Exploring and redefining Autoimmune polyendocrine syndrome type 1

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Øyvind Bruserud Bergen, October 2018

Summary

Autoimmune polyendocrine syndrome type 1 (APS-1) is a rare, monogenic, childhood-onset disorder caused by mutations in the *autoimmune regulator* (*AIRE*) gene. Multi-organ autoimmune disease and chronic mucocutaneous candidiasis (CMC) dominate the clinical phenotype, making it an important model disease for autoimmunity. The objective of this thesis was to perform a detailed clinical, genetic and immunological characterisation of Norwegian APS-1 patients and explore the mechanisms behind CMC susceptibility.

Fifty-two patients were included, revealing highly variable phenotypes. Most patients presented with one of the major disease components during childhood, hypoparathyroidism, primary adrenal insufficiency or CMC; enamel hypoplasia, hypoparathyroidism and CMC were the most frequent features. The prevalence of CMC indicates a specific immunodeficiency, which was underpinned by our finding of dysregulated immune responses to a *Candida* challenge. Specifically, monocytes produced significantly less interleukin-23p19 (IL), an important mediator in the *Candida* defence. Properly treatment of *Candida* infections is important as long-term inflammation in the oral cavity contributes to the development of oral malignancies, described here as a novel entity of APS-1.

All Norwegian patients presented tissue-specific autoantibodies, and most had reactivity against IL-17, IL-22, and interferon-ω. The most common *AIRE* mutation was c.967_979del13. The splice mutation c.879+1G>A was associated with a mild adult-onset phenotype. Possible explanations are partial activity by *AIRE* lacking exon 7 and/or a certain amount of wild-type transcripts being produced despite mutation in a conserved splice donor site. Finally, the influence of environmental factors was explored by characterizing the oral microbiome. Indeed, APS-1 patients have significantly altered oral microbiota, with a general reduction in the total number of bacterial genera and species and altered relative abundance of major phyli compared to healthy subjects.

This research has implications for the diagnosis and clinical care of patients with APS-1 and organ-specific autoimmune diseases and offers further insight into some of the mechanisms underlying autoimmune disorders and CMC.

Table of contents

Abbreviations

21OH	21-hydroxylase
AADC	Aromatic L-amino acid decarboxylase
ACTH	Adrenocorticotropic hormone
AIRE	Autoimmune regulator
ALPS	Autoimmune lymphoproliferative syndrome
APC	Antigen-presenting cell
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APS	Autoimmune polyendocrine syndrome
B cell	Bone marrow-derived lymphocyte
B2M	Beta-2 microglobulin
BCR	B cell receptor
C. albicans	Candida albicans
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
cDNA	Complementary DNA
CLR	C-type lectin receptor
CMC	Chronic mucocutanous candidiasis
Ct	Cycling threshold
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum

FOXP3	Forkhead box P3
GAD	Glutamic acid decarboxylase
HLA	Human leukocyte antigen
HP	Hypoparathyroidism
Ig	Immunoglobulin
IGFL1	IGF-like family member 1
IL	Interleukin
ILR	Interleukin Receptor
ILRA	Interleukin Receptor alpha chain
IFN	Interferon
IPEX	Immune dysregulation polyendocrinopathy enteropathy X-linked
KRT14	Keratin 14
MHC	Major histocompatibility complex
mRNA	Messanger RNA
MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cell
PAI	Primary adrenocortical insufficiency
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE	Promiscuous gene expression
PHD	Plant homeodomain zinc finger
PRR	Pattern recognition receptor
qPCR	Quantitative PCR
RAG	Recombination activation gene

RNA	Ribonucleic acid
ROAS	Norwegian registry of organ-specific autoimmune diseases
SAND	*S*p100, *A*IRE-1, *N*ucP41/75 and *D*EAF-1
SCC	Side-chain-cleavage enzyme
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
SYK	Spleen associated tyrosine kinase
T cell	Thymus-derived lymphocyte
TCR	T-cell receptor
Th cell	T helper cell
TH	Tyrosine hydroxylase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPH	Tryptophan hydroxylase
Treg	T regulatory cell
TSHR	Thyroid Stimulating Hormone Receptor
TYK	Tyrosine Kinase
WT	Wild-type

List of publications

Paper I

Bruserud Ø, Oftedal BE, Landegren N, Erichsen MM, Bratland E, Lima K, Jørgensen AP, Myhre AG, Svartberg J, Fougner KJ, Bakke Å, Nedrebø BG, Mella B, Breivik L, Viken MK, Knappskog PM, Marthinussen MC, Løvås K, Kämpe O, Wolff AB, Husebye ES. A Longitudinal Follow-up of Autoimmune Polyendocrine Syndrome Type 1. *J Clin Endocrinol Metab. 2016 Aug;101(8):2975-83.*

Paper II

Bruserud Ø, Costae DE, Laakso S, Garty BZ, Mathisen E, Mäkitie A, Mäkitie MS, Husebye ES. Oral tongue malignancies in Autoimmune Polyendocrine Syndrome Type 1. *Front Endocrinol. 2018 Aug. 9:463. doi: 10.3389/fendo.2018.00463.*

Paper III

Bruserud Ø, Bratland E, Berger A, Hellesen A, Wolff ASB, Husebye ES, Oftedal BE. The *AIRE* mutation c.879+1G>A displays a mild phenotype and alternative mRNA splicing in patients with Autoimmune Polyendocrine Syndrome Type 1. *Manuscript.*

Paper IV

Bruserud Ø, Bratland E, Hellesen A, Delaleu N, Reikvam H, Oftedal BE, Wolff ASB. Altered Immune Activation and IL-23 Signaling in Response to Candida albicans in Autoimmune Polyendocrine Syndrome Type 1. *Front Immunol. 2017 Sep. 1;8:1074. doi: 10.3389/fimmu.2017.01074.*

Paper V

Bruserud Ø, Siddiqui H, Marthinussen MC, Chen T, Jonsson RS, Oftedal BE, Olsen I, Husebye ES, Wolff ASB. Oral microbiota in Autoimmune polyendocrine syndrome type 1. *J Oral Microbiol. 2018 Feb. 26 ;10(1) :1442986. Doi: 10.1080/20002297.2018.1442986.*

Related publications

1. Fishman D, Kisand K, Hertel C, Rothe M, Remm A, Pihlap M, Adler P, Vilo J, Peet A, Meloni A, Podkrajsek KT, Battelino T, **Bruserud Ø**, Wolff ASB, Husebye ES, Kluger N, Krohn K, Ranki A, Peterson H, Hayday A, Peterson P. Autoantibody Repertoire in APECED Patients Targets Two Distinct Subgroups of Proteins. *Front Immunol.* 2017 Aug 16;8:976.

2. **Bruserud Ø**, Oftedal BE, Wolff ASB, Husebye ES. *AIRE* -mutations and autoimmune disease. *Curr Opin Immunol*. 2016 Aug 6;43:8-15.

3. **Bruserud Ø**, Husebye ES. A man in his 30s with diabetes and vitiligo. *Tidsskr Nor Laegeforen.* 2015 May 5;135(8):763-6.

1. Introduction

The immune system protects the host from foreign substances and consists of two main systems: the innate (natural, non-specific) system and the adaptive (acquired, specific) system (1). Coordinated crosstalk between these systems is needed for an effective immune response and depends on direct cell-to-cell contact and the production of numerous soluble mediators. Self-tolerance is crucial for a normal immune system and proper immune responses. However, loss of tolerance may lead to aberrant immune system responses to its own tissue, which are collectively defined as *autoimmunity* and may manifest as clinical disease. In this introduction, a brief overview of the innate and adaptive immune systems is given, and immunological tolerance and autoimmunity are defined. Then follows a detailed description of autoimmune polyendocrine syndrome type 1 (APS-1) as a severe clinical syndrome and model disease for studying immunological mechanisms.

1.1 Innate immunity

The innate immune system is the first line of defence against foreign invaders, and it consists of the cellular components of epithelial barriers and different subsets of immune cells, including neutrophils, monocytes and macrophages, natural killer cells and mast cells. Innate immune cells harbour specific pattern recognition receptors (PRRs) that recognise structures called pathogen-associated molecular patterns and damage-associated molecular patterns, which indicate microbe invasion or cellular damage, respectively (2). These PRRs are grouped into families based on their structure and function, including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like receptors, and NOD-like receptors. The most extensively studied PRRs are TLRs (3), which include both membrane-bound receptors (e.g. TLR2, TLR4, TLR6) and intracellular receptors (e.g. TLR3, TLR9). Collectively, TLRs have the capability to recognise foreign antigens of bacteria, fungi and protozoans,

and viral and prokaryotic nucleic acids. Activation of TLRs leads to the production of pro-inflammatory cytokines and chemokines.

CLRs are essential for the recognition of the *Candida* species (4) and include Dectin receptors, which recognise glucose polymers of the cell wall of fungi (5, 6). Dectin-1 receptors are mainly expressed on monocytes and macrophages and recognise the β-glucans of the *Candida* cell wall. The activated Dectin-1 receptor induces intracellular signalling through a pathway mediated by spleen tyrosine kinase (SYK), caspase activation and recruitment domain-containing protein 9 (CARD9), and the protein Cδ (7-11) as well as the signalling pathway mediated by serine-threonine kinase RAF1 (12). These intracellular signalling pathways terminate in activation of the transcription factor nuclear factor κB, which lead to cytokine production and internalisation of fungi (13). Engagement of Dectin-1 can also amplify responses of other PRRs such as TLR2 and TLR4. Figure 1 gives a schematic overview of the host innate immune response against fungal infections exemplified by *Candida albicans* (*C. albicans*). Recent evidence indicates that the Dectin-1 receptor also has the potential to induce epigenetic reprogramming and a metabolic shift in innate immune cells (14, 15). These mechanisms create a kind of immunological memory in innate immune cells, which is defined as *trained immunity* (16).

Taken together, PRRs initiate intracellular signalling pathways that terminate in expression of cytokines and other inflammatory mediators, which further induce phagocytosis of microbes, activation of the complement system and recruitment of immune cells to the primary site of infection.

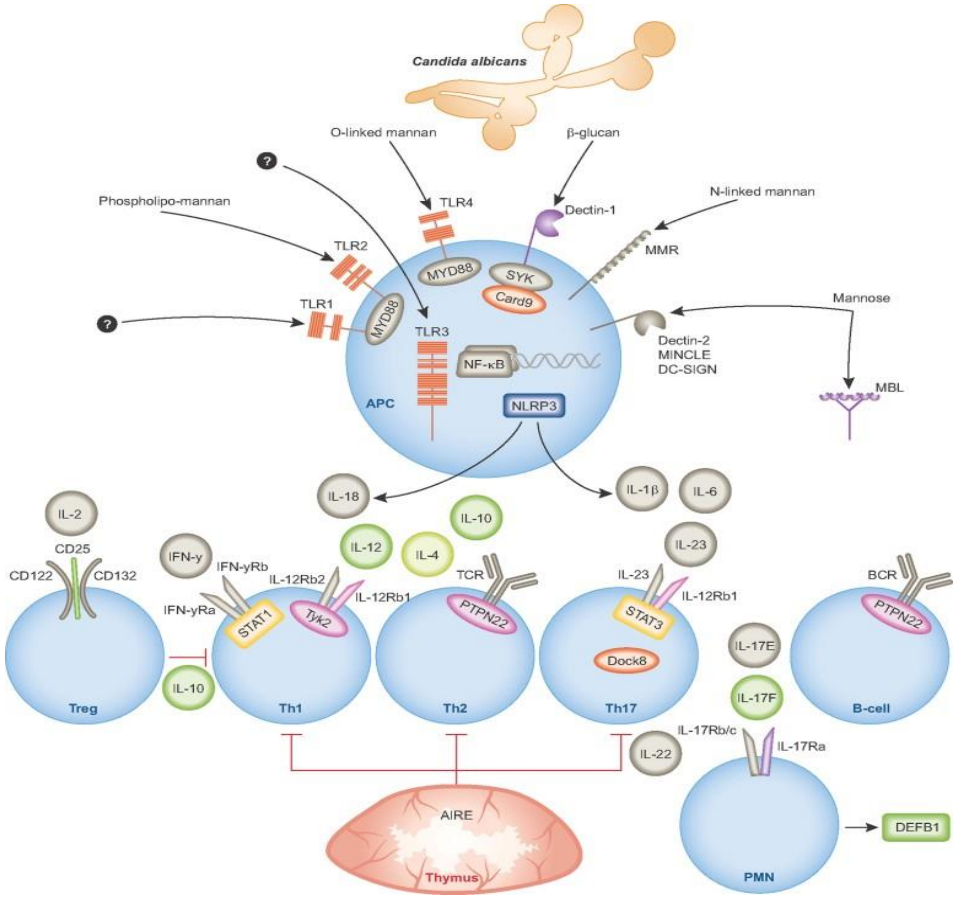

Figure 1. Schematic overview of the immune response against Candida albicans.

Candida is recognised by PPRs, including TLRs and CTLs which, upon activation, induce the production of cytokines through the activation of NF-κB. The NLRP3 inflammasome cleaves both IL-1β and IL-18 before they can be secreted. IL-2 is crucial for differentiation of all effector T cells. IL-12 and IL-18 induce the differentiation of Th1 cells whereas the differentiation of Th2 cells is promoted by IL-4 and IL-10. Th17 cell development depends on IL-1β, IL-6 and IL-23, and is maintained by DOCK8. Recruitment of neutrophils to the infected tissue is promoted by IL-17. The different cytokines bind to their receptors, which activate adaptor molecules including STAT1, STAT3 and TYK2. PTPN22 is involved in BCR and TCR signalling. APC, antigen-presenting cell; BCR, B-cell receptor, CARD9, caspase recruitment domain 9; CD, cluster of differentiation; CTL, C-type lectin receptor; DC-SIGN, dendritic cell-specific ICAM-grabbing non-integrin; DEFB1, beta-defensin 1; DOCK8, dedicator of cytokinesis 8; IL, interleukin; IFN, interferon; MBL, mannose binding lectin, MMR, macrophage mannose receptor; MYD88, myeloid differentiation factor 88; NFκB, Nuclear factor kappa beta; NLRP3, NACHT, LRR and PYD domain-containing protein 3; PMN, polymorphonuclear leukocytes; PTPN22, Protein Tyrosine Phosphatase Non-Receptor type 22; SYK, spleen-associated tyrosine kinase; STAT, signal transducer and activator of transcription; TCR, T-cell receptor; Th, T helper; TLR, Toll-like receptor. Reproduced with permission from Smeekens et al. (17).

1.2 Adaptive immunity

The adaptive immune system acts as a second line of defence and has a prolonged activation relative to the innate system. The adaptive immune response is mediated by thymus-derived T lymphocytes (T cells) and their associated T-cell receptors (TCRs) and bone marrow-derived B lymphocytes (B cells) receptors (BCRs). The recognition of antigens by TCRs and BCRs is highly specific. Importantly, the adaptive immune system has the capacity to develop immunological memory (18, 19).

1.2.1 T cells

Precursor T cells arise in the foetal liver or adult bone marrow, seed the thymus where they differentiate into mature T cells, exit the organ and migrate to secondary lymphoid organs. However, naïve T cells circulate between lymph nodes and the bloodstream. T cells play a crucial role in cellular immunity and can be divided into two major subsets based on the expression of cluster of differentiation (CD) 8 and CD4, which interact with the major histocompatibility complex (MHC) class I and MHC class II molecules, respectively. MHC class I molecules are found on all nucleated cells and express peptides processed from endogenous antigens, whereas MHC class II molecules are found on professional antigen-presenting cells (APCs), such as dendritic cells (DCs), macrophages and B cells, and express peptides from the endocytic pathway. Antigens are presented on the MHC molecules to be recognised by their specific TCRs. Appropriate TCR-peptide-MHC interactions activate the naïve T cells through complex downstream intracellular signalling cascades (20). Additional co-stimulation is required for the development of full effector functions, which is particularly dependant on interaction between the CD28 co-receptor on the T cells and the CD80/CD86 receptor on APCs as well as specific cytokine stimuli (21).

Upon activation by APCs, the naïve T cells produce interleukin (IL)-2, which acts in an autocrine manner driving T cells into clonal expansion (22). CD4+ T cells

differentiate into one of several subsets of T helper (Th) cells (e.g. Th1, Th2, Th17 and Th22) or regulatory T cells (Tregs). For example, the Th17 linage is characterised by the production of IL-17 and IL-22 and is crucial for immunity against fungal infections. Their differentiation depends on the cytokine-transforming growth factor β and the presence of IL-6, IL-21 and IL-23, together with the induction of the Retinoic-acid-receptor-related orphan nuclear receptor γt (23, 24). The naïve CD8+ T cells differentiate into cytotoxic T lymphocytes upon activation. These differentiation processes depend on cytokines and co-stimulatory signals from APCs, and effector T cells navigate to the affected area through chemokine gradients. Most activated T cells die in apoptosis, but a small population survive giving rise to a pool of long-lived memory cells. Long-lived memory cells are crucial for the immunological memory of the adaptive immune system.

1.2.2 B cells

B cells develop in the bone marrow and finally mature in the spleen before they circulate between the bloodstream and secondary lymphoid organs searching for their cognate antigen. B cells act as APCs for T cells. However, their activation depend on the recognition of naïve antigens by their transmembrane BCRs in the presence of costimulatory signals from Th cells (25). Activated B cells secrete antibodies with the same affinity as their BCRs with the potential of neutralizing toxins, activating the complement system and marking microbes to facilitate phagocytosis (26). These effector functions depend on the isotype of the antibodies/immunoglobulins (Ig) which can be divided into five major classes: IgA, which is crucial in mucosal immunity; IgD, which is an antigen receptor of naïve B cells; IgG, which is crucial for opsonisation of antigens and promoting phagocytosis, complement activation, and neonatal immunity; IgM, which contributes in complement activation and is an antigen receptor of naïve B cells; and IgE, which causes mast cell degranulation.

During activation and differentiation, B cells have the potential to change their antibody class (isotype switching) and antibodies with increasing antigen affinity

progressively dominate the response (affinity maturation). This represents a way of fine-tuning B cell responses. Specific stimuli, particularly CD40 ligation and cytokines, drive further proliferation and differentiation of activated B cells into plasma cells and memory B cells (25), the latter of which will rapidly expand upon a second challenge (26).

1.3 Mucosal immunity

Mucosal surfaces of the human body are in intimate and constant contact with the external environment, representing portals for infectious agents, allergens and carcinogens (27). Mucosal epithelial surfaces provide physical barriers and are covered by mucus and antimicrobial products, including secretory IgA, that limit epithelial contact and penetration of foreign substances. Epithelial cells also hold innate immune functions and interact with intraepithelial lymphocytes to maintain homeostasis. The underlying specialised connective tissue, namely mucosa-associated lymphoid tissue, mediates immune responses against invading organisms and provides a continuous source of memory B and T cells. Adaptive immune responses to invading microbes are initiated and amplified in the distant draining lymph nodes, with T cells being the crucial effector cells (28, 29). Importantly, immunosuppressive mechanisms, particularly those regulated by Tregs, inhibit overreaction against innocuous luminal antigens and are collectively referred to as *oral tolerance* (30).

Mucosal surfaces of the human body also home an ecosystem of microbes known as the microbiota. A substantial body of evidence suggests a potential effect of the microbiota on physiological processes, including metabolism and immune-system development and function (31). Disturbances in this ecosystem, referred to as dysbiosis, can contribute to several pathological processes including autoimmune disorders (31, 32). However, *Candida* species are also thought to induce innate

immunity through interaction with epithelial surfaces (33, 34) and lymphoid cells (35, 36).

1.4 Immunological tolerance and autoimmunity

1.4.1 Central tolerance

Immunological tolerance can be defined as a lack of response by the immune system to an antigen with the capacity to elicit an immune response, and the term central tolerance includes all mechanisms by which T and B cells learn to discriminate self from non-self. The thymus is divided into the cortex and medulla and is the anatomical site where T cells mature and central tolerance occurs. Within the cortex and under the control of the *recombination activation* gene (*RAG*), TCRs are randomly generated by the somatic, convergent recombination of the variable (V), diversity (D), and joining (J) gene segments. Double-positive T cells bearing both CD4 and CD8, whose TCRs appropriately recognise the self-peptide-self-MHC complexes expressed on cortical thymic epithelial cells receive critical surviving signals. This process of testing the TCRs specificity and avidity is known as *positive selection* (37).

