by the innate and adaptive immune responses, which include a broad range of signalling and effector proteins, and different cell-types. This is a process demanding strict control, to assure clearance of foreign agents without causing tissue damage. In some instances this control fails and the immune cells become autoreactive, resulting in an autoimmune disease [1].

Autoimmune diseases are caused by the combined effects of multiple susceptibility genes, environmental triggers and stochastic events. However, a few monogenic autoimmune diseases exist, which constitute powerful models. Among them, autoimmune polyendocrine syndrome type I (APS-I) has thought us how central tolerance is developed. In the following, a brief introduction to the immune system and autoimmunity is given, with particular emphasis on APS-I and its underlying autoimmune regulator (*AIRE*) gene.

Innate immunity

The innate immune reactions are mediated by cells like dendritic cells (DCs), macrophages, neutrophils and natural killer cells, in combination with circulating proteins of the complement system, and anti-microbial peptides [2, 3]. This immediate response utilises a limited number of germ-line encoded pattern recognition receptors (PRRs) which detect pathogen-associated molecular patterns (PAMPs) found in most microorganisms [4]. The best characterised PRRs is the Toll-like receptors (TLRs), which trigger activation of adaptive immune responses and

control them at multiple levels, e.g. the induction of T helper (Th) 1 cells, production of immunoglobulins (Igs) by B cells, and activation of cytotoxic T cells (Tc) [5].

Adaptive immunity

The adaptive immune system depends on randomly generated receptors clonally displayed on the membranes of T and B cells in order to fight down infection. These receptors have infinite specificity in their recognition of antigen, and although the adaptive immune system acts slower than the innate branch, it inhabits immunological memory [6].

B cells

When the B cell receptor (BCR) encounters its specific antigen in its native form, usually in the presence of cytokines provided by Th cells, the B cells shift to clonal expansion. Clones with identical receptor specificity are then produced; most will develop into plasma cells and produce large amounts of Igs while some develop into memory cells that can be rapidly reactivated on the next encounter with the antigen. The secreted Igs are quite similar to the BCR, only without a transmembrane region. The different isotypes have different effector functions and are named by their constant region as IgA, IgD, IgG, IgM, and IgE, and bind soluble antigens by their variable domains. Destruction of the pathogen by interaction with the secreted Igs either leads to neutralisation of the pathogen, marks it for opsonisation, or activates the complement system. In addition to the production of Igs, B cells up-regulate co-stimulatory molecules, they express antimicrobial activity and secrete factors directly mediating microbial destruction, in addition to act as antigen presenting cells (APC) for T cells [7].

Failure of establishing B cell tolerance in the bone marrow [8-12] or in the outer T cell zones of the spleen where the antibody response to foreign antigens is initiated, may result in autoreactive B cells and the production of autoantibodies [13, 14]. These autoreactive B cells are thought of as important APCs for T cells in the initiation and development of an immune response toward self-antigens driven by cluster of differentiation (CD)4^+ cells [15, 16], and may promote autoimmunity by multiple pathways and mechanisms [17].

T cells

The heterogeneous group of T cells play a central role in cell-mediated immunity. They commit to linages specified by their surface markers, as single positive (SP) CD8 or CD4 expressing T cells. These surface markers direct their interaction with the major histocompability cluster of genes (MHC), where CD8 are specific for MHC class I expressed on all nucleated cells, and CD4 for MHC class II, expressed on B-cells, dendritic cells and activated macrophages. The $CD8^+$ Tc killer cells eliminate infected cells of the body, which display peptides of their invading agent on MHC class I. The cytokine-secreting $CD4^+$ Th cells diverge into different subset, a paradigm established by the discovery of the distinct Th1 and Th2 subsets in mice in 1986 [18]. The differentiation of a naïve $CD4^+$ T cells into the different groups of (i) proinflammatory effector (Th) cells or (ii) regulatory T (Treg) cells depend on the cytokine milieu influenced by the innate immune system and type of invading organism, together with the strength and duration of the interaction between the T cell and the APC [19]. Different transcription factors characterise each Th cell subset and drive the expression of distinct soluble mediators and surface molecules in response to a specific microbe, to initiate clearance of the pathogen and tissue inflammation (Fig. 1). This initial proinflammatory response by the Th cells is followed by an activation of the Treg cells, which dampen the immune response to limit collateral tissue damage [20-22].

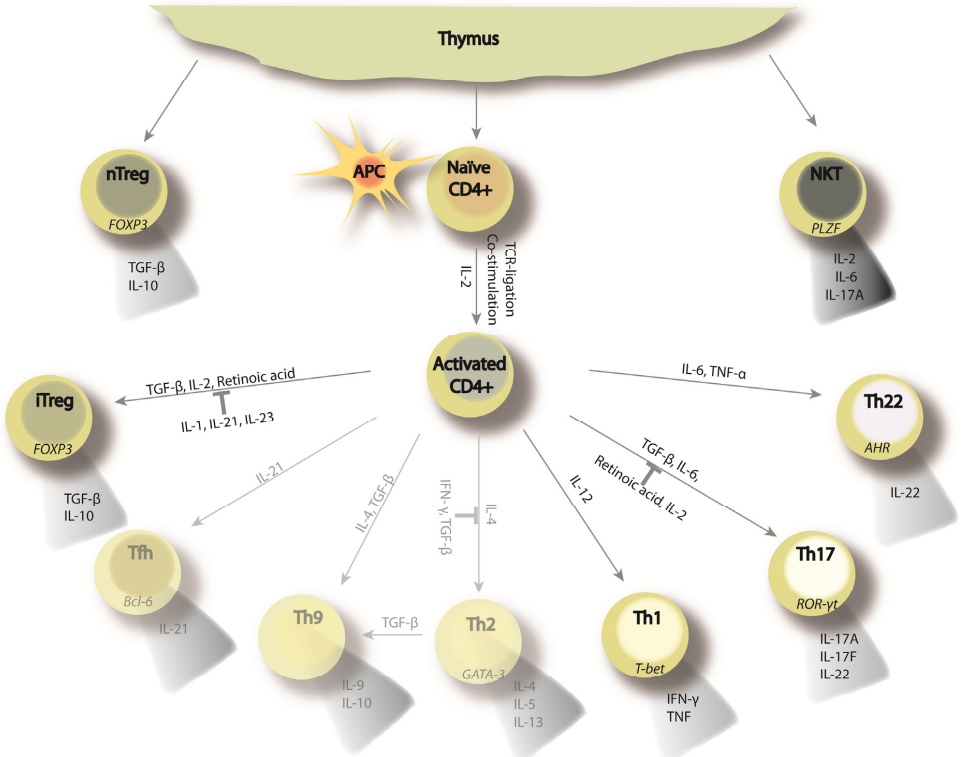


Figure 1. Overview of the CD4$^+$ T cell differentiation. The T cell-subsets that will be emphasised here are highlighted, including their specific transcription factors, and some of their secreted effector molecules. Upon interaction with an APC, the naïve CD4$^+$ cells separate into different subsets due to their developmental programs, dependent on the cytokine environment. Each subset is characterised by expression of cytokines that regulate other immune cells. Commitment to the **Th1** linage is promoted by interleukin (IL) 12 and the type I interferons (IFNs) in humans [23] to activate their signature transcription factor T-bet (T-box transcription factor) [24]. The Th1 cells produce the proinflammatory cytokine IFN-gamma (IFN-γ) to aid the clearance of viruses and intracellular bacteria, in immune reactions that may cause tissue damage [25]. The **Th17** cells are mainly promoted by tumor growth factor (TGF) β and IL-6 [26, 27], and are dependent on the transcription factor RAR-related orphan receptor C gamma (RORγt) [28] to express IL-17A, IL17-F, IL-22 and IL-26. They are responsible for the clearance of extracellular bacteria and fungi, especially at mucosal surfaces. The **Th22** subset is promoted by IL-6 and tumor necrosis factor (TNF) α to produce IL-22 and is dependent on the transcription factor aryl hydrocarbon receptor (AHR) [29-31]. The **Tregs** are recognised by surface expression profile consisting of CD25, CD62L and specific CD45 isoforms [32-34] and their transcription factor forkhead box P3 (FOXP3). They regulate the immune homeostasis and control unwanted immune responses [35-37]. Subpopulations include the functionally similar **iTregs,** which are induced in the periphery, and the natural **nTregs**, generated in thymus. The nTregs are regarded as stable, while the iTreg are induced by TGF-β, thereby sharing a signal of differentiation with Th17, establishing a balance between the proinflammatory and anti-inflammatory cell subsets. It is still unclear how to separate nTreg from iTreg cells, as are any differences in biological functions. **NKT** (natural killer T) cells have a restricted TCR-repertoire, and inhabit an activated, memory cell phenotype, possibly dependent on the transcriptional regulator promyelocytic leukemia zinc finger (PLZF) for their development [38, 39].

Autoimmune diseases

Autoimmune diseases include a wide range of conditions, differing in severity and symptoms. In the developed world, they affect five to ten per cent of the population [40]. Almost every endocrine organ is a potential target for an autoimmune attack, and several may be targeted at the same time as part of a polyendocrine syndrome. The most common endocriopathies include Hashimoto's thyroiditis, Graves' disease and type 1 diabetes [41], and their pathogenesis and etiology are still obscure, but most are thought to result from a combination of environmental and genetic factors. The last decades have brought an increase in the incidence of type 1 diabetes [42, 43], possibly due to the relative reduction in childhood infections and the development of extensive immunisation programs, proposed as the "hygiene hypothesis" [44]. The human leukocyte antigen (HLA) genes are among the most important genetic risk factors, where distinct genotypes predispose for different autoimmune diseases. Yet, the exact polymorphisms that confer risk are hard to elucidate due to the extensive linkage disequilibrium (LD) in the HLA region [45]. A few monogenic autoimmune diseases, such as APS-I and immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), have been especially powerful models in studies of the immune system and how it fails in autoimmunity.

Autoimmune polyendocrine syndrome type I

APS-I (online mendelian inheritance in man (OMIM) 240300), also known as APECED (autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy), is a monogenic, autoimmune disease leading to several endocrine and ectodermal manifestations as well as to chronic mucocutaneous candidiasis (CMC). The disease is rare (about 1: 90 000 in Norway [46]), but more common in certain populations such as the Finnish (1:25 000), Sardinian (1:14 000) and among Iranian Jews (1: 9000) [47]. The disease typically presents early in childhood, but the patients gradually develop autoimmune manifestations throughout life.