After positive selection, T cells further differentiate into CD4 or CD8 single-positive cells according to their affinity to MHC class I and II molecules, respectively (38, 39). The single-positive T cells then migrate to the thymic medulla where they interact with medullary thymic epithelial cells (mTECs) and other bone marrow-derived APCs. The transcription factors Fez family zinc finger protein 2 and autoimmune regulator (*AIRE*) independently regulate presentation of a wide range of tissue-specific self-antigens in a process called *promiscuous gene expression* (PGE) (40-42). *AIRE* has been extensively studied, and its role in central tolerance is illustrated in Figure 2. T cells expressing TCRs that react strongly to self-antigens presented by MHC molecules induce the deletion of thymocytes by apoptosis or differentiate into Tregs (37, 43). Tregs primarily develop from immature CD4+

thymocytes and differentiate into functionally competent cells within the thymus (44). However, low-avidity TCR interactions with self-peptide and MHC class I or II complexes expressed by mTECs allow survival and further differentiation of single-positive CD4 and CD8 cells to naïve T cells, which migrate from the thymus to the peripheral lymphoid organs (37). This process is referred to as *negative selection* and provides a mechanism by which developing T cells are exposed to a wide array of self-proteins, making it possible to delete autoreactive T cells from the repertoire (45).

B cells mature and go through central tolerance in the bone marrow. Immature B cells that recognise self-antigens with a high affinity are induced to change their specificity by a process called receptor editing (46). Mature self-reactive B cells that fail in rearranging their BCRs undergo apoptosis (47).

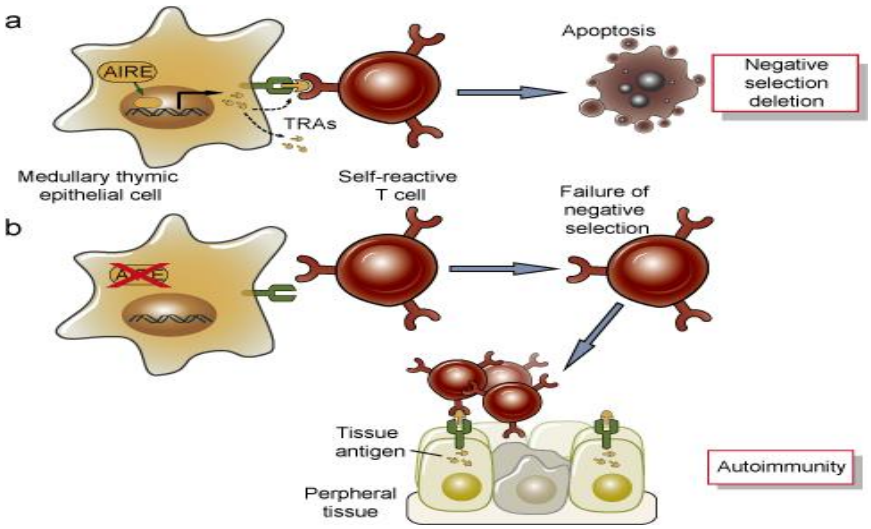

Figure 2. The role of AIRE in central tolerance.

*AIRE orchestrates the process of PGE in mTECs where a wide range of TRAs are presented for developing T cells. (**a**) In healthy individuals, T cells presenting TCRs that interact too strongly with TRAs go into apoptosis. (**b**) Due to lack of AIRE, T cells with a high affinity to self-antigens escape negative selection and are released into the periphery with the potential to cause autoimmune disease. The figure is reproduced with permission from Creative Diagnostics, US. mTEC, medullary thymic epithelial cell; PGE, promiscuous gene expression; TCR, T cell receptor; TRA, tissue restrictive self-antigens*

1.4.2 Peripheral tolerance

Some self-reactive lymphocytes will complete their maturation despite the mechanisms of central tolerance. A peripheral line of defence against these potential harmful cells is therefore needed. The handling of peripheral self-reactive T cells involves three main mechanisms (48). First, the self-reactive T cells may become anergic, or functionally inactivated by a lack of proper co-stimulatory signals; second, APCs or DCs may induce apoptosis or differentiation into peripheral Tregs (also called induced Tregs or adaptive Tregs); and third, self-reactive T cells may be suppressed by Tregs (48).

Self-reactive B cells that escape the mechanisms of central tolerance become anergic if they lack proper co-stimulatory signals from T cells (49) or are deleted in the spleen or lymph nodes upon interaction with CD4+ T cells (50, 51).

1.4.3 Systemic and organ-specific autoimmunity

Autoimmune reactions reflect an imbalance between effector and regulatory immune responses, with the overall underlying mechanism being defective elimination and/or control of self-reactive lymphocytes. Autoimmune diseases have a prevalence of about eight percent of the populations in Europe and the United States (52) and seem to increase in prevalence (53). Collectively, they cause considerable morbidity and mortality (54), creating a worldwide economic burden of comparable magnitude to that of cardiovascular diseases and cancer. Autoimmune diseases generally occur more frequently in women, and disease onset usually occurs during adolescence (54, 55).

Autoimmune disorders can be divided into two major forms based on their autoimmune responses: systemic autoimmunity and tissue-specific autoimmunity. In systemic, or disseminated autoimmunity, autoimmune responses are directed against self-antigens with ubiquitous expression patterns, typically nuclear components such

as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), histones, and topoisomerases (56). This group of disorders shows diverse phenotypic expressions, as described in, for example, systemic lupus erythematosus (SLE) (57), sclerosing skin diseases (58) and Sjögrens syndrome (59). Immune modulatory agents including cortisone, methotrexate and anti-Tumour necrosis factor alpha (TNF-α) are effectively used to inhibit disease activity. In tissue-specific autoimmunity, the autoimmune responses are directed against self-antigens present in one particular organ or tissue. A typical example is autoimmune diabetes mellitus type 1, in which the autoimmune reaction is directed against organ-specific self-antigens (Glutamate decarboxylase 65, Islet antigens-2) causing autoimmune destruction strictly limited to insulin producing beta cells in the pancreas (60). One particular self-antigen (21-hydroxylase (21OH)) is dominant in Addison's disease (61), whereas patients with autoimmune polyendocrine syndromes (APS) have autoimmune reactions against several self-antigens typically located in affected endocrine tissues (62-64). In tissue-specific autoimmunity, the autoimmune activity is self-limiting because of the complete destruction of the affected tissue, and substitution therapy is often required to compensate for the lost tissue function.

Autoantibodies

Interaction between specific TCRs and BCRs and a distinct self-antigen is needed to induce an autoimmune response. Although T cell effector functions are directly responsible for tissue damage, B cell response is better suited to monitor disease. In addition to being markers of cell-mediated autoimmunity, autoantibodies may have a direct causative role in disease development. For example in Grave's disease, the binding of autoantibodies to the thyroid stimulating hormone receptor (TSHR) causes constant receptor activation, leading to an overproduction of thyroid hormone (65). Characterisation of autoimmune targets is likely to both reveal underlying cellular mechanisms and explain the origin of the clinical disease. However, autoantibodies can easily be assessed in peripheral blood and play a key role in both the research and clinical diagnosis of autoimmune diseases (66).

1.4.4 Genetic risk factors for pathological autoimmunity

Complex inheritance

Most autoimmune diseases arise from a combination of genetic predisposition, environmental factors and stochastic events (67). Genome-wide association studies have identified numerous genetic polymorphisms involved in the development of human diseases and several hundred loci that are associated with autoimmunity (68-70). Human MHC molecules, known as Human Leucocyte Antigen (HLA) molecules (71, 72), exhibit the strongest associations across most autoimmune diseases (73). The specificity of the HLA-peptide-TCR interactions is crucial for an appropriate adaptive immune response and for maintaining self-tolerance (73). Several mechanisms affecting the HLA-peptide-TCR interaction have been suggested as contributors to the loss of self-tolerance and the development of autoimmune diseases, including altered TCR docking, low-affinity-mediated thymic escape, molecular mimicry, hybrid peptides and differences in HLA expression and stability (73). Furthermore, genetic polymorphisms in genes encoding immune signalling molecules, including the interleukin 23 receptor (IL23R) and tyrosine kinase 2 (TYK2), are associated with autoimmune disorders (74), for example, polymorphisms in the *IL23R* gene are observed in ankylosing spondylitis, Bechet's disease, Crohn's disease, psoriasis and ulcerative colitis (75). Moreover, genes encoding proteins with specific functions in immunological synapses, *HLA, CD40*; T cell regulation, *Cytotoxic T-lymphocyte Associated Protein 4* (*CTLA-4)* and *Protein Tyrosine Phosphatase Non-Receptor Type 22*; and protein coding, *Interleukin 2 Receptor alpha chain, Fc Receptor-Like Protein 3, Thyroglobulin,* and *TSHR* are associated with autoimmune thyroid diseases (76). However, multiple polymorphisms within the genome of each individual patient probably contribute to disease development in most autoimmune diseases.

Monogenic factors

While most human autoimmune disorders result from polymorphisms of multiple genes involved in immune functions, some rare diseases are caused by mutations in single genes. Relevant examples of monogenic human autoimmune diseases are APS-1 caused by mutations in the *AIRE* gene (62, 64) and immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome caused by mutations in the *forkhead box P3* (*FOXP3)* gene (77). Studies of APS-1 patients have revealed the essential role of *AIRE* in thymic negative selection and central tolerance (78, 79), and studies of IPEX-patients have highlighted the crucial role of FOXP3 in development and function of Tregs (77, 80). The *CTLA-4* gene encodes a protein receptor that acts as an immune checkpoint, downregulating immune responses. Mutations in this gene are found in families with Mendelian multi-organ autoimmune disorders and are associated with impairment of the suppressive functions of Tregs (81, 82). Similarly, mutations in genes involved in Fas-mediated signalling cause autoimmune lymphoproliferative syndrome (ALPS), which is clinically defined by lymphoproliferative disease, autoimmune cytopenia and susceptibility to malignancy (83). Protein kinase Cδ deficiency can cause both SLE and an ALPS-like disease (84-86), and some extremely rare monogenic autoimmune diseases of the endocrine system are also described (87). Monogenic diseases are powerful *in vivo* models for immunological research, and studies of these patients have already provided insight on several biological pathways in immune homeostasis.

1.4.5 Environmental factors in autoimmunity

Environmental factors contribute to the heterogeneity found in the human immune system (88), and several environmental factors and external triggers have been suggested as contributing factors in the development of autoimmune diseases. In celiac patients, an immune response against the gliadin fraction of the gluten protein, driven by enterocytes and gliadin-reactive CD4+ T cells, promotes inflammation in the upper small intestine (89). This interaction between gluten and immune, genetic

and environmental factors in celiac disease is unique because the environmental trigger which causes the disease is well-known (89, 90). Interestingly, elimination of gluten in the diet induces clinical improvement and histologic recovery in most patients.

Drugs represent another class of external triggers that may induce autoimmune disorders (91), the most documented of which is probably drug-induced SLE (92). Although the exact mechanisms involved are not well understood, continuous exposure to several different agents, such as thiazide diuretics, calcium channel blockers, terbinafine, angiotensin-converting enzyme inhibitors and immune modulators, has the potential to induce SLE (92). New cancer treatment involving blocking immune check-points (programmed cell death-1 inhibitors, CTLA-4 antibodies) and enhancing anti-tumour immunity can also cause a wide range of immune-related adverse events including autoimmune disorders (93-95).

There is also an established link between infections and autoimmune diseases (96-98), and many possible explanations have emerged including release of hidden antigens, epitope spread, molecular mimicry, the adjuvant effect and excessive PRRs activation. For example, systemic infections trigger relapses in patients with relapsing-remitting multiple sclerosis (MS) through the activation of myelin-specific T cells (96), and different microorganisms have been suggested to influence pathogenesis, relapse and disease progression (99). Vaccines are another reported external trigger for the immune system; observational studies indicated an increased incidence of narcolepsy after the H1N1 influenza pandemic and vaccination campaign in 2009-2010 (100, 101), and molecular mimicry has been theorised as a possible explanation for these findings (102).

The microbiota

A microbiota is defined as an ecosystem of microbes residing on the mucosal surfaces of the human body that co-evolves with the human host in a symbiotic manner. High throughput sequencing of the highly conserved 16S ribosomal RNA

subunit of prokaryotes have recently replaced traditional culture-based techniques for characterizing microbial communities in any given sample (103, 104). However, the intestinal microbiota is currently the most studied and its overall composition depends on both genetic (105) and environmental factors (106). Oral, gut and skin microbiota could all affect the pathogenesis of systemic and organ-specific autoimmune diseases (107). For example, studies in mice indicate that the microbiota influences the gender bias in autoimmunity (108, 109). Microbes have the potential to activate different immune receptors, including TLRs, and induce signalling through the adaptor myeloid differentiation primary response gene 88 and the TIR domain-containing adaptor inducing interferon-β (IFN), providing signals that both promote and inhibit autoimmunity (110, 111). Segmented filamentous bacteria have been of particular interest as they have the potential to breach the mucosal layers, interact with epithelial cells and, particularly through the induction of Th17 cells or Tregs, modulate host immunity and disease status (112, 113). Other suggested mechanisms through which microbiota promote autoimmunity are molecular mimicry and metabolic and epigenetic changes in host cells, especially histone acetylation controlled by microbial metabolites such as short-chain fatty acids (114, 115).

The intestinal microbiota has also been described in cohorts of human patients with autoimmune diseases such as diabetes mellitus type 1 (116), MS (117, 118), rheumatoid arthritis (119), and SLE (120). Although a general autoimmune profile has not been proven, a common feature is reduced species diversity and altered ratios between major phyli (116, 117, 119, 120). The complexity of both the bacterial community and the host immune system makes it difficult to develop robust models to investigate the molecular mechanisms of this interplay in humans. Notably, both genetic and environmental factors that affect the immune system also modulate microbiota profiles and functions, which leads to an obvious question about the cause and effect of dysbiosis. Nevertheless, the microbiotas probably influence several diseases and contribute in the pathogenesis and phenotypes of autoimmune disorders.

1.4.6 Other potential modulators of autoimmunity

Paraneoplastic syndromes are a heterogeneous group of systemic signs and symptoms caused by malignant cells and are most commonly found in patients with malignancies of the lungs, breasts, ovaries, or the lymphatic system. For example, bronchial carcinoids and small cell lung carcinomas are associated with the ectopic production of adrenocorticotropic hormone (ACTH), causing paraneoplastic Cushing syndrome (121). Moreover, a parathyroid hormone-related protein produced in malignant cells is the major mediator of humoral hypercalcemia in malignancy (122). Malignant cells may also display self-antigens that activate the immune system, leading to a break in immune tolerance, a phenomenon often seen in paraneoplastic neurological syndromes (123) where autoantibody production is also found (124). Pemphigus is another autoimmune disorder that is typically described as being part of a paraneoplastic syndrome (125). Interestingly, tumour resection and cancer treatment often cure the paraneoplastic disorders as well.

Epigenetic mechanisms such as DNA methylation, histone alterations, and RNA-based transcriptional control have the potential to regulate gene expression without changing the DNA sequence. Their sensitivity to external stimuli represent a link between environmental factors and genetics (126). Aberrant epigenetic patterns are described for several autoimmune diseases, suggesting a potential role in pathogenesis (127, 128). For example, impaired DNA methylation is found in T cells from human SLE patients (129, 130). Histone modifications in CD4+ T cells show significant alterations when compared to healthy controls and correlate with SLE disease activity (131). Noncoding RNA has also been proposed as a modulator of autoimmunity (132-134).

Other cellular mechanisms may also contribute in the pathogenesis and development of autoimmunity. Dysfunctional endosomal and cytosolic receptors including TLRs have been linked to autoimmune disorders (135). The type I IFN system plays a role in the pathogenesis of rheumatic disorders such as rheumatoid

arthritis and Sjögrens syndrome (136). Drugs targeting this pathway are currently being tested in clinical trials (137). Excessive production of type I IFNs is thought to affect both loss of tolerance and the autoimmune process in SLE (138, 139). Finally, because of the considerable sex bias characteristic of autoimmunity, with a higher incidence in females (140), gonadal hormones and direct X-chromosome effects are thought to affect the pathogenesis of autoimmune disorders (140).

1.5 Autoimmune polyendocrine syndrome type 1 (APS-1)

1.5.1 Diagnostic criteria and clinical features

APS is a group of diverse clinical conditions collectively defined by an autoimmune functional impairment of multiple endocrine glands. APS can be further categorised into APS-1 and APS-2 (64). APS-2 is a common polygenic syndrome characterised by at least two of the three following endocrinopathies: diabetes mellitus type 1, autoimmune thyroid disease and primary adrenocortical insufficiency (PAI), whereas APS-1, or Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, OMIM 240300), is a rare, childhood-onset, monogenic disease caused by mutations in the *AIRE* gene (141, 142). APS-1 affects both sexes equally and is reported to have a worldwide incidence of 1:100 000, with higher incidence among Persian Jews (1:9000) (143), Sardinians (1:14000) (144, 145) and Finns (1:25000) (62, 146). The syndrome is clinically defined by the presence of two of the three main components: hypoparathyroidism (HP), PAI, and chronic mucocutaneous candidiasis (CMC) (62, 64). The diagnosis can also be made upon identification of two disease-causing mutations in *AIRE* together with clinical manifestations or on the basis of one main manifestation combined with a sibling diagnosed with APS-1 (64).

The clinical picture of APS-1 is highly variable (Figure 3). Most patients present with one of the major disease components during childhood. HP is often the first endocrine manifestation, is the second most common component after CMC, and is present in about 80% of patients (145, 147-149). The onset of PAI normally occurs after CMC and HP, and it is reported in 60-80% of patients (145-148). Several other endocrine manifestations are also seen in APS-1 (64, 146, 150). Ovarian failure is frequently described (150), autoimmune hypophysitis and growth hormone deficiency have been reported (151), and autoimmune hypothyroidism is reported in about 30% (150). Pituitary failure and diabetes mellitus type 1 are uncommon (150), and central diabetes insipidus, gonadotropin, and ACTH deficiencies are very rare.

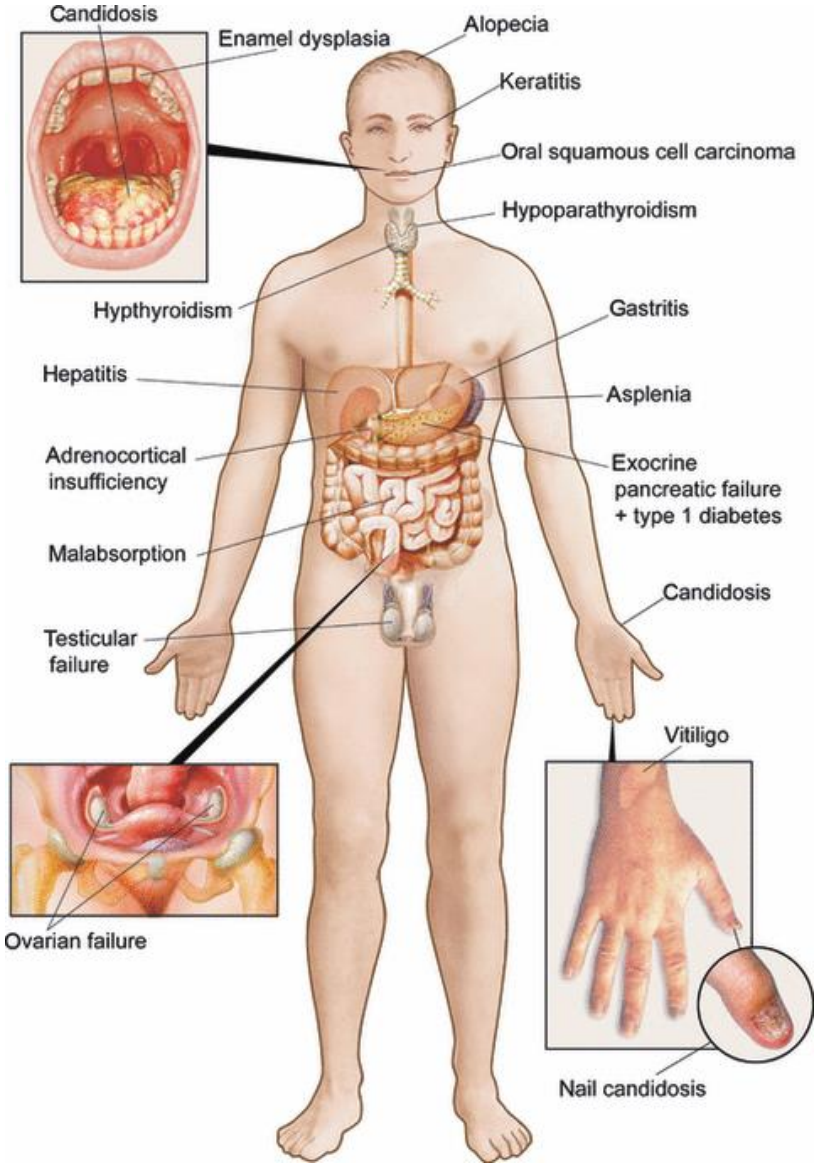

Figure 3. Homunculus illustrating the various manifestations of APS-1.

The figure illustrates the different disease components of APS-1. The syndrome is clinically defined by the presence of two of the three main components: HP, PAI and CMC. However, the clinical picture is highly variable including several minor disease manifestations. The figure is adapted with permission from Husebye et al. (150). CMC, chronic mucocutaneous candidiasis; HP, hypoparathyroidism; PAI, primary adrenal adrenocortical insufficiency.

A wide spectrum of both autoimmune and non-autoimmune gastrointestinal manifestations has been reported in association with APS-1 (152). Autoimmune gastritis with pernicious anaemia is the most common of these disorders, and autoimmune hepatitis, which can be a serious complication, is often found in young patients (150), but prevalence varies across cohorts. Other gastrointestinal manifestations possibly associated with APS-1 are gallstones (152), celiac disease (153) and pancreatic insufficiency (146). Intestinal symptoms may be caused by an autoimmune enteropathy (152). Alopecia is the most common ectodermal manifestation (150, 154), and vitiligo may be one of the first signs of the syndrome (150). Nail dystrophy and enamel hypoplasia are other ectodermal components that seem to vary between populations (146-148). Periodic fever with urticarial rash is described as a frequent manifestation, especially in childhood (146, 148). Psoriasis and cutaneous vasculitis are described in case reports (155, 156). Finally, different ocular manifestations and a Sjögren's like syndrome are reported (157-159).

Several atypical and rare features are possibly associated with APS-1, including autoimmune destruction of the spleen (146) and haematological features such as autoimmune haemolytic anaemias, hypoplastic anaemias (146) and autoimmune thrombocytopenia; pulmonary manifestations such as bronchiolitis and interstitial lung disease (160-162); autoimmune tubulo-interstitial nephritis causing terminal renal failure (163, 164); and reversible metaphyseal dysplasia with growth retardation (165). Moreover, the neurological disorders inflammatory demyelinating polyneuropathy (166) and posterior reversible encephalopathy are reported as disease features of APS-1 (167). Recently, ptosis was also reported in the context of APS-1 (168).

Typically APS-1 presents with CMC and PAI before the age of 10, and all three main disease components are often observed before the age of 15 (64). Further disease manifestations may appear throughout life, although the precise timing and sequence of the onset of disease components varies considerably (147). Components

that commonly appear at young age are CMC, PAI, HP, urticarial eruption, hepatitis and intestinal dysfunction, whereas gonadal failure, diabetes mellitus type 1, vitamin B12 deficiency and gastritis are typically late manifestations (146-148). The overall rarity of the syndrome and its heterogeneity in phenotypic expression make it difficult to recognise, often resulting in delayed diagnosis (146-148, 150). Interestingly, recent findings from an American APS-1 cohort suggested adding the minor disease components urticarial eruption, intestinal dysfunction, and enamel hypoplasia to the diagnostic criteria to facilitate earlier recognition and diagnosis (148). The overall mortality rate is high (146, 169) and the most common causes of death are acute endocrine crisis and oral and esophageal squamous cell carcinomas (170).

1.5.2 Chronic mucocutaneous candidiasis (CMC) in APS-1

Most *Candida* species are opportunistic fungal pathogens that colonise the skin and mucosa of most healthy individuals without causing tissue damage or disease. However, several well-known factors increase susceptibility to *Candida* infections. *Candida* infections are, for example, common in neutropenic patients (171, 172) and patients treated with glucocorticoids or other immunosuppressive agents (173). Treatments with broad-spectrum antibiotics change the normal bacterial flora, eliminating natural antagonisms for fungal colonisation, which may lead to *Candida* infections (174), and prolonged hospitalisation of patients and damage to the skin or mucosal barriers increase the risk (174). Recent findings have also highlighted the influence of host genetics on susceptibility to *Candida* infections (17).

CMC is defined as recurrent infections of nail beds, skin or mucosal surfaces. Oral *Candida* infections present clinically as creamy-white fungal plaques (pseudomembranous candidiasis), painful red lesions of the tongue (erythemaous candidiasis) or smooth or nodular lesions on any mucosal surface of the mouth (chronic hyperplastic candidiasis). The diagnosis of *Candida* infections depends on clinical signs and symptoms, growth of *Candida* in culture, and improvement of symptoms during anti-*Candida* therapy. CMC caused by *C. albicans* is the most

common and earliest manifestation in APS-1 and is reported to occur in 75-100% of patients (144-150). The clinical course of CMC varies from periodic to chronic; however, it usually affects the oral mucosa as angular cheilitis or the whole mouth as hypertrophic and/or atrophic lesions (146, 150, 175). Intestinal and invasive candidiasis is rare (146, 152), but *Candida* oesophagitis sometimes occurs (147).

General resistance to mucosal fungal infections is thought to be T cell-mediated at epithelial surfaces, particularly by Th17 cells (176) (Figure 1). Based on their specific cytokine profile (IL-17A, IL-17F, IL-21, IL-22, IL-26), Th17 cells have the potential to trigger production of neutrophil-recruiting and -activating cytokines and chemokines (e.g. granulocyte-colony stimulating factor and CXC chemokine ligand 8), pro-inflammatory cytokines (e.g. IL-6 and TNF-α) and anti-microbial peptides (e.g. defensins and S100 proteins) in many other cell types (177). The local environment controls the exact cytokine profile produced by Th17 cells via different receptors such as IL-23 or aryl hydrocarbon receptors (178). Both IL-17 and IL-17F are required for mucosal immunity, and IL-22 functions by critically regulating anti-microbial genes, protecting epithelial surfaces and maintaining barrier integrity (179). Th17 cells have additionally been implicated in several autoimmune diseases by the observed induction of inflammatory responses and neutrophil recruitment (180, 181).

Several studies have highlighted impaired immune functions in APS-1 patients. Neutralizing autoantibodies against the Th17-cytokines IL-17A, IL-17F and IL-22 are suggested as a possible explanation for the impairment of mucosal immunity (182, 183). However, other studies have investigated immune mechanisms in response to *Candida* challenges in APS-1 patients, describing significant differences relative to healthy subjects. Data indicate that IL-22 producing cells are reduced in APS-1 patients, whereas the data regarding IL-17 production is inconsistent (183-185). Monocyte-derived DCs from APS-1 patients produce significantly more IL-2, IFN-γ, TNF-α and IL-13 compared to healthy controls and show general impairment in maturation and activation (186). In a study of Sardinian

APS-1 patients, no significant association between genomic copy numbers of the β-defensin cluster and susceptibility to CMC was found (187). Findings in a human monocyte model indicate that AIRE modulates the formation of fungal synapses and hyphal recognition and contributes in organising an effective immune response against *C. albicans* through an interaction with the Dectin-2 receptor and SYK signalling (188). Others have reported reduced numbers of CC chemokine receptor 6 and CXC chemokine receptor 3-positive Th cells, CD16+ monocytes and Tregs in patients with APS-1 (189). However, expression levels of all PRRs involved in the defence against *Candida*, levels of plasmacytoid and myeloid DCs, and monocyte TLR2/6 expression are described as similar in APS-1 and healthy subjects (190). Collectively, these alterations in patients' immune systems are likely to contribute to a phenotype of severe autoimmunity and susceptibility to CMC.

Other factors possibly contributing to susceptibility to oral CMC in patients are reduced levels of antimicrobial peptides such as cystatin SA1 in saliva (191) and the fact that *C. albicans* isolates from patients show specific characteristics including strain specificity and resistance patterns (192-195). Mutations in the transcriptional activator of *Complementary-determining region* genes probably cause the azole resistance found in *C. albicans* isolates from patients (194). Interestingly, patients are often persistently colonised with *C. albicans* despite the relief of symptoms found in response to treatment.

The clinical finding of severe CMC as a major disease manifestation in APS-1 and the specific molecular immune defects described above clearly underpin immune deficiency as part of APS-1.

1.5.3 Immunological features of APS-1

Patients develop autoantibodies against molecular targets in affected organs and against components of the immune system. Organ-specific autoantibodies have proven to be excellent markers of organ manifestations, often preceding clinical

disease by years (196-198). Autoantibodies against type I IFNs and ILs involved in the Th17 immune response have been identified in almost all APS-1 patients and their development often precede onset of clinical disease, making their detection a suitable diagnostic tool (182, 183, 199-203) (TABLE 1).

Table 1. Clinical manifestations and associated autoantigens in APS-1.

Clinical manifestations	Autoantigens	References
Main components		
CMC	IL-17, IL-22	(182, 183)
HP	NALP5	(196)
PAI	21OH, 17OH, SCC	(61, 63, 204-208)
Other components		
Endocrine manifestations		
Ovarian failure	SCC	(63, 205-207)
Testicular failure	TGM4, PDILT, MAGEB2	(209, 210)
Type 1 diabetes	Insulin, IA-2	(63)
Autoimmune hypophysitis	TDRD6	(151)
Gastrointestinal manifestations		
Autoimmune hepatitis	C-P450, C-P450-1A2, AADC, TPH	(152, 197, 211)
Autoimmune enteropathy	TPH, HD, GAD	(152, 211, 212)
Ectodermal manifestations		
Vitiligo	SOX-9, SOX-10, AADC	(197, 213)
Alopecia	TH	(214, 215)
Rare manifestations		
Pulmonary disease	KCNRG, BPIFB1	(160, 216)
Demyelinating polyneuropathy	Myelinprotein zero	(217)
Tubular interstitial nephritis	Aquaporin-2	(218)
Non-organ specific	INF-ω, IL-22, IL-17	(182, 183, 200)

17OH, 17-hydroxylase; 21OH, 21-hydroxylase; AADC, aromatic L-amino acid decarboxylase; BPIFB1, bactericidal/permeability-increasing fold-containing B1; CMC, chronic mucocutaneous candidiasis; C-P450, cytochrome P450; C-P450-1A2, cytochrome P450 1A2; GAD, glutamic acid decarboxylase; HD, histidine decarboxylase; HP, hypoparathyroidism; IA-2, tyrosine phosphatase like protein; IF, intrinsic factor; IL, interleukin; INF-ω, interferon omega; KCNRG, potassium channel regulating protein; MAGEB2, Melanoma-associated antigen B2; NALP5, NACHT leucine-rich repeat protein 5; PAI, primary adrenal failure; PDILT, Protein Disulfide Isomerase-Like Testis Expressed; SCC, side-chain-cleavage enzyme; SOX, SRY-related HMG-box; TDRD6, Tudor Domain Containing 6; TG, thyroglobulin; TGM4, transglutaminase 4; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; TPO, thyroid peroxidase.

1.5.4 The *autoimmune regulator* (*AIRE*) gene

The underlying cause of APS-1 is mutations in the *AIRE* gene. The *AIRE* gene is located at position q22.3 on chromosome 21 and contains 14 exons that encode a protein of 545 amino acids with a molecular weight of 57.5 kD (141, 142). The AIRE protein localises in the nucleus forming discrete dot-like structures (219) and harbours several functional domains typical for proteins involved in gene transcription (Figure 4). CARD is crucial for the homo- and multimerisation of AIRE (220), and the SAND domain (named after the protein Sp100, *AIRE* -1, NucP41/75 and DEAF-1) mediates protein-protein interaction and DNA binding (221, 222). The plant homeodomain zinc finger motif 1 (PHD) is a histone code reader that recognises demethylated lysine 4 residues (223), while PHD2 is critical for interaction with protein partners involved in chromatin binding (224). The AIRE protein also contains a nuclear location signal (225) and four LXXL motifs (226).

AIRE has its highest expression in a subset of mTECs (227, 228) characterised by the expression of MCH class II and the co-stimulatory markers CD80, CD86 and CD40 (229-231). In mTECs, AIRE acts together with several protein partners (232) regulating PGE (41), a process crucial for negative selection and deletion of autoreactive T cells from the repertoire (45). Without functional AIRE, autoreactive T cells with the potential to cause autoimmune disease are released to the periphery (Figure 2). Although AIRE's function outside thymus has not been truly investigated and is not well understood, AIRE protein expression has been reported in human tissues such as lymph nodes, tonsils, gut-associated lymphoid tissues and the foetal liver (233), whereas *AIRE* mRNA transcripts are found in the spleen, B cells, granulocytes, macrophages and DCs (230, 233, 234).

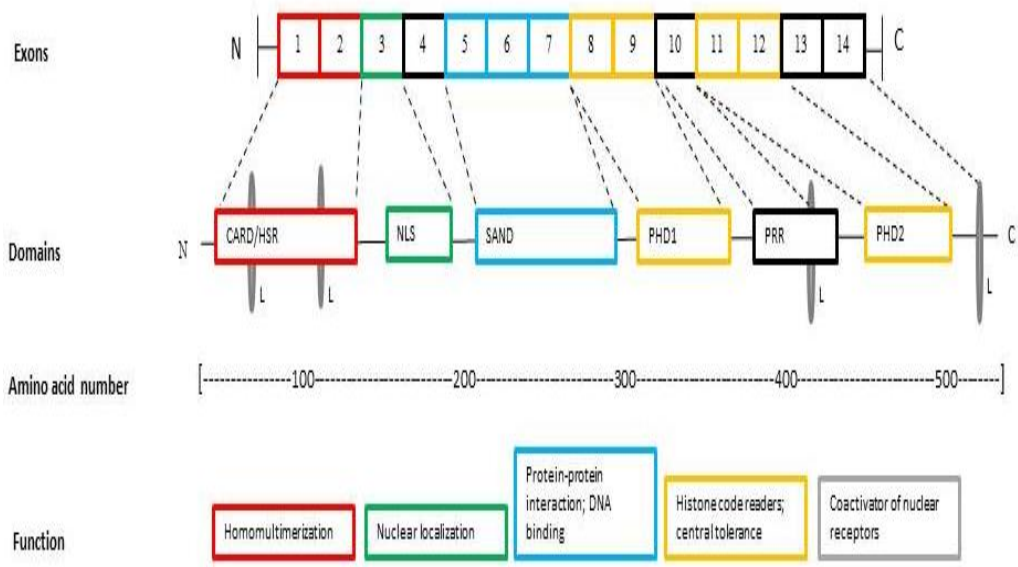

Figure 4. The AIRE protein with its functional domains.

AIRE acts as a transcription regulator, together with several protein partners, and has four major subdomains: the CARD/HSR domain, for caspase recruitment domain/homodimerisation domain or homogeneously straining region (amino acids 1-105); the SAND domain, for Sp100, AIRE-1, NucP41/75, DEAF-1 (amino acids 181-280); and the two plant homeodomain (PHD) zinc fingers (amino acids 296-343 and 434-475, respectively). The AIRE protein also holds four LXXLL domains with the potential to activate nuclear receptors (amino acids 7-11, 63-67, 414-418, 516-520, respectively) and a nuclear localisation signal (amino acids 100-189). The function of the different domains is given in the boxes below. The corresponding exons and amino acid numbers are given in the figure. The figure is modified from Bruserud et al. (235).

In humans, *AIRE* mutations cause clinical APS-1 (235). Over 120 mutations have been reported (236) varying from single nucleotide substitutions to large deletions spread out across the coding sequence (235). This includes about 15 mutations that affect mRNA splicing. The most common mutation worldwide is p.R257X/c.769C>T located in the SAND domain, whereas c.967_979del13, located in the PHD1 domain is the most common mutation in Norway (147), the British Isles (237), North America (238) and France (239). Founder mutations have been

described in Finland (p.R257X), Sardinia (p.R139X), Sicily (p.R203X), among Persian Jews (p.Y85C) and in Italy (p.W78R) (143, 145, 240, 241), all areas with a generally higher prevalence of the syndrome. *AIRE* mutations are typically passed on through autosomal recessive inheritance. However, the mutation p.G228W in the SAND domain has been shown to be dominant (242), and several mutations within the PHD1 domain are found to suppress gene expression driven by wild-type (WT) *AIRE* in a dominant-negative manner (243). Notably, one dominant mutation can cause autoimmune disease, although these patients exhibit a milder phenotype relative to classical APS-1 (243).

Significant correlation between phenotype and *AIRE* genotype has not been described except for the possible association of CMC with p.R257X (183) and low frequencies of PAI and CMC with p.Y85C (143). However, some associations between HLA class and disease components have been reported (244). Many single nucleotide polymorphisms (SNPs) in *AIRE* have been studied in human disease association studies, albeit with conflicting results (245).

Recent studies have indicated potential influence of *AIRE* beyond APS-1 and autoimmunity. For example, the expression of *AIRE* protein has been verified in human breast cancer cells and seems to be a strong prognostic factor for relapse-free survival (246). In addition, keratin 17-dependent *AIRE* expression can be induced in human and mice keratinocytes and is required for timely onset of Gli2-induced skin tumorigenesis in mice (247). However, the potential role of *AIRE* in peripheral tissues and other disorders needs to be further investigated.

2. Aims of the thesis

The overall objective of this thesis is

- To identify all Norwegian APS-1 patients, characterise the disease phenotypes, and use APS-1 as a model disease for studying immunological mechanisms of autoimmunity and CMC.

The specific aims of this thesis are

1. To describe the phenotypes of APS-1 among all known Norwegian patients; correlate the clinical features with autoantibody profiles and *AIRE* mutations during extended follow up; and search for and describe potential novel distinct entities of the syndrome (*paper I, paper II*).
2. To scrutinise genotype-phenotype relationships and explain why the c.879+1G>A splice mutation results in a mild APS-1 phenotype (*paper III*).
3. To find a molecular explanation for increased CMC susceptibility in APS-1 (*paper IV*).
4. To assess the bacterial microbiota of whole saliva in APS-1 patients (*paper V*).

3. Comments on methods

Complete descriptions of all materials and methods are given in each separate paper (*papers I-V*). Additional comments and considerations regarding patients, materials and key methods and assays follow.

3.1 Materials

3.1.1 Patients (Papers I-V)

Patients were recruited from departments of internal medicine and paediatrics in Norwegian hospitals and included in the nation-wide Norwegian Registry of Organ-specific Autoimmune Diseases (ROAS). The ROAS was first established in 1996 with particular interests in APS and Addison's disease. Its biobank contains whole blood, sera, DNA, RNA, peripheral blood mononuclear cells (PBMCs), tears and saliva from patients. In addition, the registry contains clinical information regarding disease components, medical treatments, autoantibody profiles and genotypes. The process of updating and improving ROAS is continuously ongoing.

A total of 52 APS-1 patients, representing all identified Norwegian APS-1 patients as of 2016, were described in *paper I*. Among these were seven patients identified after their deaths who were determined to have had a high probability of undisclosed APS-1. The selection of patients for *papers II-V* was based on the patient's *AIRE* mutations, clinical phenotype, and the availability of biological samples. In *paper II*, additional patients (two Finnish and one Israeli) were included for studying oral tongue malignancies.

3.1.2 Healthy controls (Papers I-V)

Healthy age- and sex-matched controls were recruited from the local blood bank at Haukeland University hospital. Whole blood was taken directly into TrueCulture

Tubes (*paper IV*), and PBMCs or plasma was isolated from heparinised blood using Ficoll-Paque PLUS and stored at -150 degrees Celsius and -80 degrees Celsius, respectively (*papers I, III, IV*). Healthy age- and sex-matched volunteers working in the hospital were recruited for saliva sampling for *paper V*. Complete procedures for sample processing are given in each paper.

3.2 Methodological considerations

3.2.1 Clinical characterisation (Papers I-V)

All patients were invited for follow up at Haukeland University hospital in Bergen for a clinical assessment including hormonal status and autoantibody profiles. All patients were seen by a senior consultant in endocrinology who followed a standard protocol including esophagogastroduodenoscopy, chest x-ray and imaging of the spleen and kidneys. Endocrinopathies were diagnosed as previously described (62). A dental and oral examination was performed by an experienced dentist, who also collected the saliva samples analysed in *paper V*. The two Finnish patients and the one Israeli patient described in *paper II* were characterised by international collaborators. Even if standardised protocols were employed we cannot rule out some variation between physicians.