Diagnosis

Clinical diagnosis is based on the presence of two of the three main components; CMC, primary hypoparathyroidism and autoimmune adrenocortical insufficiency (Addison's disease). Patients often suffer from other manifestations, like primary ovarian failure, chronic diarrhoea and malabsorption, keratitis, autoimmune hepatitis, vitiligo, alopecia and enamel hypoplasia (Fig. 2) [47-49]. One of the three major components is sufficient for diagnosis if a sibling already is identified with APS-I, or if a mutation is found in both alleles on *AIRE*. APS-I should be suspected if one of the three major components present before the age of thirty, together with at least one of the other components [50].

Left undiagnosed and untreated, APS-I may have a fatal outcome. In 1962, sixteen out of twenty-three (69.5%) patients died before the age of thirty [51], while this number decreased to four out of forty-one (10%) in a patient group followed from 1967 to 1996 [47]. Early diagnosis, close monitoring of new disease-components and suitable treatment are therefore essential for this group of patients, making the need for good diagnostic assays and awareness among the general physicians of outmost importance.

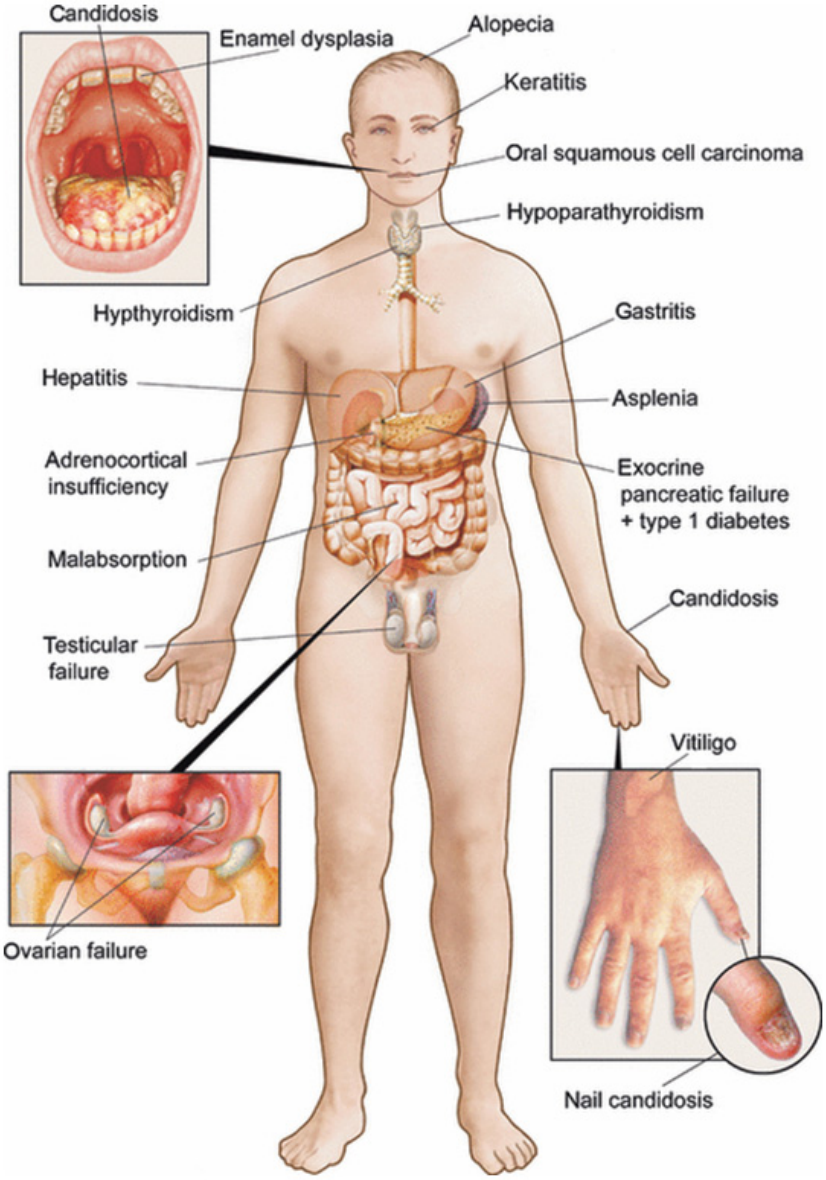


Figure 2. Illustration of the different manifestations in patients suffering from APS-I. Adrenocortical insufficiency, hypoparathyroidism and chronic mucocutaneous candidiasis are the three main symptoms of APS-I, and a wide array of different additional manifestations are usually present. Reproduced with permission from *Husebye et al. Journal of Internal Medicine, 2009* [50]

The major components

Chronic mucocutaneous candidiasis

CMC is often the first sign of APS-I, presenting in the first years of life as *Candida Albicans* infections of nails, skin, and the mucous membranes in the mouth, oesophagus and vagina. From a study of the Finnish patients, one in six was diagnosed with CMC by the age of one, which increased to ninety-eight per cent by the age of thirty [49], and corresponding numbers are found among the Norwegian patients [46]. The infections differ in severity between the patients, but are often recurrent and difficult to treat. Prolonged oral CMC may increase the risk of squamous cell carcinoma, and it is therefore important with proper and antifungal treatment [52].

Hypoparathyroidism

Hypoparathyroidism is the second most prevalent feature of APS-I, and typically presents in eighty-five per cent of the patients by the age of thirty [49], which is also representative for the Norwegian cohort regardless of age [53]. This is usually the first endocrine manifestation, and is more prevalent in women than in men [54]. Hypoparathyroidism is caused by autoimmune destruction of the parathyroid glands which decrease plasma calcium leading to paraesthesias and cramps. It is treated with vitamin D analogues and calcium and magnesium supplementation, but is the most challenging APS-I disease-component to manage [50]. The immunological target within the parathyroid cells is the NACHT leucine-reich repeat protein 5 (NALP5), and autoantibodies against NALP5 are correlated to hypoparathyroidism [55].

Adrenal insufficiency

Autoimmune Addison's disease (AAD) typically appears after CMC and hypoparathyroidism, as the third most common feature in APS-I, with a seventy-eight per cent prevalence among the Finnish patients by the age of thirty [49]. In a

Norwegian survey, eighty per cent of the cohort had this manifestation, regardless of age [53]. Addison's disease was first described by Thomas Addison in 1855 [56], and is characterised by fatigue, salt craving, weight loss and increased pigmentation of the skin and mucus membranes. The first serological sign of AAD is autoantibodies against 21-hydroxylase (21-OH) which can present up to years before any biochemical and clinical evidence of adrenal insufficiency [57, 58]. Early diagnosis is important, as untreated AAD is fatal [59]. Replacement therapy with hydrocortisone and fludrocortisone largely normalises mortality rates [60].

The Norwegian registry of organ-specific autoimmune diseases

In 1996 the Norwegian registry of organ-specific autoimmune diseases (ROAS) was established, gathering information from patients with endocrine autoimmune diseases, particularly APS-I and Addison's disease. ROAS contains information on disease components, current treatment, autoantibody profiles, and allelic variations of several genes. The biobank includes blood, sera, DNA, peripheral blood mononuclear cells, tears, and saliva regularly sampled from the patients. Both the characterisation and the collection of biological material from the Norwegian APS-I patients is on-going work. To date, ROAS includes 39 APS-I patients. In addition, records of 11 diseased patients have been collected. The registry and the corresponding bio-bank have been approved by the Norwegian data directorate and regional ethical committee.

Autoantibodies in APS-I

Organ-specific autoantibodies in APS-I

Due to their deficiency in developing tolerance to self, B cells and plasma cells from patients suffering from APS-I typically produce a variety of autoantibodies directed

against intracellular enzymes in the affected organs (Table 1). These organ-specific autoantibodies have proven to be excellent markers for autoimmune disease in the organ which they are expressed. Since they often precede clinical symptoms, they are assayed at routine basis in this group of patients [47, 61, 62].

Table 1. Common organ-specific autoantigens in APS-I, their main organ of expression relevant to APS-I, associated disease components and reported prevalence.

Autoantigen	Main expression	Disease component	Prevalence (%)*	Ref.
21-OH	Adrenal cortex	Addison's disease	66	[58, 63-65]
SCC	Adrenal cortex, gonads	Addison's disease, hypogonadism	52	[64-66]
17-OH	Adrenal cortex, gonads	Addison's disease, hypogonadism	44	[64, 65, 67]
GAD65	Pancreas	Intestinal dysfunction	37	[57, 68]
CYP1A2	Liver	Autoimmune hepatitis	8	[69]
AADC	Pancreas, liver	Hepatitis, Vitiligo	51	[70]
TPH	Duodenum	Malabsorption	45	[71]
TH	Keratinocytes	Alopecia	40	[72]
NALP5	Parathyroid gland, ovary	Hypoparathyriodism	41**	[55]

21OH; 21-Hydroxylase, SCC; Side-chain cleavage enzyme, 17-OH; 17-α-Hydroxylase, GAD; Glutamic acid decarboxylase, CYP1A2; Cytochrome P450 1A2, AADC; Aromatic L-amino acid decarboxylase, TPH; Tryptophan hydroxylase, TH; Tyrosine hydroxylase, NALP5; NACHT leucine-reich repeat protein 5

*Prevalence is based on detected autoantibodies in a cohort of Finnish, Swedish and Norwegian APS-I patients, n=90 [57].

**Prevalence is based on detected autoantibodies in a cohort of Finnish, Swedish and Norwegian APS-I patients, n=87 [55].

The adrenal, steroidogenic P450 superfamily autoantigens 21-OH, 17-α-hydroxylase (17-OH), and side-chain cleavage enzyme (SCC) catalyse chemical reactions required for the production of steroid hormones, like aldosterone and cortisol (21-OH), progesterone (17-OH), and pregnenolone (SCC) [58, 65, 67, 73]. Assay of autoantibodies against 21-OH is an excellent marker for Addison's disease, both in its isolated form and as part of a polyendocrine syndrome [74], while 17-OH and especially SCC are the main gonadal autoantigens [75]. Autoantibodies directed against tryptophan hydroxylase (TPH) and aromatic L-amino acid decarboxylase (AADC) are also highly precise in their detection of APS-I and correlate to malabsorption and hepatitis, respectively. They are rarely seen in other conditions except in a subgroup of AADC-positive Addison's patients [70, 76, 77]. Autoantibodies against tyrosine hydroxylase (TH) are correlated to alopecia in APS-I [72], and autoantibodies against TH is also seen in isolated alopecia and viteligo [78, 79]. Autoantibodies against glutamic acid decarboxylase 65 (GAD65) are commonly found in insulin dependent diabetes mellitus [80], and are also common in APS-I, although associated with intestinal dysfunction [57].

Autoantibodies against NALP5 are specific for APS-I, and a serological sign of hypoparathyroidism. NALP5 is also expressed in ovaries, and anti-NALP5 is suggested as a marker of primary ovarian failure. This is yet to be confirmed, as all investigated NALP5 positive patients had hypogonadisms in combination with hypoparathyroidism [55]. Autoantibodies against the calcium-sensing receptor (CaSR) are also related to hypoparathyroidism [81]. Although controversial [57], CaSR has been confirmed as a receptor-stimulating antigen in a limited number of APS-I patients [82, 83].

As these antibodies are quite specific for either APS-I or a specific phenotype (Table 1), a probable diagnosis can be made from any disease-component together with autoantibodies against NALP5, AADC, TPH or TH.

Cytokine autoantibodies in APS-I

The cytokine autoantibodies recently described in APS-I patients have changed the view on autoantibodies in endocrine diseases, as they no longer only are directed against organ-specific antigens, but also against components of the immune system itself. The first cytokine autoantibodies described in patients with APS-I were those against type I interferons (IFNs) [84], followed recently by Th17-derived cytokines [85, 86].

Interferons type I

The IFNs were first recognised in 1957 as substances with antiviral activity that are secreted from cells in response to a variety of stimuli [87]. Type I interferons are a large group of structurally similar cytokines, grouped into IFN-α, (with more than 13 different members), IFN-β, IFN-ε, IFN-κ and IFN-ω in humans. They are encoded by genes clustered in one locus on the human chromosome 9. IFN-α, IFN-β and IFN-ω are expressed at very high levels in plasmacytoid DCs and monocytes following viral infection, exposure to double-stranded RNA or stimulation through Toll-like receptors, and are early key players in the innate immune response [88, 89]. They signal through the Jak-Stat and mitogen-activated protein kinase (MAPK) pathways through the same receptor composed of the IFN-αR1 and IFN-αR2c subunits. In autoimmunity, the type I IFNs have a dual role, both promoting and restraining autoimmunity and inflammation [90-92]. This is best exemplified by the human diseases systemic lupus erythematous (SLE) and multiple sclerosis (MS), where the type I IFNs promote autoimmunity and inflammation in SLE [93], but have a protective and therapeutic role in MS [94]. Autoantibodies against type I IFNs, namely against IFN-α, were first described in patients suffering from myasthenia gravis (MG) and thymoma in 2001 [95]. MG is a neuromuscular junction disease, characterised by autoantibodies against acetylcholine receptor (AChR) [96], while thymomas are histological heterogeneous tumours consisting of thymic epithelial cells [97-100]. Fifteen per cent of the MG patients develop thymoma, and

autoantibodies against titin and ryanodine receptor identifies ninety-five per cent of these patients. On the other hand, fifty per cent of the thymoma patients develop MG [96].

Autoantibodies against IFN type I in APS-I

In 2006, neutralising autoantibodies against IFN-type I were detected in patients suffering from APS-I; surprisingly anti-IFN-ω was found in all the investigated patients [84]. Several studies utilising different methods, control groups, and including large number of patients from different countries have confirmed the presence of anti-IFN-ω in close to every patient diagnosed with APS-I [84, 101, 102]. These autoantibodies also seem to precede clinical symptoms, and have been detected as early as 7 month of age [103]. Utilising autoantibodies against IFN-ω has therefore the potential as a valuable diagnostic marker, and are suggested as a simpler and faster diagnostic tool preceding *AIRE* mutational analysis [101]. A probable APS-I diagnose can consequently rely on the presence of anti-IFN-ω together with any disease-components (Fig. 2) [50]. The origin and role of these autoantibodies are still unclear, but autoantibodies against IFN-α have been shown to down-regulate interferon-stimulated gene expression in blood cells from APS-I patients [104]. The autoantibodies against IFN-α and IFN-ω are also found in patients suffering from MG and thymoma, and their shared thymic dysfunction has been suggested as a possible connecting point.

Interleukin 17A, 17F and interleukin 22

The leukocyte-derived cytokines interleukin (IL) 17-A, IL-17F and IL-22 belong to a class of cytokines with a predominant effect on epithelial cells in various tissues. They are mainly produced by Th17 cells (Fig.1), which were identified as an independent linage of $CD4^+$ T cells in 2005 [105]. But also other immune cells, both adaptive and innate may produce these interleukins (Table 2).

IL-17A and IL-17F belong to a family of six identified proteins named from A to F, where the *IL-17A* and *IL-17F* genes, located on chromosome 6, share fifty per cent homology [106-109]. IL-22 is a member of the IL-10 family, together with IL-10, IL-19, IL-20, IL-24 and IL-26, and the *IL-22* gene is located on chromosome 12 [110, 111].

Table 2. The human IL-17 and IL-22 producing immune cells and their properties.

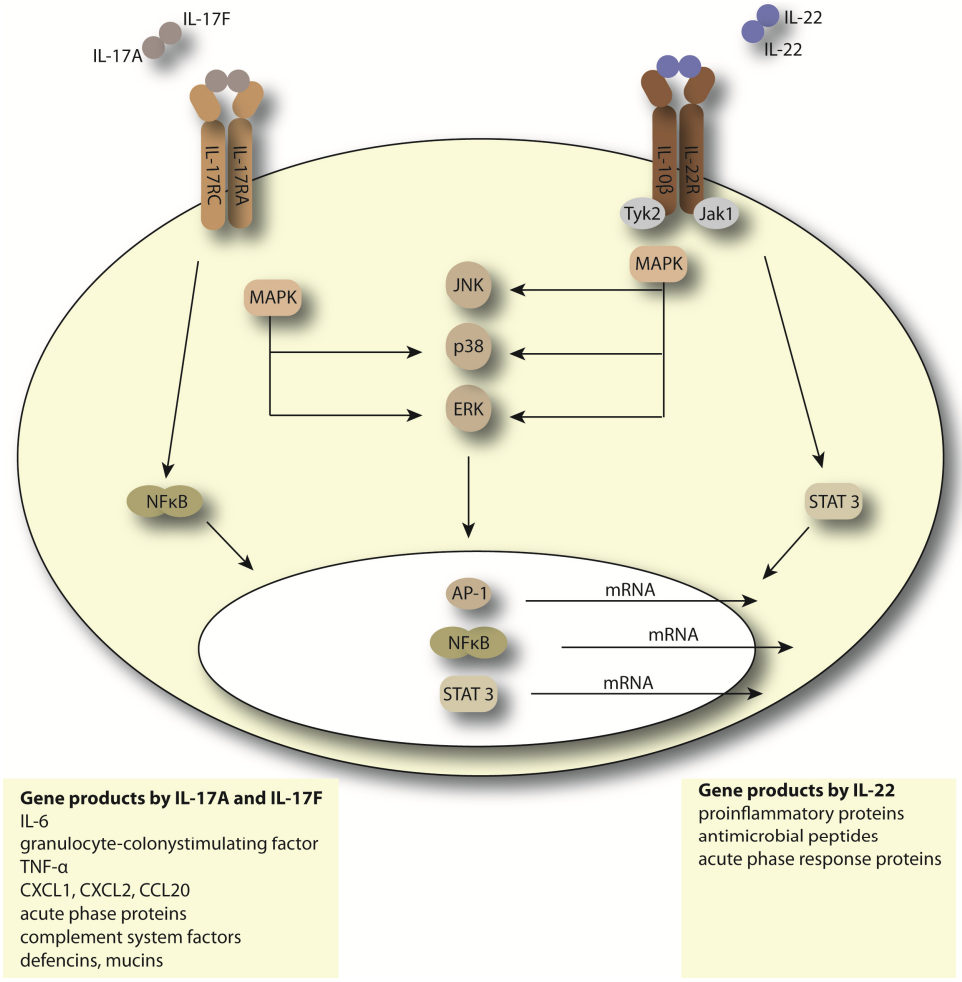
Cell type	Secreted cytokine	Other secreted factors	Transcription factor	Surface markers	Ref.
Adaptive					
Th17	IL-17, IL-22	IL-21, IL-26, TNF-α, CCL20	RORγt	$CD4^+$ $CCR4^+$ $CCR6^+$ $CXCR3^-$ $CD161^+$	[112-114]
Th22	IL-22	TNF-α, FGFs	Unknown	$CD4^+$ $CCR4^+$ $CCR6^+$ $CCR10^+$	[30, 31]
Innate					
NKT	IL-17, IL-22	IFN-γ	ROR	$CD3^+$ $CD56^+$	[115-117]
LTi	IL-17, IL-22	TNF-α, Lypmphotoxin	RORγt	$CD3^-$ $CD56^-$ $NKp44^+$ $NKp46^+$ $CD117^+$ $CD127^+$ $CD161^+$	[118]
$RORC^+$ NKp46	IL-17, IL-22		RORγt	$NKp46^+$ $NKG2D^+$ $CD117^+$ $CD127^+$	[119]
NK22	IL-22	IL-26, leukaemia inhibitory factor	RORγt	$CD3^-$ $CD56^+$ $NKp44^+$ $CCR6^+$	[120]

Modified from *Eyerich et al, Trends in Immunology 2010 [121].*

TNF-α; tumour necrosis factor alpha, CCL; Chemokine (C-C motif) ligand, CD; Cluster of Differentiation, CCR; C-C Chemokine receptor, CXCR; CXC Chemokine receptor, FGFs; Fibroblast Growth Factor, NKT; Natural Killer T cells, LTi; Lymphoid tissue inducer cell, RORγt; RAR-related orphan receptor gamma. IL-17 includes both IL17-A and IL17-F.

IL-17A, IL-17F and IL-22 have an overall function in the epithelial cells of barrier organs, where they defend the host against extracellular pathogens. They activate innate immune responses in tissue-cells, recruit immune cells by the induction of different chemokines, cytokines, inflammatory and antimicrobial proteins, and also have a regenerative effect on tissue after inflammation [121].