3.2.2 Radio-ligand binding autoantibody assays (Papers I-V)

Radio-ligand binding assays were used to screen patient sera for autoantibodies against 21OH, 17-α-hydroxylase, aromatic L-amino acid decarboxylase (AADC), glutamic acid decarboxylase 65-kDA isoform (GAD65), IFN-ω, IL-17, IL-22, melanoma antigen B2, NACHT leucine-rich-repeat protein 5, protein disulphide isomerase-like testis expressed, putative potassium channel regulator, side-chain-cleavage enzyme (SCC), sex-determining region Y-box 10, transglutaminase 4, tryptophan hydroxylase 1 (TPH1) and tyrosine hydroxylase (TH). The assays were performed as previously described (160, 210, 248). In summary, complementary

DNA (cDNA) clones of APS-1 antigens were transcribed and translated *in vitro* in the presence of ^{35}S-labelled methionine. Radio-labelled proteins were immunoprecipitated with 5 µl serum in 96-well filtration plates. Autoantibody-antigen complexes were immobilised to protein A Sepharose and radioactivity was measured in a liquid scintillation counter (197). Pooled human sera from healthy donors were used as a negative control, and the positive control was a previously analysed APS-1 serum with known medium-to-high antibody levels. Normal limits were given as three standard deviations above the mean of about 100 healthy controls. Samples were tested as duplicates, and index values were calculated as ((count per minute (cpm) sample – cpm negative control) / (cpm positive control – cpm negative control)) x 1000. All reported autoantibody assays were performed in our laboratory. Sera from the same patients taken at different time points were analysed in the same experiment to avoid variations between assays.

3.2.3 Enzyme-linked immunosorbent assays (Papers I, IV)

In *paper I,* an enzyme-linked immunosorbent assay (ELISA) was used to screen patients' sera for autoantibodies against parietal cell antigens (Euroimmun). Sera from all patients included in *paper IV* were screened for autoantibodies against IL-23 using an "in-house" sandwich ELISA. Moreover, ELISA (R&D Systems, UK) was also used to assess immune mediators of special interest (IL-17A, IL-17F, IL-22, and IL-23p19) in *paper IV*, verifying the results from the TrueCulture whole blood assay (*paper IV*).

3.2.4 *AIRE* sequencing (Paper I-V) and *HLA* allele typing (Paper I)

All living patients were screened for *AIRE* mutations (*paper I*). The DNA sequencing of *AIRE* spanning the exon-intron boundaries was performed using standard methods, and copy number analysis was performed by duplex TaqMan real-time polymerase chain reaction (PCR) (249). The HLA genotyping was performed using sequence-based SBT Resolver and Assign Software (Conexio Genomics).

3.2.5 Thymic 4D6 cell line assay (Paper III)

Due to the lack of thymic tissue from human APS-1 patients and healthy subjects, a transfected cell line assay was used to investigate the effect of different *AIRE*-mutations on the expression of AIRE-regulated genes. The human thymic epithelial 4D6 cell line was cultured, plated, and transfected with the current plasmids (*AIRE* without exon 7, c.769C>T/p.R257X and c.932G>A/p.C311Y in the pCMV6 vector) using the Fugene HD transfection reagent (Promega Corporation). Cells were further incubated for 24 hours, total RNA was extracted and cDNA prepared. AIRE-regulated genes (*keratin 14 (KRT14), IGF-like family member 1 (IGFL1)*) were analysed by quantitative PCR (qPCR) and compared to the expression of the *AIRE*-independent genes *cyclin H* and *protein arginine methyltransferase 3*. *Beta2-microglobulin* (*B2M*) was used as an endogenous control. The fold difference was calculated based on the cycling threshold (Ct) values as 2^(Ct((target gene) – Ct(B2M)) – (Ct(test sample) – Ct(calibrator sample))), with the test sample defined as the current mutation and the calibrator as WT *AIRE*. The assay was repeated two to four times, and the results shown (*paper III*) are the mean values of these replicates.

3.2.6 Immunoflourescence (Paper III)

The transfected 4D6 cell line assay was also used to investigate the cellular compartmentalisation of mutant AIRE protein without exon 7 (*paper III*). Cells were plated in a 6-well plate containing sterile cover slips and incubated overnight, transfected with current plasmids, and incubated for another 24 hours. Cover slips with cells were then washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde and washed again. Cells were then permeabilised with 0.5% Trioton-X, washed, and incubated for one hour at room temperature with 10% fetal bovine serum in PBS. Another washing step was performed before staining with phycoerythin conjugated anti-DYDDDDK (clone L5, BioLegend) in 1% bovine serum albumin in PBS for one hour at room temperature. Cover slips were then washed in PBS followed by water and attached to SuperFrost microscope slides using ProLong Gold

antifade reagent with DAPI (4',6-diamidino-2-pheylindole). Slides were incubated for 24 hours in the dark at room temperature and analysed using a Leica SP5 microscope.

3.2.7 Whole blood stimulation assays (Paper IV)

Whole blood assays probably mirror the *in vivo* conditions of immune activation and inflammation more precisely than cell assays considering the crosstalk between immune cells and the distribution of soluble mediators. Therefore, a TrueCulture whole blood assay (Myriad, RBM, US) was chosen to investigate immune activation after the *Candida* challenge in APS-1 patients. Whole blood was taken directly into TrueCulture collection and culture tubes, which contained either the supplied media (baseline) or media supplemented with *Candida* antigens (ATCC 10231). After 48 hours of incubation, the supernatants were frozen and sent to Myriad RBM's testing laboratory for analysis. The multiplex immunoassay Human InflammationMAP 1.0 panel (Myriad, RBM, US) was used for analysing 46 unique analysts or biomarkers of immune activation. Thus, each individual patient and healthy subject became their own control when comparing baseline and stimulated levels of immune mediators.

3.2.8 PBMCs stimulation experiments (Paper IV)

A cell assay was designed to investigate IL-23p19 production in monocytes. Cryopreserved PBMCs were cultured overnight, and immune stimulation and activation of monocytes was induced by adding the Dectin-1 agonist beta-1,3-glucan to the cultures. After the period of incubation, cells were washed and stained for the surface marker CD14 and the intracellular IL-23p19 and analysed on a Becton Dickinson Fluorescence-activated cell sorter Accuri C6 flow cytometer. Individual populations were gated according to forward scatter, side scatter and specific markers, and the data were subsequently analysed with FlowJo software version v.X.07.

To be mentioned, few monocytes produced IL-23p19 upon simulation with the beta-1,3-glucan and some cells were borderline positive for IL-23p19 after stimulation. However, we carefully optimised our assay regarding incubation period, concentration of beta-1,3-glucan and Brefeldin A stimulation. Samples from patients and controls were always analysed in parallel, and a consistent gating strategy was used.

3.2.9 Saliva collection (Paper V)

All saliva samples were collected by the same experienced dentist and were collected between 9 a.m. and 11 a.m. Participants were asked to not brush their teeth, eat or drink for at least two hours before sampling. No participants were regular smokers or had received antibiotics within the last month before sampling. Samples were immediately stored at -80 degrees Celsius until analysed. Unstimulated saliva flow rate was estimated based on a time period of 15 minutes.

3.2.10 High-throughput 16S ribosomal RNA sequencing (Paper V)

The hypervariable region of the 16S ribosomal RNA (rRNA) is highly conserved between different species of bacteria making sequencing techniques suitable for assessing the bacterial content of any given sample. This technique is superior to culture techniques as it recovers both cultivated and not-yet-cultivated bacteria. High-throughput 16S rRNA sequencing was therefore used to profile the oral microbiota in APS-1 patients and healthy controls. In brief, DNA was extracted from unstimulated saliva samples and the hypervariable regions V1-V2 were amplified using PCR, then sequenced on a 454 GS Junior System (Roche, Brandford, CT).

3.2.11 Statistical considerations (Papers I-V)

Sample sizes in most papers were relatively small and data did not always follow a normal distribution. This determined the statistical methods used. Data that did not follow a normal distribution was analysed using the Mann-Whitney U test (*paper IV*),

whereas t-tests was used for normally distributed data (*papers III-V*). The Fischer's exact test was used when comparing categorical data of small sample sizes (*paper I*). The Spearman's rank correlation coefficient was calculated for correlation analyses (*paper I*). The level of significance was defined as *P* values less than 0.05. These statistical analyses were performed using IBM SPSS Statistics 22/23 or Prism 7 (Graph Pad Software, Inc., San Diego, CA, USA).

In *paper IV*, hierarchical cluster analyses were performed using J-Express (MolMine AS, Bergen, Norway). The alterations analysed were standardised after the ratio after/before stimulation and log(2) transformed before unsupervised hierarchical clustering with squared Euclidean distance measure with weighted average linkage was performed.

In *paper V*, a bioinformatics pipeline was used to analyse the pyrosequencing reads of the 16S V1-V2 rRNA. Raw sequence reads were subjected to a species-level, reference-based taxonomy assignment. The Quantitative Insight Into Microbial Ecology pipeline software package version 1.9.1 was used for downstream analyses. A statistical method introduced in Metastats (http://cbcb.umd.edu/software/metastats) was then used to reveal significant differences between the oral microbiota in APS-1 patients and healthy controls. For these latter analyses, Bonferroni Correction for multiple testing was included to avoid type I errors (false positives).

3.2.12 Ethics (Papers I-V)

All patients and healthy controls signed informed consent approved by The Regional Committee for Medical and Health Research Ethics. All studies included in this thesis were conducted in accordance with the Declaration of Helsinki and approved by The Regional Committee for Medical and Health Research Ethics (Biobank FOAS: 2013/1504. Projects: 2009/2555, 2010/2069, 047.96).

4. Summary of results

4.1 Paper I: “A Longitudinal Follow up of APS-1”

The aim of this first paper was to describe the phenotype of all Norwegian APS-1 patients based on extended follow up (1996-2016) and correlate the clinical features of APS-1 with autoantibody profiles and *AIRE* mutations.

Fifty-two patients (24 females, 28 males) from 34 different families from all parts of Norway were identified. The phenotypes were highly variable, even among members of the same family. However, most patients had presented with one of the major disease components during childhood, and hypoparathyroidism and CMC were the most common initial manifestations. Overall, CMC (77%), enamel hypoplasia (72%) and hypoparathyroidism (73%) were the most frequent features. Forty percent of patients presented with the classical triad and 67% of these had developed all three major manifestations before the age of 25 years. With age, most patients exhibited three to five disease manifestations. A minority had milder phenotypes diagnosed in adulthood. Fifteen patients died during follow up or were deceased siblings with a high probability of undisclosed APS-1. The major causes of death were malignancies and adrenal and hypocalcemic crises.

IFN-ω autoantibodies were found in all patients except three, and all had organ-specific autoantibodies. Autoantibodies against 21OH and SCC were significantly correlated with PAI ($p<0.001$ and $p=0.002$, respectively); autoantibodies against IL-22 were correlated with CMC ($p=0.004$); and autoantibodies against AADC were correlated with vitiligo ($p=0.047$).

The most common *AIRE* mutation, c.967_979del13, and three patients had mono allelic *AIRE* mutations located within the PHD1 domain. Patients with the splice mutation c.879+1G>A all displayed a mild phenotype. None of the APS-1 patients with PAI carried the high-risk HLA class II haplotype DR3-DQ2/DR4.4-

DQ8, and three out of four diabetes patients carried the DQB1*0602 allele, which is otherwise extremely rare in type 1 diabetes.

Taken together, we found that the Norwegian patients present variable phenotypes where non-endocrine components such as CMC and enamel hypoplasia are common. Diagnostic workup using autoantibody analyses (e.g. IFN-ω) and *AIRE* sequencing to reveal APS-1 is highly recommended. Treatment is complicated and mortality is high. Structured follow up should be performed in a specialised centre.

4.2 Paper II: "Oral Tongue Malignancies in APS-1"

In this study, we aimed to clinically describe oral tongue malignancies in the context of APS-1. Together with international collaborators, we present a case series of four APS-1 patients from Finland, Norway, and Israel with oral tongue malignancies, an entity not previously described in detail. All patients presented disease-causing *AIRE* mutations and a typical APS-1 phenotype including severe CMC from childhood. One patient smoked on a daily basis, and two patients reported regular alcohol consumption. With the exception of early onset, the clinical presentation and the tumour histology were similar to other non-APS-1 patients with oral malignancies. However, they were treated with radical surgery resection alone or in combination with chemo-radiotherapy, or photodynamic therapy in one case. One patient died after recurrence of the tumour, while the remaining three patients are still under careful surveillance.

This case series describe oral tongue malignancies as a novel disease component of APS-1. Oral manifestations should be properly investigated to reveal early signs of oral malignancies including tongue carcinomas. Moreover, aggressive CMC treatment and elimination of additional risk factors are important to avoid the development of oral malignancies in patients.

4.3 Paper III: "The *AIRE* mutation c.879+1G>A display a mild phenotype and alternative mRNA splicing in patients with APS-1"

In this paper, we report four Norwegian APS-1 patients with the splicing mutation c.879+1G>A affecting the splice donor site of intron 7; all patients exhibit a mild phenotype compared to that of classical APS-1. Mini-gene analyses, with PCR and sequencing of *AIRE* mRNA transcripts in PBMCs from patients, reveal an altered splicing pattern including both normal transcripts and transcripts skipping exon 7. However, family members that were heterozygous carriers of the mutation presented with neither APS-1 manifestations nor typical autoantibodies in sera. Normal transcripts were consistently found in healthy controls. Using a transfected thymic cell line assay (4D6 cells) and qPCR, we demonstrated that the AIRE polypeptide deleted for exon 7 has the potential to induce AIRE-regulated genes (*KRT14, IGFL1*). Visualised by confocal microscopy, the AIRE polypeptide deleted for exon 7 show a similar nuclear localisation and dot formation typically described for WT *AIRE* and has the potential to co-localise and interact with WT protein. WT transcripts in patients are probably less than 50% as all patients display a definite APS-1 phenotype. However, the occurrence of normally spliced *AIRE* together with an alternatively spliced AIRE polypeptide with some residual AIRE function, probably prevent severe disease and contribute to the mild phenotype observed in these patients.

4.4 Paper IV: "Altered Immune Activation and IL-23 Signalling in Response to *C. albicans* in APS-1"

The aim of this study was to investigate immune responses in APS-1 patients in response to *C. albicans*. Whole blood and monocytes were stimulated with *C. albicans* antigens and the immune activation was assessed using a multiplex immunoassay and flow cytometry, respectively.

APS-1 patients displayed significantly altered immune responses relative to healthy controls. When stimulating whole blood with *C. albicans,* patient samples failed to increase several essential ILs including IL-2, IL-17A, IL-22, and IL-23. Monocytes from patients also produced significantly less IL-23p19 upon stimulation with curdlan compared to healthy subjects (p=0.005). A significant difference was also detected when comparing the percent increase in IL-23p19+ monocytes (p=0.0193). No significant differences in the CD14+ monocyte levels were found comparing patients and controls.

In summary, we found that APS-1 patients had significantly altered immune responses to *C. albicans* including dysregulation of IL-23p19 production in monocytes. This probably contributes to the selective susceptibility to CMC found in the majority of patients.

4.5 Paper V: “Oral microbiota in APS-1”

Increasing evidence suggests a potential role of skin, oral and gut microbiota in the pathogenesis of autoimmunity. No information exists regarding oral microbiota in APS-1. The aim of this study was to investigate the possible role of oral microbiota in the pathogenesis and phenotypic expression of APS-1 through assessment of the bacterial microbiota of whole saliva in APS-1 patients using high-throughput sequencing.

Five different phyla were detected in APS-1 patients with DNA sequences predominately assigned to the phyla Firmicutes (60%), Bacteroidetes (15%), Proteobacteria (10%), Actinobacteria (6%), and Fusobacteria (8%). The proportion of bacteria belonging to the major phyla Firmicutes was higher (60% vs 41%, p=0.002) than in controls, while the proportion of those belonging to the phyla Bacteroidetes was lower (15% vs 28%, p=0.007). *Streptococcus* and *Gemella* were prevalent genera in APS-1. Overall, a reduction in the total number of bacterial genera and species was detected in APS-1 compared to healthy subjects.

These findings indicate a significant altered oral microbiota in APS-1 that may contribute to both the pathogenesis and the phenotypic expression in this syndrome. However, it remains unclear whether an altered microbiota causes disease manifestations or if the altered microbiota has an effect on disease components.

5. Discussion

5.1 APS-1 as a powerful model for immunological studies

There are both challenges and benefits in studying monogenic diseases. The rarity of most monogenic diseases reduces the availability of both patients and biological samples. The Norwegian APS-1 patients described in *paper I* were geographically spread throughout the country and received regular follow-up care at local hospitals. The process of clinical characterisation, collection of biological samples and timing of experiments therefore had to be carefully planned to achieve the representative data needed for our studies. These processes depended on extensive collaborations with patients and their organisations, physicians, researchers, and technical staff, which was challenging and time consuming. Biological samples always had to be strictly prioritised, and samples from all patients were unfortunately not always available for all planned experiments. These issues have previously been overcome by broad international collaborations, for example the Finnish-German APECED Consortium (142) and projects that were part of the European Union's Research and Innovation funding programmes (e.g. EurAPS). This should probably also be the approach for future studies using APS-1 or other rare monogenic disorders as models for studying immunological mechanisms. International collaborations are also beneficial for the further characterisation of APS-1 phenotypes, as illustrated in *paper II*, where oral tongue malignancies in selected patients from Norway, Israel, and Finland were collectively described as a novel disease component of APS-1. Moreover, organising patient information into nation-wide registries such as the ROAS standardises the characterisation of patients, improves follow-up routines, and generates novel longitudinal data (*paper I*).

Studying monogenic human disorders gives the opportunity to understand the genetic and immunological pathogenesis underlying clinical phenotypes. The principles of immunological tolerance have already been revealed through studies of

APS-1 and *AIRE* (250-252), and the molecular mechanisms have been further described through the development of various *Aire* knock-out mice models (45, 232, 253). Although animal models of APS-1 have identified several targets of autoimmunity (216, 217, 254-259), there are remarkable differences in phenotype between human APS-1 patients and *Aire* deficient mice, probably because of species specific peculiarities (260-262). This underscores the importance of human studies and may also indicate that other factors, such as immunogens and environmental factors, contribute to the variable phenotypic expression seen in human APS-1 patients.

The investigation of human patient cohorts and the development of animal models together with the continuous development in immunological techniques will increase our understanding of genetic and immunological mechanisms involved in autoimmune diseases, and APS-1 has proven to be an excellent model. However, studying APS-1 patients with CMC has also increased our understanding of the genetic and immunological mechanisms underlying susceptibility to *Candida* infections, knowledge that could prove useful for future immunotherapeutic approaches. In addition, the potential role of *AIRE* in cancer development has recently been highlighted (246, 247) indicating that APS-1 and *AIRE* may have relevance beyond the field of autoimmunity and CMC.

5.2 Phenotypic variation in APS-1

Patients with APS-1 present with variable phenotypes ranging from mild to severe. Some variation in the frequency of disease components occurs among different cohorts, although longitudinal data is only available from Norwegian (*paper I*), Finnish (146), Sicilian (241), and Sardinian patients (145). The Russian APS-1 cohort is probably the largest and includes 112 patients (168). However, the American cohort displays more severe phenotypes, with early disease onset and a mean of nine disease components (148). Hypothyroidism was not found in Sardinian patients, but has been reported in several Norwegian (*paper 1*), American (148), and Apulian

patients (240). Autoimmune hepatitis was found in only two Norwegian patients (4%) but was an early and serious manifestation in one third of the Sardinian (145) and in half of American patients (148). In a study of 23 Persian Jews with APS-1 (143), 22 presented with hypoparathyroidism, while only four patients had oral CMC. Interestingly, Norwegian patients present with a variable phenotype where non-endocrine components such as CMC and enamel hypoplasia are common. Dental examinations revealed enamel hypoplasia in 72% of Norwegian patients, making this a common feature, occurring more frequently than in other cohorts. Varying practice among clinicians may influence the phenotypes reported. However, inviting other clinical specialists to join the phenotypic characterisation and follow up of APS-1 patients could reveal novel disease components and improve mapping of already well-known features.