The proinflammatory cytokines IL-17A and IL-17F are secreted as disulphide-linked homo- or hetero-dimers [106, 107, 122]. They interact with their shared homo- or heterodimeric receptor consisting of IL-17RA and IL-17RC [123], which result in transcriptional activity of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [124], or activation of the MAPK pathway, where IL17-A acts through p38, and both IL17-A and IL17-F can activate the extracellular signal-regulated kinase (ERK) [125, 126]. Genes expressed in response to IL-17A and IL-17F encode IL-6, granulocyte-colony stimulating factor, tumor necrosis factor (TNF) α, chemokines CXCL1, CXCL2 and CCL20, acute-phase proteins, complement system factors, and antimicrobial proteins like defencins and mucins (Fig. 3) [127]. Both IL-17RA and IL-17RC have a wide range of expression; IL-17RA on epithelial cells, fibroblast, macrophages, DCs, vascular endothelial cells and peripheral blood T cells [128-131], and IL-17RC on tissue cells of prostate, cartilage, kidney, liver, heart and muscle [132, 133].



Modified from *Eyerich et al, Trends in Immunology 2010 [121].*

Figure 3. Simplified presentation of the IL-17A, IL17F and IL-22 signalling. The left part of the figure represents the signalling by IL-17A and IL17F, which bind their shared receptor as disulphide-linked homo- or heterodimers. This induces transcriptional activity of NFκB, or activation of the MAPK-pathway and transcriptional activator protein 1 (AP-1), which result in the release of factors that activate the innate immune system and recruit immune cells. The response to signalling by the homodimeric IL-22 is represented on the right part of the figure, where activation of MAPK and transcriptional activity through STAT3 result in release of proinflammatory, antimicrobial and acute phase proteins.

Secreted IL-22 can create opposite effects, depending on the cytokine milieu. IL-22 on its own has a regenerative and protective effect on tissue cells, while secreted together with TNF-α, IFN-γ, and/or IL-17A and IL-17F, IL-22 increases the inflammatory immune response [30, 134]. IL-22 also forms a homodimer [135] before interacting with its heterodimeric receptor consisting of IL-10Rβ-chain and IL-22R [111]. IL-22R is exclusively expressed on tissue cells at outer body barriers, and is absent from cells of the immune system. Thus, IL-22 communicates will epithelial cells and not immune cells. This is in contrast to the usual designation of an interleukin, as immune cells are not the target for IL-22 [136]. One possible exception is one report of IL22R on monocyte-derived human macrophages [137]. The interaction of IL-22 to IL-22R enables the subsequent binding of IL-10Rβ and phosphorylation of the tyrosine kinases Jak1 and Tyk2, associated with IL-22R and IL10β, respectively. This activates signal transducer and activator of transcription (STAT) 3 [110], and the MAPK pathways of ERK, c-Jun N-terminal kinase (JNK) and p38 kinase [138-140]. This again induces expression of proinflammatory genes [141], as well as genes encoding antimicrobial peptides, acute phase response proteins with predominantly effect on cellular differentiation and mobility of keratinocytes (Fig. 3) [140, 142].

IL-17A, IL-17F and IL-22 in human disease

IL-17A, IL-17F and IL-22 in Chronic Mucocutaneous Candidiasis

In patients with isolated CMC, decreased levels of IL-17A, IL-17F and IL-22 are found after stimulation with *C. albicans*. Since these patients also have a reduced number of IL-17 producing cells, a defect in their T cell compartment is a likely explanation [143]. Patients diagnosed with autosomal-dominant hyper-IgE syndrome lack production of IL-17 and are therefore susceptible to CMC due to their mutation in *STAT3* [144], which affects the expression of the Th17-spesific transcription factor RORγt [145]. In addition, a recessive inherited mutation in *IL-17RA*, and dominant inherited mutation of *IL-17F* both result in development of CMC [146],

underlining the importance of IL-17 in defence against *C. albicans*. Among the antimicrobial proteins produced in response to IL-22, the S100 protein family and human β defensin 2 are known to protect against *C .albicans* [147, 148].

In autoimmune disorders

The effect of the Th17-derived cytokines in autoimmune diseases is best illustrated by IL-17A in Rheumatoid Arthritis (RA) where the level of IL-17A is up-regulated in the rheumatoid synovium. This promote inflammation and bone destruction *in vitro* by the pro-inflammatory cytokines TNF-α, IL-β, and IL-6. As IL-6 maintains the Th17 T cell population, a chronic inflammatory state is established [127]. The role for IL-22 in RA is more obscure, but it could have a pathogenic role by promoting inflammatory responses in synovial tissue [139]. In SLE patients, a decrease in circulating IL-22 levels is found to be correlated with disease activity [149, 150], while increased levels of IL-22 are found in inflamed region of the colonic mucosa in ulcerative colitis and Crohn's disease [138, 151]. In the autoimmune skin-specific psoriasis, high expression of IL-22 is found in psoriatic skin, and there is a correlation between the levels of IL-22 in blood and the severity of the disease [136, 140].

Autoantibodies against IL-17A, IL-17F and IL-22 in APS-I

The high occurrence of CMC in patients with APS-I was long regarded as an immune defect and not as a part of the autoimmune reaction. The current discovery of autoantibodies against IL-17A, IL-17F and IL-22 in sera from APS-I patients indicates an indirect autoimmune cause of CMC, where these autoantibodies inhibit elements in the defence against *C. albicans* [85, 86]. Interestingly, patients suffering from thymoma and CMC in combination are also positive for these autoantibodies [85]. The Th17-response towards *C. albicans* has been studied using peripheral blood

mononuclear cells (PBMC) from APS-I patients, where the production of IL-17F and IL-22 were down-regulated, while the results for IL-17A were unequivocal [85, 152].

The Autoimmune regulator gene

The autoimmune regulator gene, *AIRE,* was identified as the underlying cause of APS-I in 1997. It occupies 13 kilo bases on chromosome 21, harbouring 14 exons which encode a 545 amino acid protein [153-155]. Over 60 different mutations have been identified, among them, one dominant negative mutation [156]. Mutations are found in ninety-five per cent of the clinical APS-I cases [157], and are routinely searched for when APS-I is suspected. The high number of mutations found throughout the coding region of *AIRE* does not explain the large phenotypic variation seen in the patients, which might rely on other genetic or environmental factors [61, 158]. The only exceptions might be the association of chronic mucocutaneous candidiasis with the most common Finnish mutation, p. R257X [85], and the low frequencies of AAD and CMC in patients with the Iranian Jewish p.Y85C mutation [159].

The large numbers of mutations spread throughout *AIRE* have had an important role in revealing the properties of the AIRE protein, a field that has grown tremendously over the past couple of years.

Localisation and expression of AIRE

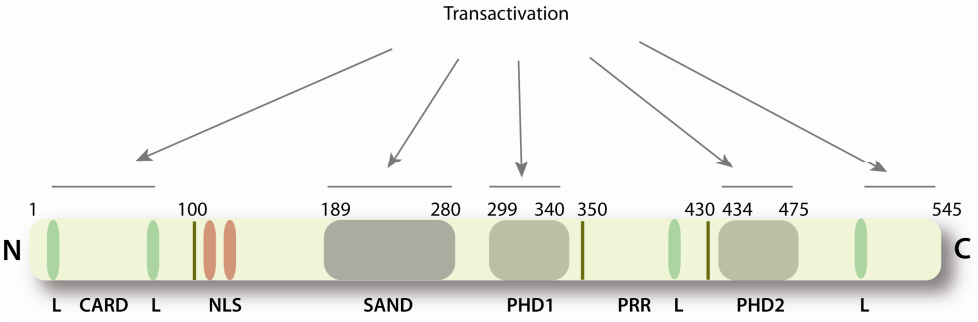
Within the cells, AIRE is found in the nucleus, in nuclear bodies, visualised as discrete dot-like structures [160-162]. AIRE is mainly expressed in the thymus in both human and mice, by a subpopulation of medullary thymic epithelial cells

(mTECs), characterised by high expression levels of MHC class II and the co-stimulatory markers CD80, CD86 and CD40 [161, 163, 164].

In the human peripheral immune system, AIRE is expressed in lymph nodes, spleen, tonsils, gut-associated lymphoid tissue and fetal liver [154, 161, 165], and by cells of the immune system, notably in B-cells, macrophages and DCs [165-167]. In mice, the results differ slightly regarding the different genetic backgrounds [168], and expression is mainly seen in lymph nodes and spleen, [164, 169-176], but also identified in liver, brain [176] and testis [171]. However, expression is only detected at mRNA-levels in immune cells, where it is found in monocyte-derived DCs, plasmacytoid DCs and myeloid DCs [175].

AIRE; protein domains and function

The observation that AIRE localises to the cell nucleus, together with the characterisation of its functional domains, were the first clues pointing to a role as a transcriptional activator. The functional domains includes a nuclear localisation signal, two plant homeodomain zinc finger (PHD) motifs, four LxxLL nuclear receptor binding motifs, a proline-rich region, a caspase-recruitment domain (CARD) and a SAND domain (named after the proteins Sp100, AIRE-1, NucP41/75 and DEAF-1) (Fig. 4) [153, 154, 177-179]. When fused to a heterologous DNA binding domain, AIRE activates transcription of a reporter gene, and the CARD domain (long referred to as a homogeneously staining domain, HSR,), mediating AIRE-AIRE homodimerization, is needed for this activation [180, 181]. AIRE binds DNA as a dimer or tetramer [182], and both PHD-domains and the SAND-domain have DNA-binding abilities *in vitro* [179].



Modified from Peterson et al, Nature Reviews Immunology 2008 [183]

Figure 4. Schematic overview of AIRE. The figure shows the functional domains and their localisation within the AIRE protein, with the corresponding number of amino acids and the regions necessary for transcriptional activation indicated above. The N-terminal caspase-recruitment domain (CARD) is necessary for nuclear dot formation and has been implicated in homo- or hetero-oligomerisation of the protein. The nuclear localisation signal (NLS) is targeting the protein to the cell nucleus, while the SAND domain is a putative DNA binding domain. AIRE has two plant homeodomains (PHD) zinc fingers, the first (PHD1) has been shown to interact with unmetylated histone H3 lysine4 and with the DNA-dependent protein kinase complex, and both interactions are required for proper transactivation. PHD1 has also been shown to have E3 ubiquitin ligase activity, which is an enzymatic process where an ubiquitin molecule is added to a target protein, as a marker for degradation. The two PHD domains are also found to bind to specific DNA motifs. Four LXXLL motifs (L) are found throughout AIRE which is a small motif found in many transcriptional co-activators. Its function in AIRE is still to be discovered together with the function of the proline-rich region (PRR).

Studies from mice have been particularly powerful in elucidating the function, localisation, and structural organisation of its human counterpart, and have proved Aire to be a key transcriptional regulator of the immune system [169, 184-186]. Aire binds *in vivo* to specific DNA-sequence motifs and regulate the thymic expression of a broad spectrum of genes, including autoantigens, cytokines, transcription factors and posttranslational modifiers [187].

AIRE in the thymus and in tolerance

Thymus and central tolerance

The thymus is the main organ for lymphoid cell differentiation and selection, both which are processes that rely on a dynamic relocation of developing lymphocytes within the thymic microenvironment. Thymocyte survival and commitment to linage is dependent upon discrimination of self- versus non-self, and may induce cell death through positive or negative selection. When lymphoid progenitor cells enter the thymus, $CD4^+CD8^+$ double positive (DP) thymocytes are generated in the outer cortex, with their αβT cell receptor (TCR) present on the surface. Here, they undergo positive selection, where the DP thymocytes displaying TCRs with no affinity to MHC undergo apoptosis, while those able to bind MHC class I or II survives and commits to the $CD4^+$ or $CD8^+$ lineages. These SP thymocytes then migrate to the thymic medulla where they interact with mTECs which expose them to a wide array of peripheral tissue antigens (PTAs). Thymocytes expressing TCRs with high affinity for self-antigens are deleted, in the process of negative selection. The presentation of PTAs by the mTECs and the elimination of self-reactive T cells are crucial to induce immunological tolerance and to prevent autoimmune disease. This stringent selection process leaves only one to three per cent of the lymphoid cells to escape into the periphery [188, 189].

Aire in central tolerance

The great repertoire of PTAs exclusively expressed in the mTECs are continuously transcribed during the period of T cell output [163], and are conserved between humans and mice [190]. They are diverse in their gene ontology, tissue specificity and chromosomal location, and tissue restricted genes are overrepresented [191, 192]. These observations pointed early to the involvement of epigenetic mechanisms in controlling the transcription of PTAs [193]. Some of the PTAs, e.g. thyroid peroxidase, thyroglobulin, the P450 cytochrome subfamilies and IA-2, are found to

be autoantigens in patients with APS-I, and linked AIRE to the regulation of PTA transcription [190]. In Aire knockout models a reduction or total loss of transcription of Aire-regulated PTAs were seen [194], leading to the escape of autoreactive T cells into the periphery [191].

Aire possesses multiple roles in the activation and transcription of genes [195]. At the epigenetic level, Aire's PHD1-domain interacts with unmethylated lysine 4 of histone 3 (H3K4me0), a marker of transcriptionally inactive chromatin [193, 195-197]. Transcriptional activity is then induced when Aire associates with factors promoting elongation of the transcript, as part of the early-acting elements of the non-homologous end-joining DNA repair machinery. This complex resolves DNA-supercoiling by creating single- and double-stranded breaks in the DNA, promoting unwinding and subsequent religation. Aire is also involved in the processing of pre-mRNA to mature mRNA, which is thought to take place in the nuclear speckles. By these mechanisms, Aire enhances transcription of weakly expressed peripheral tissue antigens in MECs, peptides from which can be presented via MHC class I or II to immature thymocytes. High avidity interactions between these peptide/MHC complexes and the T cell receptors of self-reactive T cells will eventually result in their apoptosis [195]. The molecular basis of how AIRE regulates transcription of PTAs in mTECs has yet to be confirmed in non-transgenic settings and in humans.

AIRE and DCs

The APS-I patients display normal levels of plasmacytoid and myeloid DCs in blood [104, 198]. However, after stimulation with *C. Albicans*, the monocyte-derived DCs from APS-I patients expressed less cytokines, in particular IL-10, TNF-α and CXCL10, compared to controls. In addition, the expression of genes involved in immune regulatory pathways of cell-cell signalling and cytokine production was reduced [167]. In transgenic mice, Aire mediate thymic deletion of $CD4^+$ T cells independent of PTA expression, by indirect presentation of mTEC-derived PTAs by

bone marrow derived cells. Therefore, some antigens rely on DCs for presentation to maturing thymocytes, where Aire is necessary for the transfer of antigen from the mTECs to the DCs [199-201].

Aire in peripheral tolerance

Although tightly controlled, some autoreactive T cells escape thymic negative selection and are released into the periphery. Peripheral tolerance is aimed to delete or inhibit activation of these T cells when they encounter their tissue-restricted self-antigen [202-205].

A role for Aire in peripheral tolerance is emerging, but the details are still unclear. A new subset of Aire-expressing extrathymic cells (eTACs) were identified in the T cell- B cell boundary regions in lymph nodes and spleen in a transgenic model system in 2008 [174]. Although they share certain characteristics with mTECs, they are a distinct cell type, as they do not express the co-stimulatory molecules CD80 and CD86, and the genes regulated by mTECs and eTACs show little overlap [174]. Naïve T cells are frequently trafficking the secondary lymphoid organs, and PTA-expression is therefore likely to occur there [206]. The eTACs probably play an important role in the deletion of peripheral autoreactive T cells and the maintenance of tolerance, although Aire-independent expression of tissue-restricted mRNA has been identified in stromal cell subsets in the lymph nodes [207, 208].

Treg cells in peripheral tolerance

The Treg cells are a specialised $CD4^+$ T cell linage, crucial for maintaining peripheral tolerance, as they counteract Th1 and Th17 responses in order to avoid tissue damage (Fig.1). Their suppressive activities rely on activation of their TCR by APCs. Once activated, a Treg cell can suppress a broad range of immune reactions, independent

on the initial, activating antigen [209]. The underlying suppressive mechanisms are still poorly understood, but probably involve direct cell-contact and secretion of inhibitory cytokines [210].

Their critical role in preventing autoimmunity is best demonstrated by the fatal, multi-organ autoimmune disease IPEX, characterised by the occurrence of diarrhoea, type 1 diabetes, thyroiditis, and eczema. IPEX is caused by mutations in the Treg transcription factor gene, *FOXP3*, resulting in defective Treg cells [211]. Deficient Treg cells are also associated with more common autoimmune diseases, like type 1 diabetes, MS and RA [209, 212]. Treg cells from APS-I patients have decreased levels of FOXP3-expression, and a limited clonal expansion in the TCR-repertoire is seen, together with failure to activate the Treg cells [213, 214].

Th17, Treg cells and autoimmunity

There is shown to be a balance between the Th17 and the Treg cells (Fig. 1), and the plasticity between these two lines of Th cells may tip the scale in favour of the effector Th17 subset and autoimmunity. The anti-inflammatory cytokine transforming growth factor (TGF) β is needed for the generation of both Th17 and Treg cells from naïve $CD4^+$ cells, and expressed together with IL-6, TGB-β will promote the Th17 subset [215]. IL-6 is expressed during injuries, infections and inflammation, and inhibits expression of Foxp3 in mice [215]. Both retinoic acid and the Treg growth factor, IL-2, promote Treg differentiation, while preventing the expression of Th17 cells [216, 217]. At the molecular level, the respective transcription factors of Th17 and Treg bind each other, thereby inhibiting each other's function [218, 219]. The balance of Th17 and Treg cells and the proper expression of FOXP3 are crucial to avoid promotion of autoimmunity [220, 221].

Information gained from the study of APS-I

As a monogenetic disease, the study of APS-I and *AIRE* have proved to be particularly informative regarding the establishment of tolerance and the transcription of PTAs. Defects in both central and peripheral tolerance pathways that contribute to autoimmunity has been recognised [157], and the identification of *AIRE* has expanded our knowledge about immune tolerance and emphasised the importance of a functional thymic environment in the prevention of autoimmunity [222]. This is of relevance for more common endocrine autoimmune disorders, exemplified by the thymic PTA-expression in type 1 diabetes, where the transcription levels of insulin in thymus correlates to lower risk of disease [223, 224].

Only a few autoimmune diseases follow a monogenic inheritance pattern and the study of these diseases has revealed valuable information about their molecular pathogenesis and about affected and normal physiology. A further understanding of the genetics and the molecular mechanisms behind autoimmunity will hopefully lead to new therapeutic strategies [225].

Aims of study

A translational approach to APS-I and *AIRE* is important to expand our knowledge not only on this rare autoimmune disease, but also to understand general mechanisms of autoimmune diseases.

In this work we addressed the lack of rapid and robust assays for APS-I. Moreover, we sought to find new mutations in *AIRE* and to see if variations in *AIRE* were involved in more common autoimmune diseases. Finally, we explored cellular immunity by studying immune cell subsets in order to understand the immune defect involved in multi-organ autoimmunity.

The specific aims were:

I. To develop specific diagnostic assays for the detection of APS-I patients. Radioimmunoassay is a well-established method for the analysis of autoantibodies, and was chosen as the technique for establishing novel cytokine autoantibody assays detecting patients with APS-I.

II. To investigate if genetic modifications of *AIRE*, both variations in copy number and single nucleotide polymorphisms, were associated with AAD.

III. To learn more about cellular immunity in APS-I by studying immune cells in Norwegian APS-I patients, their relatives and healthy controls.

Methodological considerations

Patient samples (*Paper I, II, III, and IV*)

The Norwegian patient and control material used in these studies were gathered from ROAS, comprising samples from 50 APS-I patients (11 diseased), and ~500 patients with AAD and APS-II. Regarding APS-I, their age, gender and time of diagnosis together with their clinical phenotype and mutational status are described (for details, see Appendix I). Anonymous healthy controls were recruited from the blood bank at Haukeland University Hospital; one group completely anonymous, and one group with a few known variables, like age and gender. All patients and controls signed an informed consent according to the declaration of Helsinki when included in ROAS.

AAD-patients with APS-I-like features (*Paper I*) were selected on one of the following criteria

- AAD onset before the age of 20 years
- autoantibodies against TPH, TH, AADC or SCC
- presence of ectodermal manifestations associated with APS-I

In *Paper II*, blood sera from a total of 48 APS-I patients of Norwegian (n=32) and control sera from their first-degree relatives (n=10), patients with AAD (n=354), type I diabetes (n=38), and healthy controls (n=150) were included from ROAS. In addition, Finnish (n=6), Swedish (n=7) and North-American (n=3) APS-I sera were included, together with control sera from Swedish patients with Down syndrome (n=45), all recruited through our international collaborators.