APS-1 patients develop a wide range of typical autoantibodies against molecular targets. Defining autoantibodies is important in order to understand the mechanisms of autoimmune disorders as they are markers of cell-mediated autoimmunity. Autoantibodies are easily accessible in peripheral blood and are useful for clinical diagnosis. Prevalence of typical autoantibodies and their association with various disease components were investigated in a cohort of 90 Scandinavian APS-1 patients (63). Autoantibodies against 21OH and SCC were associated with PAI, whereas hypogonadism was exclusively associated with reactivity against SCC (63). Autoantibodies against TPH and GAD65 were associates with intestinal dysfunction, and reactivity against TPH was shown to be the best predictor for autoimmune hepatitis (63). The association of 21OH and SCC autoantibodies with PAI has been verified in both the American (148) cohort and among Norwegian patients (*paper I*). In addition, reactivity against TPH, but not GAD65, was associated with intestinal dysfunction in American patients. Reactivity against AADC correlated with vitiligo in Norwegian patients (*paper I*). The majority of patients presented with autoantibodies against Th17 cytokines, which correlated with CMC (182, 183). In *paper I,* autoantibodies mainly remained positive over time, but, interestingly, 10

patients lost reactivity against 21OH, and fluctuation in indices were found for several other autoantibodies including TH, SCC, IL-22 and GAD65.

The Norwegian cohort (*paper I*) represents a population with a greater genetic heterogeneity compared to the Finnish (146), Sardinian (145) and Sicilian (241) patient cohorts, which are all populations with strong founder effects. However, the only association between phenotypes and the *AIRE* genotypes previously described is a higher prevalence of CMC in patients with the mutation p.R257X compared to patients with other mutations (183, 244) and low frequencies of PAI in patients with p.Y85C (143). Interestingly, Norwegian patients harbouring the splicing mutation c.879+1G>A had a milder phenotype compared to classical APS-1, with a generally late disease onset and extended time intervals between onset of new disease components. The mutation is located in the splice donor site of exon 7, and *AIRE* mRNA transcripts in PBMCs from patients show an altered splicing pattern including normal transcript and transcripts lacking exon 7. *AIRE* polypeptide lacking exon 7 has a potential to induce *AIRE*-regulated genes and exhibits nuclear localisation similar to that of WT *AIRE* (*paper III*) in a transfected cell line assay. This probably contributes to the mild phenotype seen in these patients and also indicates that altered mRNA splicing is dependent on specific *AIRE* genotypes and has the potential to modulate phenotypic expression in APS-1.

In APS-1, PAI is associated with HLA-DRB1*03 and alopecia with HLA-DRB1*04-DQB1*0302, whereas type 1 diabetes is negatively associated with HLA-DRB1*15-DQB1*0602 (244). Among Sardinian patients, an association between HLA-DRB1*0301-DQB1*0201, liver-kidney microsome autoantibodies (anti-CYP1A2) and autoimmune hepatitis is described (145). Ten Norwegian patients with PAI carried HLA class II alleles known to protect against PAI in the general population (HLA-DRB1*DQB1*0501-DQA1*01015; HLA-DRB1*1301-DQB1*0603-DQA1*0103; HLA-DRB1*1302-DQB1*0604-DQA1*0102; HLA-DRB1*07-DQB1*0201-DQA1*0201) (263), whereas none of the Norwegian APS-1

patients with PAI carried the HLA class II haplotype DR3-DQ2/DR4.4-DQ8 (*paper I*), which is by far the strongest known predisposing genetic factor for autoimmune PAI (263). Three out of four Norwegian APS-1 patients with type 1 diabetes were found to carry the DQB1*0602 allele, which is otherwise extremely rare in type 1 diabetes. Notably, the number of patient samples analysed for HLA associations was relatively small, which may explain some of the inconsistency in these reports. Although *AIRE* mutations are a definitive contributing factor in APS-1 development, the highly variable phenotype may indicate that *HLA* genotypes and environmental factors also influence disease onset and progression.

Several environmental triggers have the potential to modulate the pathogenesis of autoimmune disorders. As *AIRE* mutations and circulating autoantibodies are present before patients develop clinical features of APS-1 (200, 203), investigating the role of environmental triggers is particularly important for understanding disease onset and progression. Factors such as genetics, sex and diet directly affect the immune system, influencing the risk of autoimmunity. However, they may also indirectly affect risk through modulation of microbiota profiles and functions. Experiments in animal models have shown that changes in the microbiota can affect inflammation and disease severity (264-266). *Paper V* gives novel data on significantly altered oral microbiota in APS-1 patients. The most striking differences seen in patient microbiota were a higher proportion of Firmicutes and a reduction of Bacteroides, together with reduced species diversity, a pattern that has previously been described in the intestinal microbiota in several autoimmune diseases (116, 117, 119, 120). At the genus level, *Streptococcus* and *Gemella* were increased in patients. Only a few studies have investigated gastrointestinal microbiota in APS-1 (267-269). APS-1 patients develop early and sustained responses to gut microbial antigens reminiscent of Crohn's disease associated with Treg defects (269), and patients with gastrointestinal manifestations show significant enrichment in segmented filamentous bacteria (267). However, further investigation of oral, skin and gut microbiota in APS-1 could reveal associations between microbial taxa and disease components and

provide a better understanding of the possible links between microbiota composition and autoimmunity.

Based on the above, both genetic components and environmental triggers probably contribute to the highly variable phenotypic expression of APS-1. The TCR affinity of autoreactive T cells that escape negative selection due to lack of *AIRE* adds a stochastic factor that is also likely to be determinative for autoimmune responses in APS-1.

5.3 CMC in APS-1

Recent evidence indicates that genetic variation in the host plays an important role in susceptibility to *Candida* infections (17). Both monogenic primary immunodeficiencies and more common SNPs in genes of the immune system are associated with fungal infection. Studies of patient cohorts with severe and recurrent *Candida* infections have revealed several monogenic disorders associated with immunodeficiency and increased susceptibility to fungal infections. Most of these genes encode proteins critical for host defence against fungal infections (Figure 3). CARD9 is a central mediator in the anti-*Candida* host defence involved in downstream signalling of several PRRs (270), and homogenous mutations in this gene cause susceptibility to both mucosal and invasive *Candida* infections (10). Likewise, signal transducer and activator of transcription 1 (STAT1) is a signalling molecule downstream of both type I and type II IFN receptors (271), IL-23 receptor and IL-12 receptor (272), and mutations in this gene result in a primary immunodeficiency associated with *Candida* infections (273). Hyper IgE syndrome (274) is associated with *Candida* infections of the skin and can be caused by several different mutations, including mutations in *STAT3* (275), dedicator of cytokines 8 (276) and *TYK2* (277). Mutations in *IL-17RA* and *IL-17F* are found in patients with otherwise unexplained CMC (278). IL12Rb1 deficiency has been linked to both mucocutaneous and invasive *Candida* infections and deletions in the *CD25* gene associate with oesophageal candidiasis (279). Finally, common genetic variants

including SNPs in *TLR1* (280), *TLR2* (281), *TLR4* (282), *IL-4* (283), *IL-10* and *IL-12b* (284) have been shown to influence susceptibility to *Candida* infections.

In APS-1, most patients present with CMC in combination with an extreme autoimmune phenotype (146-148), and patients are probably almost persistently colonised with *C. albicans* (194). Neutralizing autoantibodies against immune signalling molecules probably represent a key factor in determining APS-1 patients' susceptibility to CMC (182, 183, 285). However, recent findings in a murine oropharyngeal candidiasis model suggest that additional genetic or environmental susceptibility triggers are needed to precipitate clinical disease (286). Another longitudinal study of six APS-1 patients investigating the association between CMC and anti-cytokine autoantibodies questions the importance of their role in CMC in APS-1 because some patients with CMC lack these autoantibodies (287). The hypothesis in *paper IV* was that other immune mechanisms contribute to CMC in APS-1 patients, and this study reveals several significant differences in immune activation upon *Candida* challenge in patients compared to healthy controls. Collectively, these alterations have broad implications on the quality of patients' immune response to *Candida*. Mediators involved in the crosstalk between innate immune cells and T cells, including IL-1β, IL-10, IL-12p40, IL-18, and IL-23, were all found to be significantly altered, indicating that several cellular mechanisms are potentially involved. Patients also failed to up regulate IL-17A and IL-23, which are critical for optimal host defence against cutaneous candidiasis (288).

Other studies have also reported altered immunologic responses in subsets of immune cells from APS-1 patients (185, 186, 190, 289). Although controversial, an extrathymic role of AIRE is reported in monocytes where it interacts with CARD9, SYK, and Dectin-1 (290). AIRE also has the potential to regulate the STAT1 protein levels in CD14+ monocytes (291). In *paper IV*, we showed that monocytes from patients produce less IL-23 upon stimulation with the Dectin-1 agonist curdlan. The data included in this thesis, together with the studies discussed above, clearly indicate

that APS-1 patients have significantly altered immune responses to *Candida* antigens, which collectively contribute to the susceptibility to CMC found in these patients.

Monogenic disorders are rare, and the vast majority of *Candida* infections are probably due to a combination of complex genetics and environmental factors. However, studying monogenic disorders including APS-1 will increase our understanding of the genetic mechanisms underlying *Candida* infections, which is crucial for the development of future immunotherapeutic approaches to patient treatment.

5.3.1 CMC and malignacies in APS-1

Most APS-1 patients presenting with malignancies are in their thirties and have metastatic disease at diagnosis (170). CMC and smoking are risk factors (170). Squamous cell carcinomas of the oral or oesophageal mucosa are the most frequently seen malignancies in APS-1 (146, 147); it has previously been disputed whether these malignancies are a complication or component of the syndrome (170). However, these malignancies definitely occur, in the context of APS-1, more frequently and with an earlier onset relative to the general population. Specific characteristics of *C. albicans* isolated from APS-1 patients (192-195) and long exposure to it might be carcinogenic (194). For example, *C. albicans* isolated from human APS-1 patients have the potential to produce mutagenic amounts of acetaldehyde (292), which has carcinogenic effects, and increased saliva concentrations associate with other risk factors such as tobacco smoking, alcohol intake, and poor oral hygiene. Studies in mice have suggested that autoreactive CD4+ T cells and chronic fungal infections cause inflammation and tissue injury, which further drive carcinogenesis (293). A contribution from inflammation and CMC is also underpinned by the consistent co-localisation of mucositis and malignant lesions. This was the case for the patients described in *paper II* who all had severe CMC since childhood and developed oral tongue malignancies at relatively young ages.

Interestingly, recent findings indicate that *AIRE* may play a role in the development of malignancies in peripheral tissues without *C. albicans* exposure. For example, *AIRE* protein expression in human breast cancer cells are a strong prognostic factor for relapse-free survival (246) and *AIRE* expression in keratinocytes is associated with skin tumorigenesis in mice (247).

5.4 Screening and diagnosis in APS-1

The rarity of and highly variable phenotype in APS-1 makes the syndrome hard to recognise (*paper I*), often causing a significant delay in proper workup and diagnosis. It has previously been estimated that at least one child every other year is born with APS-1 in Norway (294), but only one child has actually been diagnosed the last 11 years (147). The exact reason for this is unclear, but two possible explanations are that disease components are diagnosed and treated while the syndrome goes undiagnosed or that patients die during childhood without diagnosis, as indeed was observed in seven Norwegian patients (*paper I*). Unrecognised components of APS-1 such as PAI, HP and diabetes mellitus type I may be fatal. Dissemination of information about ROAS, recommended screening and follow-up routines could be improved, aiming to reach more physicians, patients, and families.

Based on published APS-1 cohorts, the following screening procedure/flow chart is suggested for screening and diagnosis in APS-1 (Figure 5) (64): Patients presenting with particularly suspicious manifestations should be considered for APS-1 workup, and *AIRE* sequencing is recommended in patients presenting two of the three major disease components: PAI, CMC and HP. Screening for INF-ω autoantibodies should be considered if a patient presents with one of the suggestive manifestations CMC, PAI, HP, premature ovarian failure, enamel hypoplasia, periodic fever with rash in childhood, non-infectious keratitis or autoimmune hepatitis, especially at a young age. *AIRE* sequencing is recommended in all patients presenting with INF-ω autoantibodies. The detection of two disease-causing mutations in *AIRE* meets diagnosis criteria for “classical APS-1”, whereas one

dominant mutation indicates "non-classical APS-1" (243). If no mutations in *AIRE* are revealed, imaging for thymoma or/and *RAG* sequencing should be considered to search for a combined immunodeficiency or Omenn syndrome (295). Finally, if no mutations in *AIRE* or *RAG* are found, the patient probably has an "APS-1–like" clinical condition. When a patient is diagnosed, all siblings should be investigated, and the family should be offered genetic counselling.

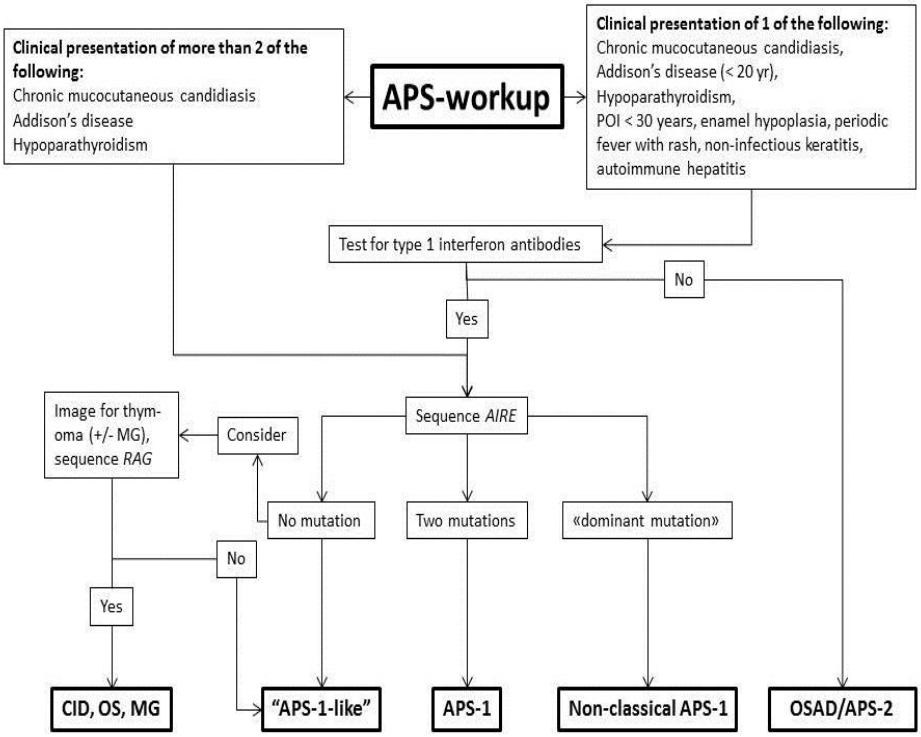

Figure 5. Flow chart for APS workup.

Patients presenting with particular suggestive manifestations should be considered for APS-1 workup including AIRE sequencing and IFN-ω autoantibodies screening. The detection of two mutations indicates the diagnosis of classical APS-1. If no mutations are found, imaging for thymoma or/and RAG sequencing should be considered, searching for combined immunodeficiency or Omenn syndrome. The figure is adapted from Husebye et al. (64). APS, autoimmune polyendocrine syndrome; CID, combined immunodeficiency; IFN, interferon, MG, myasthenia gravis; OS, Omenn syndrome; OSAD, organ-specific autoimmune disease POI, primary ovarian failure; RAG, recombination activation gene.

5.5 Treatment and follow up of APS-1

Treatment of APS-1 is complicated and needs to be optimised for each individual patient. However, it typically includes hormone-replacement therapy and specific treatment of complications. CMC should be treated aggressively using oral mycostatin and oral amphotericin (150). In addition, other risk factors for oral malignancies such as smoking and alcohol consumption should be identified and minimised. Treatment of HP requires oral vitamin D derivatives in combination with calcium and magnesium supplementation. Other manifestations including pneumonitis and hepatitis may require immunosuppressive treatment (64). Vaccination against pneumococcus, meningococcus, *Haemophilus influenza* type b, and influenza is recommended due to asplenia (64).

Patients with APS-1 are at constant risk for developing new disease components throughout life, and most receive complex treatments. Therefore, patients should be followed every 6-12 month in a specialised centre by a senior consultant in endocrinology, experienced with APS. Other specialists such as dentists, gastroenterologists and paediatricians may be involved if needed. Patients should be screened for new disease components by a proper clinical examination and serum autoantibody analyses. An oral examination should be included, focusing in particular on CMC and the development of oral malignancies.

Finnish APS-1 patients have reported impairment in quality of life based on general health, emotional well-being and vitality measurements (296). Overall mortality is increased (146, 169), and life expectancy probably depends on the severity of the disease, patient compliance, how they cope with disease components and treatments.

6. Final conclusions

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

- Norwegian APS-1 patients present with highly variable clinical phenotypes. Most patients present with one of the major disease components during childhood, and enamel hypoplasia, HP and CMC are the most frequently observed features.
- Malignancy of the tongue is a novel entity of APS-1 and is probably caused by longstanding, chronic inflammation of the oral cavity caused by CMC infections. Oral tongue malignancies in APS-1 have an early onset compared to other patients with oral malignancies.
- All Norwegian APS-1 patients present with typical autoantibodies, where autoantibodies against IFN-ω are the most frequent, followed by autoantibodies against 21OH and IL-22.
- The most common *AIRE* mutation in Norwegian APS-1 patients is c.967_979del13.
- The *AIRE* mutation c.879+1G>A is associated with a mild phenotype and causes altered mRNA splicing patterns including mRNA transcript with exon-skipping and normal transcripts. Transcripts with exon-skipping produce a protein with residual *AIRE* function.
- APS-1 patients show altered immune activation in whole blood upon stimulation with *Candida*, relative to healthy controls. This includes the dysregulation of several cytokines including IL-2, IL-17A, IL-22, and IL-23.
- Monocytes from patients produce significantly less IL-23p19 upon exposure to a *Candida*-specific antigen compared to monocytes from healthy subjects. This is probably a contributing factor in susceptibility to *Candida* infections.

- APS-1 patients have significantly altered oral microbiota, with a general reduction in the total number of bacterial genera and species. The proportion of the major phyla Firmicutes is higher and Bacteroidetes lower in APS-1 patients compared to healthy subjects.

7. Future perspectives

Monogenic diseases have proven to be excellent *in vivo* models for genetic and immunological studies. Studying APS-1 and *AIRE* has expanded our knowledge of central immunological tolerance and how negative selection of T cells takes place in the thymus. However, further development of immunological techniques, especially for the study of the human immune system in health and disease, is needed in order to understand human disease and translate results into clinical practice. In the following, some future perspectives are briefly discussed.

Published patient cohorts and case reports from different geographical areas add knowledge of both pathogenesis and phenotypic variation in APS-1. The obvious extension of *paper I* is the inclusion of more patients and extension of follow-up care. Compiling and comparing patients from different countries could both reveal novel disease components (*paper II*) and provide data to establish more robust correlations between clinical features, autoantibody profiles and *AIRE* mutations.

Although a mild phenotype seems to be associated with the c.879+1G>A splice mutation, genotype-phenotype relationships are not well described in APS-1. It could be of great interest to further explore the molecular mechanisms of splicing using *AIRE* as a model, beyond the findings described in *paper III*. The development of an animal model could be beneficial for further characterisation of the mechanisms involved in *AIRE* splicing.

Several immune mediators in whole blood from patients with APS-1 were found to be dysregulated when challenged by *C. albicans* (*paper IV*). Other mediators or pathways could be further investigated, for example, using intracellular flow cytometry. Significantly altered numbers of specific subpopulations of immune cells have already been reported in APS-1 (189). However, introducing new methods such as mass cytometry or single cell RNA sequencing could improve the phenotypic

mapping of immune cells subgroups in patients. Other areas requiring further investigation are the fields of epigenetics and metabolomics and the related mechanisms of *trained immunity* in the innate immune system. Such research would also add to knowledge about the mechanisms involved in both autoimmunity and immunity against *Candida* infections.

Novel data regarding the oral microbiota in APS-1 is presented in *paper V*. Our profiling should be reproduced in a larger cohort, and it would be interesting to also characterise the skin and intestinal microbiota of patients. Profiling a lager cohort of patients could allow the correlation of taxa to disease components, autoantibody profiles or *AIRE* mutations.

The rarity of APS-1 makes studying it complicated. There are relatively few patients, and the availability of biological samples is limited. An executive aim should therefore be to organize patients into larger, international patient cohorts, thereby generating more representative data and increasing statistical power.

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9. APPENDIX

I

A Longitudinal Follow-up of Autoimmune Polyendocrine Syndrome Type 1

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Context: Autoimmune polyendocrine syndrome type 1 (APS1) is a childhood-onset monogenic disease defined by the presence of two of the three major components: hypoparathyroidism, primary adrenocortical insufficiency, and chronic mucocutaneous candidiasis (CMC). Information on longitudinal follow-up of APS1 is sparse.