Analyses of the *AIRE* gene (*Paper I*)

SNP analysis of the *AIRE* gene

Variations of single nucleotides polymorphisms (SNPs) within the DNA-sequence have been shown to cause different human diseases. When used in association studies, a polymorphism must occur more or less frequently than what is expected by chance. By utilising LD, association between alleles at different loci are measured, expecting that the frequency of finding two loci together is different from the frequencies of the respective loci. As the common variations in the human genome can be arranged as haplotypes within blocks of strong LD, they reduce the need of multiple tests over single SNPs. In this candidate-gene association study (*Paper I*), the occurrences of SNPs within and in close-proximity to *AIRE* was investigated in 311 patients with AAD, either isolated or as part of APS-II, and in 521 healthy controls to reveal any association between polymorphisms and disease. Nine SNPs covering *AIRE* and its flanking regions were chosen based on Hap Map data and earlier work by Turunen et al. [226]. All primers and probes used in the Taqman allelic discrimination assay were commercially available from Applied Biosystems (Carlsbad), and DNA for all genetic analysis was extracted from frozen blood samples using standard molecular procedures.

Copy number variation (CNV assay) of *AIRE*

Alteration of DNA in a genome might result in an abnormal number of copies of one or more sections of DNA within the cells of the organism. These relatively large regions of DNA, ranging from one kilo base to several mega bases, are either deleted (low copy number) or duplicated (high copy number). Duplex Taqman real-time PCR assay can detect variations in copy number of *AIRE* between patients with AAD or APS-II and controls. For each patient sample run in duplicate, fluorescent signals were detected using probes for the target gene *AIRE* and for the reference gene *Rb-1* (Retinoblastoma-1) [227], known to have a copy number of two in the genome. The

relative relationship between *AIRE* (unknown number of copies) and *Rb-1* (two copies) gives the copy number of *AIRE* using a standard curve for each gene. Primers and probes were designed using Beacon Designer v. 2.1, (Premier Biosoft International), and all reagents were available from Applied Biosystems.

Genetic analysis and statistics

Data from SNP analysis were analysed by ABI 7900HT Genetic Analyser and SDS 2.1/2.2 software (Applied Biosystems). Data from haplotype mapping (Hap Map) were used to tag all known variations with a 5% minor allele frequency. A pairwise tagging method and cut-off were used, and $r^2 \geq 0.8$ by the Haploview software [228]. Single point analysis and estimation of haplotype were performed using Haploview and Unphased software.

Immunological methods (*Paper I, Paper II and Paper IV*)

Radioimmunoassay of autoantigens

Radioimmunoassay (RIA), also termed radio-ligand binding assay (RLBA), is a well-known and well-used method to determine the presence of autoantibodies, and may be utilised on a variety of fluids from patients, including blood, sera, and saliva. RIA is a fluid-phase immune precipitation method, based on *in vitro* transcribed and translated antigens, labelled with ^{35}S methionine. When added to patient sera, the antigen will be bound by autoantibodies if present. This complex is then captured by immune precipitation with protein A-sepharose and will yield a radioactive signal quantified in a scintillation counter [70]. In the development of the anti-IFN-ω assay (*Paper II*), 0.1% dithiothreitol (DTT) was included when incubating the sera with antigens overnight, to reduce the two disulphide bonds present in IFN-ω and to prevent the formation of intra- and inter- molecular disulphide bonds between the

cysteine residues in the proteins. This yielded better discrimination between positive and negative control-sera, hence expanding the range of detection without introducing false positives (FP).

The samples from patients and controls were tested as duplicates (*Paper IV*) or triplicates (*Paper I, II and III*). IgG were purified as described by Ey et al. [229] (*Paper II*), and blood sera were used in the detection of autoantibodies (*Paper I, II, III and IV*).

Design of fusion proteins for the detection of anti- IL-17F and anti-IL-22 (*Paper IV*).

To overcome the low incorporation of ^{35}S methionine obtained from the *in vitro* expression of IL-17F and IL-22, a method expressing dimers was chosen, as these interleukins also are shown to be biological active as dimers. In an attempt to detect both interleukins with one method, based on their shared correlation to CMC, an additional chimeric DNA-construct of IL-17F and IL-22 was made (*Paper IV*). In general, the 5' end of the first part in the fusion-protein cDNA was inserted into the expression vector pcDNA3, using primers where the stop-codon was replaced by base-pairs coding for methionine. The procedure was then repeated with the second cDNA with the original stop-codon, fulfilling the dimer-and chimeric constructs. The constructs were verified by sequencing to ensure no mismatch in the open reading frame before utilising them for protein expression.

Analysis of data, determination of normal range and validation of the RIAs (*Paper II and IV*)

The results from the different RIAs were expressed as index-values {(counts per minute (cpm) sample – cpm negative control/cpm positive control – cpm negative

control) × 1000}, where pooled human serum from healthy donors was used as negative control, and an APS-I serum with medium/ high antibody levels against the antigen tested, as positive control. The normal limit for each assay was calculated as the mean of at least fifty healthy controls + three standard deviations (SD). Coefficient of variation (CV) {(CV (%) = (SD/mean) × 100} and inter- and intra-variability were calculated based on two APS-I sera tested at least three times.

To confirm the specificity in which each assay detected its designated cytokine, one APS-I serum was pre-incubated with increasing amounts of non-radioactive antigen; IFN-ω (BMS308, Bender MedSystems), IL-17F (1335-IL-025/CF, R&D Systems) or IL-22 (782-IL-101/CF, R&D Systems). Ovalbumin (A 5503-1G, Sigma-Aldrich) was used as control. For each assay, the sensitivity {true positives (TP) / (TP + false negatives (FN))} and specificity {true negatives (TN)/(TN + FP)} in which the assays detected APS-I (*Paper II*) and CMC (*Paper IV*) were calculated.

Flow cytometry (*Paper III*)

To analyse the different subsets of immune cells from APS-I patients, blood mononuclear cell immunephenotyping of 19 patients with APS-I, 18 first degree relatives and corresponding sex- and age-matched controls were analysed by the multi-parametric method flow cytometry (*Paper III*). PBMC from patients and controls were isolated from EDTA-blood using Lymphoprep (Axis-Shield PoC AS), and a total of 2×10^5 cells in a volume of 100 µl were incubated with labelled monoclonal antibodies (Becton Dickinson (BD) Biosciences) against human cell markers. The isolated PBMCs were washed, before they were fixated in 1% paraformaldehyde. Four markers were analysed simultaneously by FACS Calibur (BD Sciences) and FLOWJO version 7.2.5 (Tree Star).

To estimate the number of type-1 myeloid dendritic cells, type-2 myeloid dendritic cells and plasmacytoid dendritic cells, the human blood dendritic cell enumeration kit from Miltenyi Biotech (Bergisch Gladbach) was employed.

Statistic methods *Paper II, Paper III, Paper IV*

Differences between immune cell subsets from APS-I patients and the controls (age and sex-matched), and between the family members and controls (age and sex-matched) were calculated by Mann-Whitney test in SPSS v.15 (SPSS Norway AS) and/or GraphPad v.5 (GraphPad software Inc). *P*-values below 0.05 were considered statistically significant (*Paper III)*. Correlations between RIA and ELISA (*Paper IV*) were calculated by Spearman r-test, confident interval and P-value using Graphpad v.5.02. Fisher's exact test was used to calculate statistical association of IFN-ω antibodies in APS-I patients compared to the control groups by SPSS (*Paper II*).

Summary of results

Paper I

AAD is often associated with other autoimmune symptoms as part of an autoimmune polyendorine syndrome, and several genes have been associated with AAD, in particular *MHC* [230-234], *cytotoxic T lymphocyte antigen 4* (*CTLA4*) [235-237] and *protein tyrosine phosphatase non-receptor 22* (*PTPN22*) [238]. This paper investigated whether variations in *AIRE* could be involved in increased susceptibility to AAD in a three-pronged approach: (i) association analysis using nine SNPs within and in close proximity of the *AIRE* gene, (ii) sequencing the *AIRE* gene in candidate AAD patients in order to look for rare polymorphisms, (iii) investigate whether CNV of *AIRE* could be involved. By analysing nine SNPs covering *AIRE* in 311 patients with AAD and in 521 healthy controls, the pattern of LD was found to be similar to the Hap Map data. Thus, the nine SNPs analysed identified most of the common variation seen, and none of the polymorphisms were associated with AAD. Twenty-five AAD-patients with APS-I-like features were selected for sequencing of the 14 exons of *AIRE*, identifying three novel polymorphisms, two which were considered as possible pathogenic variations; (i) c.1411C > T, p.Arg471Cys and (ii) c.1507G > A, p.Asp503Asn. Together with a previously described polymorphism, c.901G>A, p.Val301Met [239], these were genotyped in 311 AAD patients and in at least 107 healthy controls in order to investigate their frequencies.

CNV analysis of exon 2 in 161 investigated AAD patients revealed no variation in copy number when compared to 39 healthy controls. However, 14 APS-I patients were included as a control group, and large deletions in *AIRE* were detected in two patients, together with a disease-causing mutation on the other allele. The first patient had a large deletion on one allele, reaching from at least exon 1 to intron 8, which

was also found in the healthy mother and two healthy siblings. The second patient had a deletion stretching from exon 2 to exon 8.

Taken together, genetic variations in *AIRE* were not associated with AAD, and the new identified SNPs were unlikely to cause an APS-I like phenotype in a heterozygote state as they were also seen in healthy controls, however, no healthy individual carried the c.901G>A variant. The finding of large deletions in APS-I patients underpin the importance of DNA analysis of parents when APS-I patients are found to be homozygote for an *AIRE* mutation, in order to avoid faulty diagnosis.

Paper II

Since anti-IFN-ω appeared as a universal diagnostic marker of APS-I, we aimed to develop a reliable, fast and high-throughput immunologic assay to determine the prevalence of this autoantigen in a considerably large cohort of APS-I patients of different geographic origins. The established RIA detected anti-IFN-ω positive patients with high specificity, demonstrated in competition experiments with non-radiolabelled antigen. The assay discriminated well between APS-I patients and controls, and their anti-IFN-ω showed little variance over time. One patient with AAD had high levels of anti-IFN-ω; a deceased male patient diagnosed with AAD and prolactinoma. No genetic abnormalities in *AIRE* were found, neither were any MG and thymoma-associated antibodies, i.e autoantibodies against acetylcholine receptor and titin. A new APS-I patient was detected using this assay, in who subsequent mutational analysis of *AIRE* revealed a homozygote 13 base pair (bp) deletion in exon 8, the most common Norwegian mutation, and no abnormalities in CNV. This study established a robust immunoassay and confirmed the high prevalence of autoantibodies against IFN-ω in an expanded cohort of APS-I patients from different countries, underpinning its diagnostic value.