Objective: To describe the phenotypes of APS1 and correlate the clinical features with autoantibody profiles and autoimmune regulator (*AIRE)* mutations during extended follow-up (1996–2016).

Patients: All known Norwegian patients with APS1.

Results: Fifty-two patients from 34 families were identified. The majority presented with one of the major disease components during childhood. Enamel hypoplasia, hypoparathyroidism, and CMC were the most frequent components. With age, most patients presented three to five disease manifestations, although some had milder phenotypes diagnosed in adulthood. Fifteen of the patients died during follow-up (median age at death, 34 years) or were deceased siblings with a high probability of undisclosed APS1. All except three had interferon-ω) autoantibodies, and all had organ-specific autoantibodies. The most common *AIRE* mutation was c.967_979del13, found in homozygosity in 15 patients. A mild phenotype was associated with the splice mutation c.879+1G>A. Primary adrenocortical insufficiency and type 1 diabetes were associated with protective human leucocyte antigen genotypes.

Conclusions: Multiple presumable autoimmune manifestations, in particular hypoparathyroidism, CMC, and enamel hypoplasia, should prompt further diagnostic workup using autoantibody analyses (eg, interferon-ω) and *AIRE* sequencing to reveal APS1, even in adults. Treatment is complicated, and mortality is high. Structured follow-up should be performed in a specialized center. **(*J Clin Endocrinol Metab* 101: 2975–2983, 2016)**

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Abbreviations: AADC, aromatic L-amino acid decarboxylase; AIRE, autoimmune regulator; APS1, autoimmune polyendocrine syndrome type 1; CMC, chronic mucocutaneous candidiasis; GAD65, glutamic acid decarboxylase 65-kDA isoform; HLA, human leukocyte antigen; IFN, interferon; MAGEB2, melanoma antigen B2; 17OH, 17-α-hydroxylase; 21OH, 21-hydroxylase; PAI, primary adrenocortical insufficiency; PDILT, protein disulfide isomerase-like testis expressed; SCC, side-chain-cleavage enzyme; TGM4, transglutaminase 4.

Autoimmune polyendocrine syndrome type 1 (APS1) is a monogenic disease, also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (OMIM no. 240300). Clinically, APS1 is defined by the presence of two of the three major components: hypoparathyroidism, primary adrenocortical insufficiency (PAI), and chronic mucocutaneous candidiasis (CMC) (1). However, the syndrome also includes many less known disease components, and the clinical presentation is highly variable (2). One major manifestation combined with a sibling with APS1 also qualifies for the diagnosis. The disease usually presents in childhood and adolescence, but many patients are not diagnosed until adulthood or not at all (3). APS1 patients have an increased risk of cancer and increased mortality compared with the general population (4). The diagnosis can also be made by finding two disease-causing mutations in the autoimmune regulator (*AIRE*) gene together with clinical manifestations (5, 6). About 115 mutations have been reported so far (7). AIRE is almost exclusively expressed in the thymus (8) and plays a crucial role in negative selection of self-reactive T cells and development of regulatory T cells (9, 10).

The highest prevalence is found among Persian Jews (1:9000) (11), Sardinians (1:14 000) (12), and Finns (1: 25 000) (1). The prevalence in Norway was previously reported at 1:90 000 (3). Recently, patients with monoallelic *AIRE* mutations with dominant inheritance, characterized by a later disease onset and often milder phenotypes, were reported (13). These nonclassical forms may be much more prevalent because monoallelic *AIRE* mutations have a prevalence in the general population of about 1:1000 (13).

Most patients have autoantibodies against autoantigens expressed in the affected tissue (14), eg, the steroidogenic enzymes 21-hydroxylase (21OH) in the adrenal cortex and side-chain-cleavage enzyme (SCC) in the gonads and adrenal cortex. Recently, several novel autoantigens have been identified using proteome arrays, including the prostate-specific enzyme transglutaminase 4 (TGM4) associated with male infertility and prostatitis in *Aire*-knockout mice (15, 16). Other autoantigens identified using this technique include protein disulfide isomerase-like testis expressed (PDILT) and melanoma antigen B2 (MAGEB2), which are both expressed in testicular germ cells (17). In addition, almost all patients display autoantibodies to interferons (IFNs) and interleukins (ILs) (18, 19); they typically appear years before the corresponding clinical symptoms. Mutational analysis and assay of anti-IFN-ω autoantibodies are suggested as diagnostic options (20).

Information on longitudinal follow-up of APS1 patients is sparse, with only a few series published (21–23). Building on previous surveys of the Norwegian cohort and our National Registry of Autoimmune Diseases (3, 24), we here provide a longitudinal follow-up of the Norwegian cohort spanning two decades and presenting the natural course including mortality, autoantibody profiles, and correlations to genotype.

Patients and Methods

Patients

Patients were recruited from departments of medicine and pediatrics from hospitals in Norway and were included in our National Registry of Autoimmune Diseases initiated in 1996 (3). All fulfilled the diagnostic criteria for APS1 given above. The Regional Committee for Medical and Health Research Ethics approved the study, and all participants gave informed consent.

Definitions and clinical data

The patients were assessed at least annually, including hormonal status and autoantibody profiles. All patients alive were screened for *AIRE* mutations. A dental and oral examination was performed in 31 patients, and most patients underwent esophagogastroduodenoscopy, chest x-ray, and imaging of the spleen and kidneys. Endocrinopathies were diagnosed as previously described (1). The diagnostic criteria for other disease manifestations are given in Supplemental Table 1.

Autoantibody assays

Autoantibodies against 21OH, 17-α-hydroxylase (17OH), aromatic L-amino acid decarboxylase (AADC), glutamic acid decarboxylase 65-kDA isoform (GAD65), IFN-ω, IL-17, IL-22, MAGEB2, NACHT leucine-rich-repeat protein 5, PDILT, putative potassium channel regulator, SCC, sex-determining region Y-box 10, TGM4, tryptophan hydroxylase 1, and tyrosine hydroxylase were assayed by radio-binding ligand assay as described previously (15, 16, 25). All autoantibody assays were performed in our laboratory, and sera spanning a time period were analyzed in the same experiment to avoid between-assay variations in the indices. Parietal cell antigen autoantibodies were assayed by ELISA (Euroimmun).

Mutational analysis of the *AIRE* gene

DNA sequencing of *AIRE* spanning the exon-intron boundaries was performed using standard methods. Primer sequences are available upon request. Copy number analysis was performed by duplex TaqMan real-time PCR as previously described (26).

Human leukocyte antigen allele typing

The sequence-based human leukocyte antigen (HLA) genotyping was performed using SBT Resolver and Assign Software (Conexio Genomics).

Statistical analyses

Fischer's exact test with two-sided significance performed in a 2×2 contingency table was used (IBM SPSS Statistics 22), testing each autoantibody against the presence of different disease components. Similarly, the associations between pheno-

Table 1. Basic Demographics of the 52 Norwegian APS1 Patients

No. of females/males	24/28
No. of families	34
Age at onset of first component, y	0–43 (median, 8.5)
Age at death (n = 15), y	3–64 (median, 34)
AIRE mutations found (alleles, n = 92)	92% (85/92)
Autoantibodies (n = 45)	
Organ specific	100% (45/45)
IFN-ω	93% (42/45)

types, *AIRE* mutations, and different HLA alleles were tested. Specifically, we also investigated the correlation between PAI in APS1 patients and HLA class II risk genotypes for PAI in the general population as defined previously (27).

Results

APS1 patients ascertained

Applying our diagnostic criteria and the National Registry of Autoimmune Diseases, we included 52 individuals (28 males, 24 females) from 34 families, including three patients (Supplemental Table 2, family XXIX, patients no. 45–47) with monoallelic *AIRE* PHD1 mutations (Table 1). These patients represented all known Norwegian APS1 patients. Fifteen patients died during the follow-up period, including seven who were identified only after their death. Most of the patients participated in two earlier surveys (3, 24), but 12 identified after 2007 (3) were added to the current survey.

Clinical manifestations and the classic triad

The clinical picture was highly variable, even among members of the same family. An overview of the prevalence of the most common disease components, including sex distribution and age at presentation, is given in Table 2 and Supplemental Table 2. The most common initial manifestations were hypoparathyroidism (17 patients, 32%) and CMC (13 patients, 25%), but other components occasionally presented first. Among patients with all three major components, 14 (67%) had developed the triad by 25 years of age. In general, the disease components increased in prevalence by age, although the time courses differed markedly (Figure 1). The median number of disease components was five (range, one to eight) (Supplemental Figure 1).

Two-thirds of the patients with hypoparathyroidism were diagnosed before the age of 15 years. One young female patient (patient 38) died of seizures at age 3 years, probably from undiagnosed hypoparathyroidism.

Thirty patients with PAI (91%) were diagnosed before age 25 years, including 21 (64%) before the age of 15 years. Two young females died in acute adrenal failure during the follow-up period (Supplemental Table 3).

The clinical course of CMC varied from periodic to chronic. Fourteen patients (27%) developed CMC before

Table 2. Prevalence of the Most Common Disease Components, Gender, and Age at Onset (n = 52)

Disease Components	Prevalence, % (n/Total n)	Females/Males, n	Median (Range) Age at Onset, y
Classic triad			
Hypoparathyroidism	73 (38/52)	18/20	9 (1–60)
PAI	63 (33/52)	11/22	13 (4–55)
CMC	77 (40/52)	15/25	7,5 (0–64)
All three	40 (21/52)	6/15	14 (4–64)
Other endocrine disorders			
Gonadal failure	33 (8/24)[a]	8/0	18 (15–25)
Diabetes mellitus	8 (4/52)	1/3	33 (23–54)
Hypothyroidism	19 (10/52)	8/2	22 (13–51)
Skin disorders			
Alopecia	31 (16/52)	5/11	19 (4–41)
Vitiligo	15 (8/52)	4/4	20 (15–51)
Gastrointestinal disorders			
Pernicious anemia or vitamin B12 deficiency	15 (8/52)	4/4	38 (13–63)
Malabsorption	23 (12/52)	5/7	21 (10–39)
Autoimmune hepatitis	4 (2/52)	0/2	5,5 (0–11)
Eye disorders			
Keratoconjunctivitis	12 (6/52)	1/5	22 (11–25)
Others			
Enamel hypoplasia	72 (18/25)[b]	9/9	
Nail dystrophy	13 (7/52)	3/4	
Asplenia	16 (5/31)	2/3	

[a] Percentage of female patients.

[b] Percentage of patients examined by dentist.

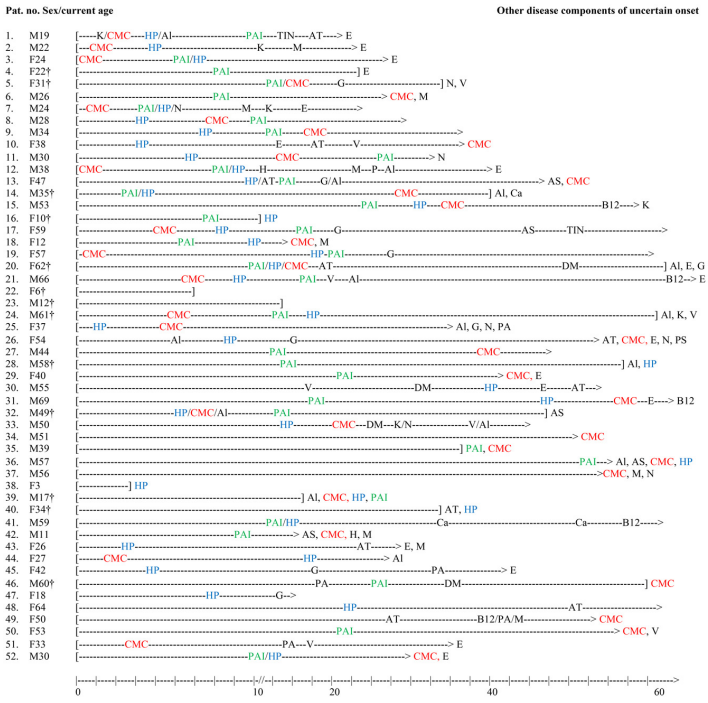

Figure 1. Disease histories of Norwegian APS1 patients. The lines start at birth and end at death (bracket) or at current age (>). Age at appearance of disease component is indicated by a symbol of the disease. Disease components of uncertain time of onset are listed at the end of the line. The major disease components CMC, HP, and PAI are marked in red, blue, and green, respectively. AI, alopecia; AS, asplenia; AT, hypothyroidism; Ca, cancer; DM, diabetes mellitus; E, enamel hypoplasia/defects; G, hypogonadism; H, hepatitis; HP, hypoparathyroidism; K, keratoconjunctivitis; M, malabsorption; N, nail dystrophy; P, pancreas failure exocrine; PA, pernicious anemia; PS, psoriasis; TIN, tubule interstinal nephritis; V, vitiligo.

age 10 years and only three after the age of 30 years. Eleven had angular cheilitis at the time of examination and another 10 reported previous episodes. Eight patients (15%) were diagnosed with candida esophagitis, sometimes without typical symptoms or coexisting oral candidiasis. One patient (patient 34) developed stenosis of the esophagus requiring endoscopic dilation. Twelve of the 31 patients examined by a dentist tested positive for *Candida albicans* by culture. One patient (patient 51) suffered from severe candida otitis.

Other endocrinopathies

Hypothyroidism was the third most frequent endocrinopathy followed by gonadal failure. No male hypogonadism was found. Diabetes mellitus type 1 was rare (n = 4) and had a relatively late onset.

Oral cavity and teeth

Six of 31 examined patients had extensive composite dental restorations, most probably secondary to underlying enamel defects. Another 18 (72%) had enamel hypoplasia typical for APS1 (Figure 2), and five had enamel hypomineralization without enamel hypoplasia, which could be of different etiology. The enamel hypoplasia varied in extent and location. Nine patients had gingivitis, and eight patients presented pathologically low (below 0.1 mL/min) unstimulated salivary flow.

Skin diseases

Vitiligo was variable in extent and location, from spots to almost universal, but was not classified into segmental or nonsegmental forms. The time of diagnosis of alopecia was highly variable, and the clinical picture varied from chronic to periodical.

Gastrointestinal manifestations

Chronic diarrhea was interpreted as malabsorption. Pernicious anemia and vitamin B12 deficiency typically presented late but were also seen in some young individuals (patient 51). Autoimmune hepatitis was found in two young male patients (patients 12 and 42). Generally, the gastrointestinal manifestations were of variable intensity and duration.

Ocular disease

Three patients had keratoconjunctivitis, two iridocyclitis, and one blepharoconjunctivitis. A female patient (patient 43) was diagnosed with optic neuritis at the age of 22 years.

Other manifestations

Two patients were diagnosed with tubulointersitial nephritis. Patient 1 was diagnosed with IgA nephritis at age 15 years, and patient 17 presented with nephrotic syndrome at age 48 years. Biopsy showed local segmental nephrosclerosis. Her kidney disease is now stable after steroid treatment. Deafness was found in one female patient (patient 51).

The natural course

Most patients follow the classic course already described for APS1: the first disease component, often one of the classic triad components, presented in childhood, with additional disease components occurring at different time intervals (Figure 1 and Supplemental Table 2). However,

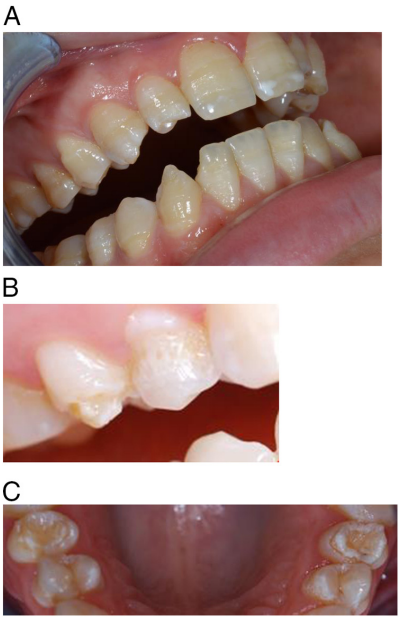

Figure 2. Typical enamel hypoplasia in APS1. A, Hypomineralization (white areas on front teeth) and enamel hypoplasia revealed by horizontal hypoplastic bands. B, Severe enamel hypoplasia with loss of normal enamel structure. C, Enamel hypoplasia varying in size and location, affecting both front teeth and molars.

only 14 of 21 patients developed the full triad before the age of 25 years. Early onset was associated with a more severe phenotype, and the disease components increased in prevalence with age. Hypoparathyroidism, PAI, CMC, and autoimmune hepatitis appeared early, whereas hypothyroidism, B12 deficiency, and pernicious anemia mainly had a late onset. Furthermore, the vast majority of patients had enamel defects or hypoplasia, probably with onset in adolescence. Atypical late presentations and long intervals between components contributed to delayed diagnosis (patients 30, 31, and 46).

Mortality and cancer

Fifteen patients died during the follow-up period, and seven patients were identified after their death (Supplemental Table 3). The major causes of death were malignant disease and adrenal and hypocalcemic crises. The median age at death was 34 years. Supplemental Table 4 gives an overview of the different malignant conditions found.

Distribution of autoantibodies

We assayed a large panel of autoantibodies related to APS1, including the recently identified autoantigens TGM4, PDILT, and MAGEB2 (Supplemental Figure 2). All 45 patients tested presented organ-specific autoantibodies. In total, IFN-ω autoantibodies were found most frequently (42 patients, 93%), followed by autoantibodies against 21OH (71%) and IL-22 (71%). Notably, the prostate-specific antigen TGM4 was found only in males (Supplemental Figure 2 and Supplemental Table 2). Assay of autoantibodies over time revealed a pattern dominated by stable positivity. However, 10 patients lost reactivity against 21OH during the follow-up period, and fluctuation of indices were found for several other autoantibodies such as tyrosine hydroxylase, SCC, IL-22, and GAD65 (Supplemental Figure 3).

AIRE genotype vs phenotype

We detected *AIRE* mutations in 44 patients. Two had no mutations or copy number variations. Six deceased patients were not tested, but genotypes could be deduced based on their siblings. The most common mutation was c.967_979del13 found in 45% of the alleles, followed by c.769C>T, and c.879+1G>A (Figure 3 and Supplemental Table 2). We then grouped them according to genotype, namely patients homozygous for missense mutations (genotype 1; seven patients), patients homozygous for mutations giving a truncated protein (genotype 2; 32 patients), and patients with one missense mutation and one mutation giving a truncated protein (genotype 3; five patients). The splicing mutations were included in group 1. The two patients without mutations (patients 49 and 50) were excluded. The median number of disease manifestations was five in all groups. However, patients with genotype 1 had a later disease onset (median age, 19 years) than genotypes 2 and 3 (median age, 7 and 11 years, respectively). Among patients with genotype 2, 90% had CMC, and 75% had PAI. All patients with asplenia had genotype 2. Three patients homozygous and one heterozygous for the splicing mutation c.879+1C>G (patients 29, 30, 31, and 48) presented a mild phenotype with late disease onset (Figure 3 and Supplemental Table 2).