Paper III

Previous reports regarding characterisation of immune cell subsets in APS-I patients have included few patients, and we aimed to characterise immune cell subset in a larger cohort of Norwegian APS-I patients and in their close relatives. This was performed by extensive blood mononuclear cell immunotyping using flow cytometry. Among cell populations with immune regulatory functions, the patients showed significantly lower proportions of Treg cells ($CD4^+CD25^+FOXP3^+$; $P = 0.029$, and $CD3^+CD4^+CD25^+CD127^-$; $P = 0.028$). Interestingly, in the effector/memory T cell subset the Th ($CD4^+$) and Tc ($CD8^+$) T cells were similar in patients and controls, except for one family, who's members had significantly lower frequencies of memory Th cells ($CD4^+CD45RA^-CD45RO^+$) compared to healthy controls ($P = 0.023$). Th cell subsets with homing properties according to differentially expressed chemokine receptors CCR6 and CXCR3 were also investigated as $CCR6^+$ cells are attracted to epithelial surfaces and $CXCR3^+$ cells to inflammatory tissue. No alteration was seen in $CD4^+CD45RA^-CCR4^+CCR6^+$ lymphocytes (containing IL-17A-secreting Th17 cells), but the percentage of Th cells expressing both CCR6 and CXCR3 (includes among others IFN-γ and IL-17A coproducing cells) were significantly decreased in patients ($P = 0.035$). In general, we found no alterations in the frequencies of DCs in this study, while the monocyte subpopulation $CD14^+CD16^+$ cells was decreased in APS-I patients ($P = 0.028$). The Norwegian APS-I patients exhibit a reduced number of Treg cells, $CCR6^+CXCR3^+$ co-expressing Th subpopulation and $CD14^+CD16^+$ monocytes, while the previous reported variations seen in invariant natural killer T (iNKT) cells and monocytes could not be confirmed in this study.

Paper IV

Autoantibodies against IL-17F and IL-22 are shown to be associated with CMC; however, various assays have differed in their sensitivity detecting these autoantibodies. Therefore, we aimed to make a reliable RIA detecting these

interleukins both individually and together using a chimeric protein. Furthermore, to overcome the generally low incorporation of radioactivity in these short proteins, dimeric proteins were designed and tested. The dimers and the chimeric constructs regularly incorporated more radioactive methionine than the single interleukins; all showed reliable detection of autoantibodies, with satisfying discrimination between positive and negative samples. The specificity in their antibody-detection was ensured by competition with unlabelled protein. Finally, the results from the RIAs were compared to results obtained by ELISA, which revealed satisfying equivalence. Unfortunately, the dual detection of IL-17F and IL-22 in one assay, using the dimeric protein, could only be verified for IL-22, as all patients with anti-IL-17F also were positive of autoantibodies against IL-22. The previously reported correlation between IL-22 and the presence of CMC could not be confirmed by this study; instead a trend towards association between CMC and IL-17F was seen.

General discussion

Studying a monogenetic disease

The main challenge to overcome when studying a rare disease is the availability of patients and samples, which result in studies with hampered statistical power. The extensive use of biological samples require good-will, patience and cooperation from our limited APS-I cohort. In Norway, their spread geographical distribution makes the sampling of fresh blood for subsequent cell-based analysis challenging, time-consuming and expensive. It also makes it demanding to obtain accurate patient records characterising phenotype and disease status from the different physicians treating them.

Extensive international collaboration may resolve some of these problems, as we are able to increase the number of patients included in the studies. However, one should consider that the genetic background of different populations might influence the results. Hence, controls from the same genetic background should be included in genetic studies, as exemplified by the great variance seen within European populations regarding allele frequencies of the *PTPN22* risk-allele [240].

In spite of these challenges, studies of APS-I and the corresponding mouse models have led to many important discoveries regarding the establishment and maintenance of immunological tolerance. Studies of various Aire knock-out mice have provided excellent molecular explanations of how Aire regulate expression of the PTAs in thymus, and how Aire is a crucial factor establishing and maintaining central and peripheral tolerance [176, 191, 195, 241]. Aire-deficient mice develop multi-organ autoimmunity, but the major APS-I manifestations seen in man are not found in the

mouse models [176, 191, 241, 242]. Hence, there is a need to study APS-I in both men and mice to understand the pathogenesis of the human disease.

Genetics in autoimmune diseases; APS-I and AAD

Genetic variations of *AIRE* have previously been associated with systemic sclerosis when combined with thyroiditis [243], and with alopecia [244]. With the function of AIRE in regulating expression of PTAs in mind, we investigated whether variations in *AIRE* were associated with AAD, one of the three main components in APSI-I (*Paper I*). Except a c.901G>A polymorphism resulting in a change from a valine to a methionine at amino acid 301 (Val301Met) previously reported in one AAD patient, we did not find such association. Similar findings were reported in a study of association with AIRE and type 1 diabetes [226]. The c.901G>A mutation lies within the DNA-binding PHD1-domain of AIRE, and several of the mutations in this area are reported as single mutations, i.e. only one mutation has been found in each patient [46, 103, 245]. This indicates that a number of unrecognised dominant negative mutations could be present among APS-I patients, in a similar manner as the dominant pattern of inheritance previously described in the SAND domain of AIRE [156].

In addition, we found novel deletions of *AIRE,* stretching from exon 2 to exon 8 in two of the APS-I patients previously thought of as homozygote for the associated mutation on the other allele (*Paper I*). For one of the patients, information for exon 1 and intron 8 could not be obtained, as the microsatellites were homozygous in these areas. These findings emphasise the importance of CNV-analysis when sequence analysis fails to detect abnormalities in patients with a clinical APS-I picture.

Analysis of autoantibodies

The presence of autoantibodies is a distinctive and characteristic feature of autoimmune diseases. Circulating organ-specific or cytokine autoantibodies is an early phenomenon in autoimmune diseases, and can be detected years before clinical disease is evident, which makes these autoantibodies excellent markers of disease. Autoantibody-analysis of IFN-ω, 21-OH, SCC, AADC and NALP-5 constitute the panel of autoantigens which new patients recruited to ROAS are screened for.

Organ-specific autoantibodies

Whether the organ-specific autoantibodies are merely markers or serve as mediators of disease is still unclear [246, 247], but there are data pointing at a role as enhancers of the T cell response against 21-OH [248, 249]. Some autoantigens are shown to inhibit the activity of the target enzyme *in vitro*, like antibodies against AADC [250], 21OH [251] and GAD65 [252],

Prospective studies show that autoantibodies against 21-OH positively predict development of AAD within few years in young APS-I patients [46, 253, 254]. A more functional role has been implicated for autoantibodies against the extracellular CaSR; A subgroup of APS-I patients were shown to have CaSR-stimulating antibodies, leading to lower calcium and hypoparathyroidism, thus reflecting a different way of autoantibody action [255].

The pathogenic role of the organ-specific autoantibodies and their underlying immunological mechanisms remain to be determined, but their use for diagnostic purpose is invaluable.

Cytokine autoantibodies

The origin and function of the cytokine autoantibodies are largely unclear, but the autoantibodies against IFN-ω have shown to be an excellent marker detecting APS-I.

We confirmed IFN-ω as an antigen in all patients in a relatively large cohort of APS-I patients by utilising a novel method ready for high-throughput screening (*Paper II*). This assay is now a routine analysis provided by our research laboratory as part of clinical practice, offered through the Hormone Laboratory at Haukeland University Hospital. From 2007 until today, we have identified new patients using anti-IFN-ω, where the subsequent genetic analysis of *AIRE* has confirmed APS-I (manuscript in preparation). Among these, we found one family consisting of four affected individuals with an unusual dominant inheritance pattern (Erichsen, poster, Endocrine Society, 2011) The reliability of the assay and of anti-IFN-ω as a sensitive and predictive marker for APS-I was further confirmed in a multicentre-study including 174 European patients [101]. Antibodies against IFN-ω is shown to be a universal marker of APS-I, regardless of phenotype and *AIRE* mutations, and the presence of anti-IFN-ω in sera is now suggested as a diagnostic tool preceding mutational analysis of *AIRE* [101].

The presence of CMC as one of the three major components of APS-I has puzzled scientists for years. The identification of the Th17 derived cytokines as autoantigens in APS-I identified CMC as part of the autoimmune reaction and not as an immune defect. Due to technical difficulties in expressing high amount of radiolabelled IL-17F and IL-22 in use for RIA, different dimers were designed in this project, and thought to mimic their biological function, as these interleukins bind their receptor as dimers. Including the monomers, dimers and the fusion protein of IL-17F and IL-22, a total of five RIAs were established, all shown to be highly sensitive and specific in their detection of these interleukins (*Paper IV*).

Choice of immunological methods

The cytokine autoantibodies have mainly been discovered by utilising antiviral interferon neutralising assay (AVINA). A major drawback with this method is the use of cytolytic virus, demanding a restrictive laboratory procedure. It is also proven difficult to standardise the method between laboratories. The biological activity exerted by the individuals' autoantibodies is detected with this assay [84], but as the level of viral infections seem to be normal in APS-I, these assays are suggested to exaggerate the neutralisation potential of the autoantibodies [256]. The final result, measured by AVINA or RIA, appeared to be highly consistent when a third of the patients included in the large European study were analysed by both methods [101], and this was also the result in *Paper II*.

Enzyme-linked immunosorbent assay (ELISA) is an extensively used immunologic method to detect antigen-antibody binding. The results from different reports employing ELISA of anti-IL-17F and anti-IL-22 in APS-I sera were divergent, which could possibly reflect both under- and over estimation of the presence of these autoantibodies (*Paper IV*) [86, 256]. As ELISA often depends on commercial proteins, the source and the purity of the protein might account for variations in the outcome, together with any variations of the secondary antibody used for detection. Autoantibodies against IL-22 were identified with higher sensitivity using AVINA compared to ELISA, which could reflect a requirement and specificity for naïve epitopes [256]. ELISA separated poorly the samples with intermediate AVINA results, while the results for the same samples in RIA were consistent with AVINA [101]. RIA (*Paper II, Paper IV*) has the obvious drawback of utilising ^{35}S radiolabelled methionine. However, RIA is a reliable method, yielding consistent results over time, and is easy to standardise with low inter- and intra- variations, which makes it ideal for high-throughput screening.