Immunotype vs phenotype

Seventeen (49%) of the patients with hypoparathyroidism had autoantibodies against NACHT leucine-rich-repeat protein 5. Among patients with PAI, 93% had autoantibodies against 21OH, 63% against SCC, and 43% against 17OH. All of the patients with autoantibodies against 17OH also had autoantibodies against 21OH. Both autoantibodies against 21OH and SCC correlated significantly to PAI ($P < .001$ and $P = .002$, respectively). Thirty patients with CMC (81%) had autoantibodies against IL-22, giving a significant correlation ($P = .004$). Autoantibodies against GAD65 were found in 22 patients, including three patients with diabetes mellitus type 1. Seven patients with vitiligo had autoantibodies against

A

Mutation type	Protein effect	No. of alleles	Percentage	No. of homozygous	No. of heterozygous
Missense		**9**	**9.9**		
c.22C>T	p.R8C	3	3.3	0	3
c.290T>C	p.L97P	1	1.1	0	1
c.934G>A	p.P312N	3	3.3	0	3
c.977C>T	p.P326Q	1	1.1	0	1
c.1336T>G	p.C446G	1	1.1	0	1
Nonsense		**14**	**15.2**		
c.769C>T	p.R257*	14	15.2	2	10
Splicing		**7**	**7.6**		
c.879+1G>A	-	7	7.6	3	1
Deletions		**43**	**46.7**		
c.402delC	-	1	1.1	0	1
c.967-979del13	-	41	44.5	15	12
large del [26]	-	1	1.1	0	1
Insertions		**12**	**13**		
c.1242_1243insA	-	3	3.3	0	3
c.1244_1245insC	-	3	3.3	1	2
c.1249dupC	-	4	4.3	0	4
c.1163_1164insA	-	2	2.1	0	2
Not found		**7**	**7.6**		
Total no of alleles		**92**	**100**		

B

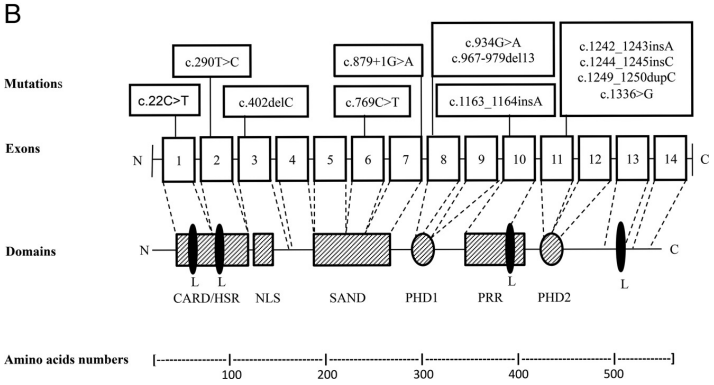

Figure 3. AIRE mutations in Norwegian APS1 patients. A, Overview of the identified mutations and their frequencies. B, Location of *AIRE* mutations in Norwegian APS1 patients together with a schematic representation of the AIRE protein and its functional domains. Boxes 1–14 represent the exons, and the different mutations are given in the text boxes above. CARD/HSR, Caspase recruitment domain/homodimerization domain (amino acids 1–105); NLS, nuclear localization signal (amino acids 100–189); SAND, Sp100, AIRE-1, NucP41/75 (181–280), DEAF-1; L (LXXLL), nuclear receptor-binding motifs (amino acids 7–11, 63–67, 414–418, 516–520); PHD, plant homeodomain type zinc fingers (amino acids 296–343 and 434–475); PRR, proline-rich region (amino acids 350–430).

AADC (88%), proving a significant correlation (P = .047). Figure 4 presents a heat map with clinical manifestations and previously reported correlated autoantibodies grouped together.

HLA vs phenotype

The HLA class II genotype stratified to the risk of PAI is given in Supplemental Table 2. However, 10 patients with PAI had HLA class II alleles known to be protective in the general population. We also found significantly more alopecia in this group compared to the rest of the cohort. None of the APS1 patients with PAI carried the high-risk HLA class II haplotype DR3-DQ2/DR4.4-DQ8 (27).

Discussion

This longitudinal follow-up of the 52 Norwegian APS1 patients demonstrates the clinical variability from very mild to severe disease, occurrence of new components over time, and an overall increased mortality from adrenal crisis and cancer. Dental examination revealed that enamel hypoplasia was present in most patients and, together with CMC and hypoparathyroidism, is one of the three most common manifestations. Longitudinal data have previously been presented from the Finnish, Sicilian, and Sardinian patient cohorts (with 91, 15, and 22 patients, respectively) (21–23), which all represent populations with strong founder effects (21, 23). The Norwegian population displays a greater genetic heterogeneity and may therefore be more representative for the situation in most countries.

The clinical variation and rarity of APS1 makes the syndrome hard to recognize. Since our latest report in 2007, only one child has been diagnosed, which is unexpected because we estimate that at least one child every other year is born with APS1 in Norway (3). The reason is unclear, but most likely disease components are being recognized and treated, whereas the syndrome goes undiagnosed. Alternatively, patients die during childhood without diagnosis, as indeed was observed in seven of our patients. This underscores the importance of increased awareness and early diagnosis. APS1 should be considered in all patients presenting one of the major clinical manifestations, especially when it presents in childhood. Moreover, when a new patient is diagnosed, all siblings should be offered genetic counseling.

Some variation in the frequency of disease components occurs among APS1 cohorts. We report several with hypothyroidism, which is also found in Apulian APS1 pa-

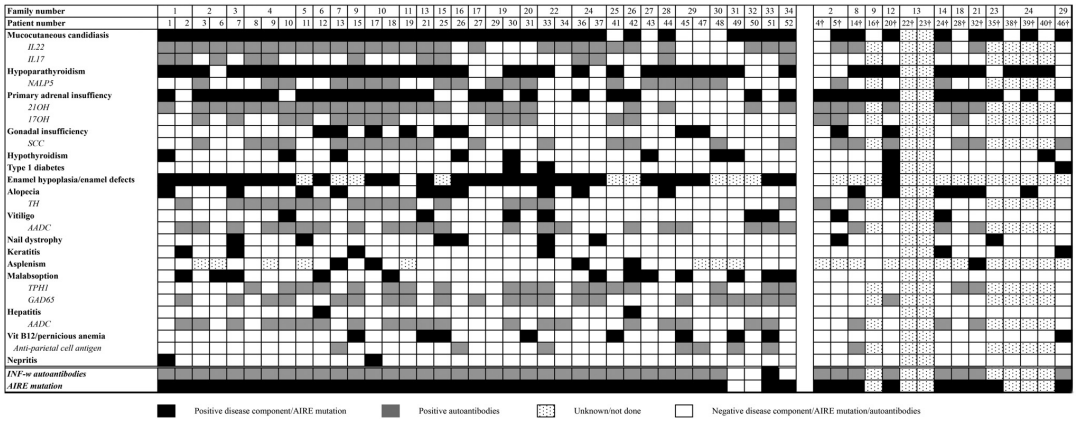

Figure 4. Heat map of clinical manifestations and autoantibodies in Norwegian APS1 patients. Family number and patient number are given in the first two rows. A black square represents a disease manifestation, and a gray square represents positive autoantibodies. If the square is dotted, the examination/analyses are not performed; a white square represents negative disease component/AIRE mutation/autoantibodies that are tested for.

tients (28). However, hypothyroidism was not seen in the Sardinian patients (23), although late onset cannot be excluded (2). Although autoimmune cause was not proved, it is probably caused by autoimmunity given the propensity for autoimmunity in APS1. Furthermore, autoimmune hepatitis was described as a serious and early feature in 27% of the Sardinian patients (23), whereas we found hepatitis in only two (4%). In a study of 23 Persian Jews (11), 22 presented with hypoparathyroidism, and only four patients had oral CMC. The Sardinian patients displayed the most severe phenotype, with a mean of seven disease manifestations per patient and early disease onset (23). Besides the different *AIRE* genotypes, other immune genes might potentially affect the phenotype (29), and environmental factors and varying practices among clinicians may also have influence.

Poorly treated or undiagnosed endocrinopathies as part of APS1, especially PAI, hypoparathyroidism, and diabetes mellitus type 1, can be fatal. Adherence to therapy, especially in teenage patients, is challenging. An increased death risk and altered cancer incidence pattern have been described (4). Identifying risk factors for malignancies and minimizing these by treatment of CMC and avoidance of smoking are probably important (21). In addition, pneumococcal vaccination must be performed in patients with asplenia and should probably be offered to all APS1 patients.

All of the Norwegian patients had organ-specific autoantibodies. Typically, the presence of autoantibodies correlates to clinical manifestations but may appear years before the corresponding clinical manifestation (3, 14, 18, 19). A correlation between gonadal failure and autoantibodies against SCC is reported (14, 30), which was also found in five of the nine female patients with gonadal failure in this study. In total, SCC autoantibodies were found in 21 patients. Autoantibodies against GAD65 are normally known to correlate with diabetes mellitus type 1 (31), but this is not the case in APS1 (14). We found GAD65 autoantibodies in three of four diabetic patients and in 19 patients without diabetes. No correlation with autoantibodies against GAD65 and vitiligo or malabsorption was found, in conflict with an earlier report (14). However, autoantibodies against AADC correlated with vitiligo, and autoantibodies against 21OH and SCC correlated with PAI. Autoantibodies against the prostate-specific enzyme TGM4 were only seen in the males, consistent with a recent report (15).

We found antibodies against IFN-ω in a similar proportion to that in other APS1 cohorts (21–23). These autoantibodies are often found in the earliest samples; they persist for decades and show a high specificity for APS1 (19, 32). IFN autoantibodies can also be found in low titer in diseases causing an increased IFN production (ie, systemic lupus erythematosus, human immunodeficiency virus, and hepatitis C virus infections), as well as in myasthenia gravis (33). Two female patients did not present *AIRE* mutations or IFN-ω autoantibodies but fulfilled the clinical criteria. They may have mutations either in the regulatory parts of the *AIRE* gene or in other genes in the same pathway, or they may be phenocopies. Another patient presented all three major disease components from childhood and was compound heterozygous (c.22C>T/c.967_979del13) for two *AIRE* mutations, but autoantibodies against IFN-ω were not found.

The cohort presented here is older and has a much greater genetic heterogeneity compared to other APS1 co-

horts. Of particular interest was the c.879+1C>G splice mutation found in three homozygous patients (patients 29, 30, and 31), who all had ancestors in a particular district of Western Norway. Their phenotypes were characterized by late disease onset and generally a milder phenotype. The three patients had their first manifestation at 15, 19, and 23 years of age. The two patients with hypoparathyroidism developed hypocalcemia at 43 and 60 years of age. It is not known whether this implies that the splice defect is not complete and that some residual AIRE function is present.

We found 10 patients with PAI carrying HLA class II alleles known to protect against PAI in the general population (27). Patients in this group also had significantly more alopecia ($P < .05$). In contrast, none of the APS1 patients with PAI carried the HLA class II haplotype DR3-DQ2/DR4.4-DQ8, which is by far the strongest predisposing genetic factor for autoimmune PAI. Moreover, three out of four patients with diabetes carried the DQB1*0602 allele, which is otherwise extremely rare in type 1 diabetes. Although many of these patients are relatives, this indicates that the known risk stratification for HLA is overridden by the effect of the *AIRE* mutations, in contrast to previous findings (29).

In conclusion, the increasing knowledge about clinical variation, *AIRE*, and autoimmunity seems to expand and redefine APS1. However, the diagnosis should be considered in all patients presenting one of the major clinical manifestations, especially when it first presents in childhood. Nonendocrine components such as enamel hypoplasia and CMC are common and should trigger further diagnostic workup. Autoantibodies against IFN-ω are usually present, but their absence does not exclude the diagnosis. The *AIRE* gene should be sequenced if clinical suspicion is high. When a patient is diagnosed, all siblings should be investigated because late onset is common. We recommend regular surveillance in a specialized center because it can reduce morbidity and mortality.

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II

frontiers in Endocrinology

CASE REPORT
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Oral Tongue Malignancies in Autoimmune Polyendocrine Syndrome Type 1

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Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) or Autoimmune polyendocrine syndrome type-1 (APS-1) (APECED, OMIM 240300) is a rare, childhood onset, monogenic disease caused by mutations in the *Autoimmune Regulator* (*AIRE*) gene. The overall mortality is increased compared to the general population and a major cause of death includes malignant diseases, especially oral and esophageal cancers. We here present a case series of four APS-1 patients with oral tongue cancers, an entity not described in detail previously. Scrutiny of history and clinical phenotypes indicate that chronic mucocutaneous candidiasis and smoking are significant risk factors. Preventive measures and early diagnosis are important to successfully manage this potentially fatal disease.

Keywords: Autoimmune polyendocrine syndrome type 1, oral malignancies, chronic mucocutaneous candidiasis, endocrinology, *Autoimmune Regulator* gene

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INTRODUCTION

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) or Autoimmune polyendocrine syndrome type-1 (APS-1) (APECED, OMIM 240300) is a rare, childhood onset, monogenic disease caused by mutations in the *Autoimmune Regulator* (*AIRE*) gene. It is clinically defined by the presence of two of the three main components: hypoparathyroidism (HP), primary adrenocortical insufficiency (PAI), and chronic mucocutaneous candidiasis (CMC) (1, 2), but several less known organ-specific manifestations are also part of the syndrome making the clinical phenotype highly variable (2–4). The overall mortality is increased (5, 6) due to different complications such as acute adrenal crisis (3, 5), severe pneumonitis with respiratory failure (7–9), fulminant autoimmune hepatitis (4), and interstitial nephritis causing renal failure (10, 11).

Malignancies are not uncommon in APS-1, and squamous cell carcinoma (SCC) of the oral or esophageal mucosa is the most common entity (3, 5). In a case series by Rautemaa et al., most patients were in their thirties and had metastatic disease at diagnosis (12). In one of the cases the carcinoma affected the tongue, and CMC and smoking were associated with the malignancies (12).

Oral malignancies typically develop in middle-aged or older individuals, often in the fifth or sixth decade of life, and there is a well-established association between both smoking and heavy drinking, and SCC (13–15). Other disorders affecting the oral and gastrointestinal mucosa, such as infections and atrophic gastritis, may also contribute to the development of malignancies (16, 17). Notably, oral leukoplakia has the potential of malignant transformation (18). Oral tongue SCC is the most common type of oral malignancies and its diagnosis is based on clinical examination combined with proper imaging using computer tomography (CT) or magnetic resonance imaging (MRI) together with histology of a tissue biopsy. The primary treatment approach for oral malignancies is wide surgical resection with clean margins, as marginal infiltration is associated with risk of recurrence and impaired survival (19, 20). Postoperative treatment typically consists of radiotherapy or chemo radiotherapy depending on the disease stage and surgical outcome.

Recently, we have come across several APS-1 patients diagnosed with oral tongue cancers, which seems to be a distinct entity associated with APS-1. Here we highlight their clinical presentation, diagnosis, treatment, and follow up. We also briefly discuss the biological aspects of oral malignancies in the context of APS-1.

BACKGROUND

Basic APS-1 characteristics of the patients including *AIRE*-mutations are summarized in **Table 1**. In the following text, we briefly describe each patient focusing on the onset, diagnosis, and treatment of their tongue SCC.

Patient #1

This Finnish female patient (born 1967) was diagnosed with HP at the age of 18 months. She presented with CMC in the mouth and esophagus from the age of three years; regular antifungal medication had not been used. The APS-1 diagnosis was established in early childhood by *AIRE* sequencing. She has smoked regularly from the age of 14 years (currently 1–4 cigarettes a day), but only consumed 4–5 units of alcohol per year.

At the age of 37 years endoscopic esophagus dilation was performed because of stenosis. At the age of 45 years she presented with a 1 cm ulceration on the right side of the tongue. Histology revealed locally invasive SCC of World Health Organization (WHO) Grade 1 without positive neck nodes (T1N0M0, Stage I). A radical resection was performed. No postoperative radiotherapy was given. She is now disease free after an uneventful five-years follow up.

Patient #2

This Finnish female patient (born 1965) was diagnosed with HP at the age of two years and has had oral CMC since the age of 10 years. The APS-1 diagnosis was made based on clinical manifestations and confirmed by *AIRE* sequencing. Renal transplantation was performed at the age of 24 years because a tubulointerstitial nephritis causing end-stage renal failure. She presented with particularly severe CMC infections from the age of 40 years. The yeast was fluconazole and itraconazole resistant, but amphotericin B sensitive, and she received local treatment with this medication. She has never been a regular smoker and reported current alcohol use of about four units per week.

At the age of 30 years she was diagnosed with carcinoma *in situ* of the right side of the tongue and a radical surgical resection was performed. However, a local recurrence of SCC (T1N0M0, Stage I) occurred one year after the initial treatment and a hemiglossectomy with a radial forearm free-flap reconstruction

TABLE 1 | Characterization of the APS-1 patients.

Pat. no.	Sex	DoB	Age of onset	Classic triad	Other manifestations	*AIRE* mutations	Autoantibodies
1	F	1967	1	Hypoparathyroidism (1), Chronic mucocutaneous candidiasis (3), Primary adrenal insufficiency (16)	Enamel hypoplasia (6), hypogonadism (13), Vitiligo (13), alopecia (27), vitamin B12 deficiency (28), malabsorption (30), diabetes mellitus type 1 (31), asplenism (39), autoimmune thyroiditis (47)	R257X/R257X	SCC, NALP5, INF-ω
2	F	1965	2	Hypoparathyroidism (2), Primary adrenal insufficiency (5), Chronic mucocutaneous candidiasis (10)	Enamel hypoplasia (5), alopecia (10), hypogonadism (15), tubulointerstitial nephritis (19), autoimmune thyroiditis (32)	R257X/R257X	17OH, SCC, NALP5, IL22, INF-ω
3	M	1996	11	Primary adrenal insufficiency (11), Chronic mucocutaneous candidiasis	Hepatitis(0), malabsorption(0), asplenism	c.967_979del13/ c.967_979del13	21OH, 17OH, AADC, IL22, SCC, TPH1, INF-ω
4	M	1970	3	Hypoparathyroidism (5), Primary adrenal insufficiency, Chronic mucocutaneous candidiasis	Alopecia(3), hepatitis, vitiligo, asplenism	A374G/A374G	21OH, TPO

The age at diagnosis for each disease component is written in parentheses. The age of onset denotes the age at which the first APS-1 main component appeared. Abbreviations: 21OH, 21-hydroxylase; 17OH, 17-α-hydroxylase; AADC, aromatic L-amino acid decarboxylase; DoB, date of birth; IL22, interleukin-22; INF-ω, interferon-omega; NALP5, NACHT leucine-rich-repeat protein 5; Pat no, patient number; SCC, side-chain-cleavage enzyme; TPH1, tryptophan hydroxylase 1; TPO, thyroid peroxidase.

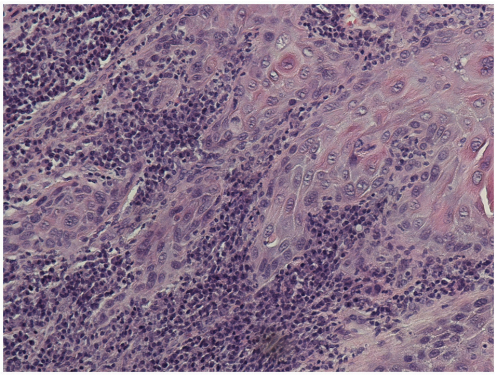

FIGURE 1 | Invasive squamous cell carcinoma in the mobile tongue of patient #2 with APS-1 (x 200 magnification).Histological picture showed a well differentiated SCC with a rich lymphocytic inflammatory infiltrate at the tumor front.

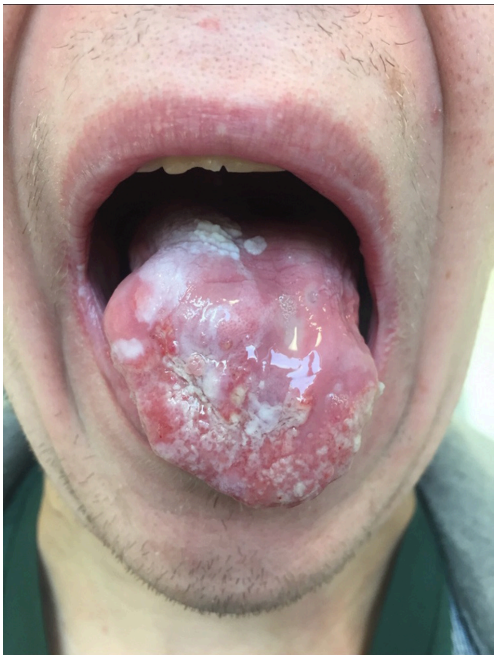

FIGURE 2 | A picture of the tongue of patient #3 at time of diagnosis. The patient presented with severe CMC, glossitis, and severe pain in the tongue. Extensive, non-homogenous changes in the form of speckled leucoplakia were observed covering the whole dorsal side of the tongue which was sensitive and indurated at palpation and functionally compromised with limited movements.

was performed (**Figure 1**). No postoperative radiotherapy was given. During follow up, several biopsies were taken revealing dysplastic changes including signs of SCC *in situ*. Seven years after the first diagnosis, microinvasive carcinoma was diagnosed in the right mandibular gingiva in the region of molar 46–47. The lesion was treated with photodynamic therapy (PDT) with 5 mm clinical margins. PDT was delivered as an alternative postoperative treatment option due to the history of recurrent multifocal SCC, assuming that repeated surgical resections and conventional radiotherapy would have caused a higher risk for further impaired oral function. During a 16-year follow up, two granulomatotic gingival lesions have been resected 13 years after the primary diagnosis; histology showed mild dysplasia, gingivitis and fungal infection.