Ethical aspects of autoantibody-screening

As the cytokine autoantibodies in APS-I (*Paper II and Paper IV*) are detectable from a very early age and before any clinical manifestations [103], they, together with anti-21-OH, predict a possible onset of disease years before any clinical signs. Therefore, they could be suggested as a screening tool in populations with high prevalence of inherited autoimmunity, raising ethical questions of population screening.

The importance of autoantibody-screening is exemplified by diabetes type 1, where the detection of autoantibodies against islet cell antigens and GAD65 following reference standards, are strong predictors of disease [257]. The screening for these autoantibodies in different groups including infants, school children and adults aim to predict development of the disease and to reveal contribution of environmental triggers [258]. In life-threatening but treatable diseases like AAD, type 1 diabetes and thyroiditis where autoantibodies precede the clinical symptoms, autoantibody-screening could be beneficial for the patients and cost saving for the community [259]. Yet, as there is no way to avoid progression of these diseases, the risk of over-treatment and the possible lower quality of life by living with a prospective diagnosis should be addressed.

Aire, thymoma, CMC and cytokine autoantibodies

IFN-ω, thymoma and AIRE

We (*Paper II*) and others [84, 101, 102] have confirmed IFN-ω as a universal marker of APS-I and investigated its diagnostic potential. Interestingly, autoantibodies against IFN-ω and other type-I IFNs were first described in patients with MG and thymoma [260], where they are present in sixty per cent of the patients [261].

Most human thymomas lack expression of AIRE, but AIRE is still present in the remaining tissue of the thymus and in lymph nodes of thymoma-patients, and only five per cent develop symptoms characteristic for APS-I [262, 263] (submitted manuscript). Forty per cent of the thymoma patients develop MG [264], which is not part of the clinical picture seen in APS-I. Interestingly, some thymoma patients also display the more common tissue restricted autoantibodies typically seen in APS-I, while the reverse is not the case, as anti-Titin and anti-AChR were not found in patients with APS-I (submitted manuscript).

The reason why only a few thymoma patients develop an APS-I like clinical picture might be due to their normal expression of AIRE in the fetal period, which seems to be important in the establishment of tolerance [265], and by their expression of AIRE in the periphery and in the residual thymus. This could possibly reflect different roles of AIRE throughout life, and support the importance of peripheral tolerance. The similarities between the two disorders in terms of cytokine autoantibodies and sometimes in clinical picture, indicates a role of AIRE and the thymus in maintaining central tolerance even in adult life, and suggest the thymus to be responsible for development of the cytokine autoantibodies. Thymoma represent a potential of studying the lack of AIRE in thymus in a human setting different from APS-I.

IL-17F, IL-22, thymoma and CMC

Similar to the autoantibodies against the type-I IFNs, autoantibodies against the Th17 derived cytokines are a shared feature between APS-I patients with CMC and patients with thymoma and CMC. Autoantibodies against IL-17F and IL-22 are reported in five to ten per cent of thymoma and CMC patients, and respectively in seventy-five and ninety-one per cent of the APS-I patients [256]. In *Paper IV*, we could not confirm these autoantibody-levels, as autoantibodies were detected in thirty-six (anti-IL-17F) and eighty (anti-IL-22) per cent of the Norwegian APS-I patients. Our results from *Paper IV* indicates a correlation between IL-17F and CMC, and the previous

reported correlation between IL-22 and CMC could not be confirmed [85, 256]. Our results are more in line with the increased susceptibility to CMC due to impaired IL-17 immunity in patients with inherited mutations in *STAT3*, *IL-17RA* or *IL-17* [144-146]. Conversely, patients with *STAT3* mutations have severe staphylococcal infection which is not seen in APS-I [266]. Taken together, inefficient signalling, autoantibodies targeting the cytokines, or the lack of cytokine expression, all result in malfunctions in the Th-17 compartment which predispose for CMC, and the common malfunction of the thymus shared between APS-I and thymoma patients is a plausible cause for the origin of these autoantibodies.

Immune cells in APS-I

The balance between Th17 and Treg cells is of particular interest in the view of autoimmunity. Their shared dependence of TGF-β is an effective mechanism to regulate the effect of the pathogenic Th17 cells [215, 267, 268]. Treg cells were shown to be down-regulated in APS-I by us (*Paper III*) and others [213], and the lack of *AIRE* is suggested as the cause of their impaired suppressive function [214]. Taken together, the malfunction of AIRE impairs the function and development of Treg cells, making them likely to play a part in the pathogenesis of APS-I. The disrupted homeostasis and activation of Treg cells is thought to take place in the periphery due to the lack of AIRE [214]. This could be a likely situation, as patients with MG and thymoma have normal levels of Treg cells in the periphery, while a decreased number Treg cells is found in the residual thymus [269]. However, the status of Treg cells in patients with MG and thymoma are still to be established [270-272]. The level of $CCR4^{+}CCR6^{+}$ cells, which includes IL-17A producing cells, were found to be unaltered in patients compared to controls (*paper III*), which is in agreement with the unchanged IL-17A response reported in APS-I [85], and isolated CMC [143]. Hence, these data emphasise the role of autoantibodies against IL-17F and IL-22 as the main mediators of CMC. As the number of Treg and Th17 cells should be balanced, a reduced number of Treg cells might imply increase in the levels of Th17 cells. However, as we found the levels of Th17 cells in APS-I to be normal (*Paper III*), the

lack of up-regulation of the Th17 cells could reflect the clinical status of CMC, as decreased function and number of Th17 cells are seen in patients with isolated CMC [143].

The number of iNKT cells has previously been reported to be decreased in APS-I patients [273], but this finding could not be confirmed in the Norwegian APS-I cohort (*Paper III*). This might be due to the relatively low amounts of iNKT cells in blood, which make the data less robust.

The frequency of monocytes in APS-I patients (*Paper III*) varied between the patients, but overall, no significant increases could be detected, as previously observed [198, 274]. However, the monocyte sub-population of $CD14^{+}CD16^{+}$ cells was found to be decreased. These monocytes are predicted to inhabit an increased APC-activity as they express high levels of HLA-DR [275]. Distribution of high doses of methylprednisolone to patients with MS and healthy controls resulted in a ninety-five per cent decrease in the levels of $CD14^{+}CD16^{+}$ cells after five days, possibly reflecting selective depletion of these cells by glucocorticoids [276, 277]. This could explain the observed decreased frequency, as eighty per cent of our patients are diagnosed with AAD and therefore treated with hydrocortisone and fludrocortisone. In addition, the levels of $CXCR3^{+}CCR6^{+}$ Th cells, which co-produce IFN-γ and IL-17A, were reduced in our study, which could reflect the persistent CMC infection; increased homing of these cells to inflammatory tissue by binding of the interferon-induced chemokine CXCL10 previously shown to be increased in APS-I [104], which might decreased their frequency in circulation.

Conclusions

In accordance with the specific aims of this study, the following conclusions can be drawn:

- The susceptibility to develop AAD in a Norwegian population was not influenced by common polymorphisms in *AIRE* (*Paper I*).

- CNV-analysis should be performed in APS-I patients found to be homozygote for an *AIRE* mutation as deletions may be present (*Paper I*).

- The Norwegian APS-I patients exhibit a reduced number of Treg cells, $CCR6^{+}CXCR3^{+}$ co-expressing Th subpopulation and $CD14^{+}CD16^{+}$ monocytes which may contribute to the observed autoimmunity (*Paper III*).

- RIA was established as a robust, highly specific method detecting anti-IL17F and anti-IL-22 based on monomer or dimers of the interleukins (*Paper IV*).

- The high prevalence of autoantibodies against IFN-ω in patients suffering from APS-I was confirmed in an expanded cohort of patients from different countries including a large number of different controls (*Paper II*).

- The diagnostic value of IFN-ω was confirmed in an assay that is easy to standardise and ready for high-throughput analysis (*Paper II*), which is now implemented in the routine analysis.

Future perspectives

Patients with APS-I have circulating autoantibodies directed against target enzymes in their body, as well as against components of their immune system. As already emphasised, these autoantibodies are important markers and predictors of disease. Therefore, the identification of additional molecular target within organs or the immune system itself is of outmost importance to aid serological diagnosis and may shed light on the molecular mechanisms of APS-I. The most widely used, and so far most successful method, has been to utilise sera from patients to screen cDNA library of different tissues for autoantigens, a method that is well established in our lab. The utilisation of more modern techniques, like proteomics, could complement and facilitate identification of new autoantigens.

Patients recruited to ROAS are analysed against a panel of different autoantigens, which have the potential to be analysed together by the development of new immunologic tools. Approaches like flow cytometric bead-based technology or peptide arrays have the benefits of reduced sample volumes and allow analysation of multiple autoantigens in a single sample, and could also include antigen-markers of more common autoimmune endocrine disorders. Using these techniques coupled with prospective follow-up of patients could provide new information on the prospective value of autoantibody assays.

The underlying cause and function of the autoantibodies remain obscure, and their study in patients with APS-I compared to their presence in patients with MG and thymoma, might reveal new clues on the pathogenesis of autoimmunity and the establishment and maintenance of tolerance. In addition, patients with inherited CMC pose an additional human disease with common features which could shed new light on immunological pathways.

ROAS is still growing, as is the number of APS-I patients included in the registry. Among them are several families which potential in studies of the genome and proteome as well as cell-based studies are still to be fully exploited.

The role of epigenetic mechanisms and the newly discovered molecular partners of AIRE in the thymus pose novel targets to be analysed both in APS-I and in other autoimmune diseases. Even though these studies in humans only are possible outside the thymus, it should be interesting to investigate the partners of AIRE in different autoimmune diseases. Further, to mimic different disease-causing mutations of AIRE in cell-culture studies could pinpoint the exact molecular flaw or flaws of nature underlying APS-I, with possible implications for explaining the large phenotypic variations and severity of the disease.

To date, a combinational approach, utilising autoantibodies and genetic markers is the best method to predict the presence and development of APS-I and its components. Early diagnosis is crucial, as it gives the opportunity of early intervention, by immunomodulation or immunosuppressive treatment.

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