Patient #3

This Norwegian male patient (born 1996) presented with autoimmune hepatitis and severe malabsorption during the first year of life. He also had recurrent CMC infections in early childhood. PAI was diagnosed at 11 years of age. The APS-1 diagnosis was confirmed by *AIRE* sequencing. His gastrointestinal manifestations have been treated with mycophenolate mofetil and tacrolimus with a good response. The patient has also been diagnosed with asplenism. He neither smokes nor uses alcohol.

At the age of 21 years he developed severe glossitis and pain in the tongue (**Figure 2**). A constantly elevated lymphocyte count in peripheral blood was also present. Initial biopsy revealed stromal inflammation and hyperkeratosis without signs of malignancy. However, the pain continued and, 2 months later, new biopsies showed areas with epithelial hyperplasia, hyperkeratosis (**Figure 3A**), and stromal inflammation dominated of plasma cells (**Figure 3B**), and invasive SCC with a various histologic appearance from well (**Figure 3C**) to poorly differentiated lesions (**Figure 3D**) at five different locations. The invasive tumor front showed non-cohesive cancer foci, tumor cords, and single cells, indicating an aggressively invasive lesion (**Figure 3E**). Hemiglossectomy and a reconstruction using a radial forearm free flap were performed. Moreover, investigation of the surgical specimen revealed metastasis into one lymph node (**Figure 3F**). The tumor was classified as T3N1M0, Stage III. He received postoperative cisplatin-based chemotherapy and radiotherapy because of an incomplete surgical resection and has no signs of residual disease after 7 months follow up.

Patient #4

This male patient with APS-1 (born 1970) was the son of Persian Jews who were first cousins. He presented, at the age of three years, with alopecia areata, which advanced during the next 4 years to alopecia totalis. HP was diagnosed at the age of five. During the following years additional diseases developed including PAI, vitiligo, bilateral cataract, keratitis, pernicious anemia, hepatitis, and asplenism. He had CMC since childhood and had numerous episodes of oral and oesophageal candidiasis

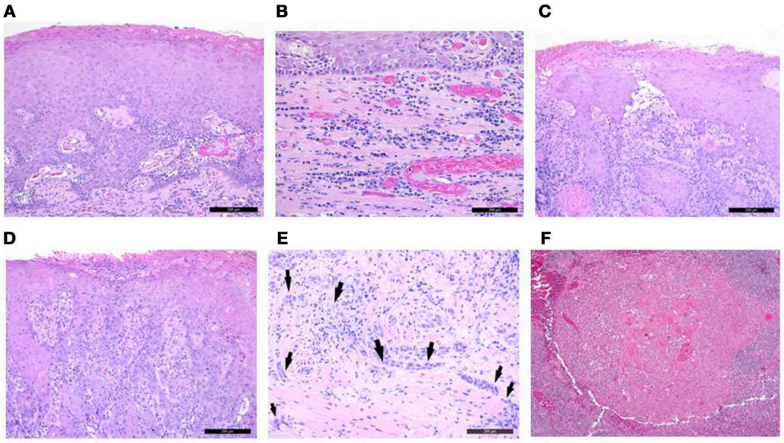

FIGURE 3 | Histological images of several biopsies taken from the tongue of patient #3. **(A)** epithelial hyperplasia with hyperkeratosis (x 100 magnification); **(B)** stromal inflammation dominated of plasma cells (x 200 magnification); **(C)** well differentiated SCC (x 100 magnification); **(D)** poorly differentiated SCC (x 100 magnification); **(E)** Non-cohesive cancer foci, tumor cords, and single cells (black arrows) observed at the invasive front indicate a highly aggressive SCC lesion (x 200 magnification). Note the lymphocytic inflammatory infiltrate toward the more central area of the tumor, but its lack at the very edge of the invasive tumor front; **(F)** Histological analysis of the lymph nodes removed at the time of hemiglossectomy revealed squamous cell carcinoma metastasis spread to one lymph node (x 100 magnification).

which was treated with nystatin, ketoconazole and fluconazole. There was no history of smoking or alcohol consumption.

At the age of 38 years, a 2 cm mass was observed on the right side of the tongue. Biopsy revealed SCC. Partial glossectomy with selective neck dissection was performed and postoperative radiotherapy to the primary site and to the neck was delivered. Unfortunately, two years later, the tongue tumor recurred. Chemotherapy was initiated, but without effect on tumor growth and the patient died a few months later due to *Staphylococcal aureus* septicaemia.

DISCUSSION

We here report four APS-1 patients with oral tongue SCC at a relatively young age treated with radical surgical resection alone or in combination with chemo-radiotherapy, or PDT in one case. All patients had severe CMC since childhood; some in combination with other well-known risk factors for oral malignancies such as smoking and alcohol use. Apart from early onset, the clinical presentation and histology of the tumors was similar to other patients with oral malignancies. Based on our findings, a prevalence of oral tongue malignancies in the entire APS-1 cohort can be estimated to about 1–2 percent. However, our case series highlights the importance of aggressive CMC treatment and regular follow up examination of the oral mucosa in APS-1 patients.

All four patients presented in childhood with CMC as part of their initial APS-1 manifestations, probably causing a longstanding chronic inflammation in the oral cavity. CMC is a common and early main manifestation of APS-1 (2, 21). It usually affects the oral mucosa as angular cheilitis or the whole mouth causing hypertrophic and/or atrophic lesions (4, 5, 21). In APS-1, the chronic inflammation of CMC changes the microenvironment of the oral cavity causing gingivitis and glossitis (3) and it is proposed that patients are almost persistently colonized with *Candida albicans* despite the relief of symptoms found in response to treatment (22). Oral mycostatin or oral amphotericin B is recommended to manage oral CMC in APS-1 to avoid the problem of drug resistance and the inhibition of steroidogenesis associated with continuous use of azole preparations (2). Studies in mice have suggested that autoreactive CD4+ T cells and chronic fungal infections cause inflammation and tissue injury, which further drive carcinogenesis (23). Although a synergistic effect of other risk factors such as smoking and alcohol use is possible, these factors were not prominent in two patients (#3; #4) and only one patient (#1) reported to smoke on a daily basis.

The third patient had severe CMC and glossitis. Initial biopsies did not reveal malignancy but stromal inflammation and hyperkeratosis. However, the clinical examination and visual findings gave a strong suspicion of malignancy and repeated biopsies showed SCC. This highlights the crucial role of the clinical examination including a visual inspection of the oral cavity which should be included in the regular surveillance of patients with APS-1. Using endoscopes with a "narrow-band imaging" modality to screen oral and pharyngeal mucosa should be considered in postoperative follow up and in high risk patients, as this tool seems to increase the detection rate of dysplastic and carcinoma lesions (24). This case also points out the importance of selecting the area for biopsy and that several biopsies might be necessary in case of extensive non-homogenous mucosal

changes, as for this patient where biopsies from different areas showed a histological appearance varying from no dysplasia to poorly differentiated carcinoma (**Figure 3**). No general histologic feature predictive for carcinogenesis could be recognized. Nevertheless, the clinical suspicion for malignancies should be high to secure proper and timely diagnosis.

All our patients described here had typical APS-1 manifestations and disease-causing *AIRE* mutations. Recent studies have investigated the potential role of *AIRE* in cancer and malignancies. For example, the expression of AIRE protein has been verified in human breast cancer cells and seems to be a strong prognostic factor for relapse-free survival (25). Moreover, in human and mice keratinocytes, *AIRE* expression is inducible in a keratin 17-dependent manner which is required for timely onset of Gli2-induced skin tumorigenesis in mice (26). However, genome-wide gene expression profiling of tongue SCCs using RNA-sequencing has not revealed *AIRE* expression (27, 28). Another recent elegant study in mice showed that *Aire*-deficiency decreased the thymic expression of phosphoribosyl-anthranilate isomerase (TRP-1), which is a self-antigen in melanocytes and a cancer antigen in melanomas (29). This leads to defective negative selection of TRP-1-specific T cells and elevated T-cell immune responses that were associated with suppression of melanoma outgrowth (29). In addition, transplantation of *Aire*-deficient thymic stroma was sufficient to confer more effective immune rejection of melanoma in otherwise *Aire* wild-type hosts (29). Taken together, *AIRE* probably has functions beyond thymic negative selection of T cells and may play a role in the development of malignancies. This also underpins the value of APS-1 as a powerful model disease for studying immunological mechanisms in both autoimmunity and malignancies.

CONCLUDING REMARKS

Oral manifestations in APS-1 should be properly investigated to reveal initial signs of oral malignancies including tongue carcinomas. This malignancy seems to be a hitherto undescribed distinct entity associated with APS-1. CMC should be aggressively treated, and the risk factors reduced, to avoid development of oral malignancies in these patients. A regular investigation of the oral cavity is recommended as part of the annual follow up.

ETHICS STATEMENT

Written informed consent has been obtained from all patients alive and from the next of kin of the deceased patient for publication of the case report and accompanying images.

AUTHOR CONTRIBUTIONS

All authors contributed in the clinical characterization of the patients and in writing and critically reviewing the manuscript. D-EC described the histologic pictures.

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III

IV

frontiers in Immunology

ORIGINAL RESEARCH
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Altered Immune Activation and IL-23 Signaling in Response to *Candida albicans* in Autoimmune Polyendocrine Syndrome Type 1

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Objective: Autoimmune polyendocrine syndrome type 1 (APS-1) is a rare, childhood onset disease caused by mutations in the *autoimmune regulator* (*AIRE*) gene. Chronic mucocutaneous candidiasis (CMC) is one of the three major disease components and is, to date, mainly explained by the presence of neutralizing auto-antibodies against cytokines [interleukin (IL)-17A, IL-17F, and IL-22] from T helper 17 cells, which are critical for the protection against fungal infections. However, patients without current auto-antibodies also present CMC and we, therefore, hypothesized that other immune mechanisms contribute to CMC in APS-1.

Methods: Whole blood was stimulated with *Candida albicans* (*C. albicans*) in a standardized assay, and immune activation was investigated by analyzing 46 secreted immune mediators. Then, peripheral blood mononuclear cells were stimulated with curdlan, a Dectin-1 agonist and IL-23 inducer, and the IL-23p19 response in monocytes was analyzed by flow cytometry.

Results: We found an altered immune response in APS-1 patients compared with healthy controls. Patients fail to increase the essential ILs, such as IL-2, IL-17A, IL-22, and IL-23, when stimulating whole blood with *C. albicans*. A significantly altered IL-23p19 response was detected in patients' monocytes upon stimulation with curdlan.

Conclusion: APS-1 patients have an altered immune response to *C. albicans* including a dysregulation of IL-23p19 production in monocytes. This probably contributes to the selective susceptibility to CMC found in the majority of patients.

Keywords: autoimmune polyendocrine syndrome type 1, chronic mucocutaneous candidiasis, monocytes, IL-17, IL-22, IL-23

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INTRODUCTION

Autoimmune polyendocrine syndrome type 1 (APS-1) or autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (OMIM 240300) is clinically defined by the presence of two of the three major disease components primary adrenal insufficiency, hypoparathyroidism (HP), and chronic mucocutaneous candidiasis (CMC) (1). However, the phenotypic expression of the

syndrome is highly variable and includes many minor disease components (2). All patients present auto-antibodies against autoantigens expressed in the affected tissue and/or against immune mediators (3–5). The *autoimmune regulator* (*AIRE)* gene is the disease-causing gene (6–8). AIRE acts as a transcription factor and is almost exclusively expressed in the thymus (9), where it orchestrates the process of negative selection of self-reactive T cells and contributes to the development of regulatory T cells (Tregs) (10, 11).

Candida albicans (*C. albicans*) is an opportunistic yeast, colonizing the skin and mucosa of most healthy individuals without causing tissue damage or disease (12). However, it may cause superficial mucocutaneous or systemic infections; often in individuals with impaired immune functions. In APS-1, CMC caused by *C. albicans* is the most common and earliest main manifestation (13) and is reported in 75–100% of patients (1, 4, 14–17). The clinical course varies from periodic to chronic and usually affects the oral mucosa as angular chelitis, or the whole mouth with hypertrophic and/or atrophic lesions (1, 2, 13). Skin, nails, and genital mucosa in females may also be affected. Susceptibility to candidiasis maps to mucosal, not systemic, disease in APS-1 (1).

Interleukin (IL)-23 is required for differentiation, function, and maintenance of T helper 17 (Th17) cells, and this signaling axis plays a central role in host defense against cutaneous candidiasis (18). In APS-1, neutralizing autoantibodies against the Th17 cytokines, IL-17A, IL-17F, and IL-22, are suggested to explain the impairment in mucosal immunity (19, 20). Noteworthy, patients without autoantibodies also present CMC (4), and therefore, it remains disputable whether the Th17 cytokine-neutralizing autoantibodies are sufficient to precipitate CMC. To gain a better understanding of the molecular mechanism of CMC in APS-1, we investigated the immune activation in response to *C. albicans* in both whole blood and monocytes of patients finding a generally altered immune activation including a dysregulation in the IL-23/Th17 pathway.

MATERIALS AND METHODS

Patients and Clinical Data

Patients ($n = 18$) were included from our National Registry of Organ Specific Autoimmune Diseases and were previously described in the Norwegian cohort (4, 21, 22). All fulfilled the diagnostic criteria of APS-1 (2). Patients received appropriate hormone replacement therapy of endocrine deficiencies at physiological doses. In HP, normal levels of calcium were maintained with oral administration of cholecalciferol and calcium. However, these treatments should not have significant immunomodulatory effects. None of the patients or healthy controls was pregnant, had acute infections, or received vaccinations at the time of sampling. An overview of the patients' data is given in **Table 1**. Samples from all patients were not available for all experiments. Healthy age and gender matched controls ($n = 31$) were recruited from the local blood bank at Haukeland University Hospital. All participants gave informed and written consent, and the study was approved by The Regional Committee for Medical and Health Research Ethics for Western Norway.

Measurement of Immune Mediators

In vitro production of immune mediators in response to *C. albicans* was characterized using the TrueCulture assay system (Myriad, RBM, USA). One milliliter of whole blood was taken from APS-1 patients ($n = 11$) and age- and sex-matched healthy controls ($n = 13$) into TrueCulture collection and culture tubes (Myriad, RBM) that contained either the supplied media (baseline response) or media supplemented with *C. albicans* (ATCC 10231; Myriad, RBM). Following a 48 h activation period at 37°C, the supernatants were frozen and sent to Myriad RBM's testing laboratory where 46 unique analytes or biomarkers of immune activation were assessed using the multiplex immunoassay Human InflammationMAP 1.0 panel (Myriad, RBM). Mediators of special interests (IL-17A, IL-17F, IL-22, and IL-23p19) were also assayed with enzyme-linked immunosorbent assay (ELISA) (R&D Systems, UK) for all patients included in this experiment ($n = 11$) and an extended group of healthy controls ($n = 19$). These ELISAs were done on supernatants from the corresponding TrueCulture tube. Standard sandwich ELISA was performed on sera from all patients ($n = 18$) searching for auto-antibodies against IL-23 (PeproTech, USA).

Isolation and Culture of Cells

Peripheral blood mononuclear cells from APS-1 patients ($n = 6$) and healthy controls ($n = 12$) were isolated from heparinized blood by Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation and stored at −150°C. Cryopreserved peripheral blood mononuclear cells (PBMCs) were used in all cell experiments. PBMCs (10^6 cells/mL) were cultured overnight in RPMI-1640 medium (Lonza) supplemented with 10% human AB serum (Sigma, USA) and 1% penicillin–streptomycin (Sigma) at 37°C with 5% CO_2. For the immune stimulation and activation of monocytes, 10 μg/mL of the Dectin-1 agonist beta-1,3-glucan (Curdlan AL, InvivoGen, USA) was added to the cell cultures. Lipopolysaccharide from *Salmonella abortus equi* (Sigma-Aldrich) in a concentration of 1 μg/mL was used as a non-*Candida* positive control for monocyte activation. The protein transport inhibitor Brefeldin A (BioLegend, USA) was added at 1 μg/mL 90–120 min after the start of incubation.

Flow Cytometry

After the incubation period, cells were washed with 2 mL buffer [phosphate-buffered saline (Sigma) containing 5% fetal bovine serum (Life technologies)] and centrifuged at 350 × *g* for 5 min. Cell surface staining of monocytes was done in 0.1 mL buffer for 30 min on ice with fluorescein isothiocyanate-conjugated antibody to human CD14 (clone HCD14, BioLegend) at a dilution of 1:20. Cells were then washed and fixed in 0.5 mL/tube Fixation Buffer (BioLegend) in the dark for 20 min at room temperature before centrifugation at 350 × *g* for 5 min and then washed again. The fixed cells were re-suspended in 0.1 mL Intracellular Staining Perm Wash Buffer (BioLegend) and stained with 10 μL phycoerythrin-conjugated antibody to human IL-23p19 (clone #727753; R&D Systems) in the dark for 20 min at room

TABLE 1 | Characterization of the autoimmune polyendocrine syndrome type 1 (APS-1) patients.

Patient number	Family number	Sex	Year of birth (YoB)	Age of onset	Classic triad	Other manifestations	Autoimmune regulator (AIRE) mutations	IFNω auto-antibodies	Other auto-antibodies
1	I	M	1995	3	CMC(3), HP(4), PAI(12)	AI(4), TIN(15), AT(16), E	c.967_979del13/c.769C>T	Positive	21OH, IL-17, IL-22, TGM4
2	I	M	1992	2	CMC(2), HP(4)	K(11), M(15), E	c.967_979del13/c.769C>T	Positive	AADC, GAD65, IL-17, IL-22, TGM4, TH
3	II	F	1958	5	CMC(5), HP(9), PAI(14)	G(18), AS(43), TIN(47), E(53)	c.967_979del13/large del	Positive	21OH, 17OH, IL-22, MAGEB2, NALP5, SCC, TH
4	II	F	2002	7	PAI(7), HP(10), CMC	E, M	c.967_979del13/ c.967_979del13	Positive	21OH, 17OH, AADC, GAD65, IL-22, MAGEB2, NALP5, SCC, TH, TPH1
5	III	M	1948	7	CMC(7), HP(9), PAI(16)	V(17), AI(21), B12(63), E	c.769C>T/c.769C>T	Positive	21OH, AADC, IL-17, IL-22 MAGEB2, SCC, SOX10, TGM4
6	IV	F	1960	9	HP(9), CMC	AI(6), G(17), AT, E, N	c.22C>T/c.290T>C	Positive	NALP5, PCA
7	V	M	1970	12	PAI(12), CMC(42)	E	c.967_979del13/ c.967_979del13	Positive	21OH, GAD65, IL-22, SCC
8	VI	F	1974	23	PAI(23), CMC(23)	E	c.879+1G>A/c.879+1G>A	Positive	21OH, 17OH, NALP5
9	VI	M	1959	43	HP(43), CMC	V(15), DM(32), E(49), AT(51)	c.879+1G>A/c.879+1G>A	Positive	21OH, 17OH, AADC, GAD65, NALP5, TH, TPH1
10	VII	M	1964	14	HP(14), CMC(22)	DM(23), K(25), N(25), V(41), AI(41), E	c.769C>T/c.1249dupC	Positive	AADC, GAD65, IL-22, PCA, PDILT, TGM4, TH, TPH1
11	VII	M	1963	nk	CMC?	E	c.769C>T/c.1249dupC	Positive	AADC, IL-22, SOX10, TGM4
12	VIII	F	1988	3	HP(3)	AT(24), E, M	c.967_979del13/ c.967_979del13	Positive	NALP5
13	IX	F	1987	2	CMC(2), HP(15)	E(24), AI, E	c.1163_1164insA/ c.1249_1950dupC	Positive	21OH, AADC, IL-17, IL-22, MAGEB2, NALP5, SOX10
14	X	F	1971	5	HP(5)	G(19), B12(35), M(39), E	c.934G>A/not found	Positive	NALP5, AADC, GAD, PCA
15	XI	F	1976	4	HP(4), C	E(14), AT(20), V(25)	c.967-979del13/c.967-979del13	Positive	21OH, 17OH, NALP5, TH, TPH, AADC, GAD, SCC, MAGEB2, SOX10, PDILT, IL-22
16	XI	M	1980	9	HP(9), PAI(12), C(16)	E	c.967-979del13/c.967-979del13	Positive	21OH, SCC, TH, AADC, GAD, NALP5, TGM4, IL-17, IL-22
17	XII	M	1958	Not known	PAI(55), HP, C	AI, AS, E	c.967-979del13/c.967-979del13	Positive	GAD, TPH, MAGEB2, IL-17, IL-22
18	XIII	F	1982	5	CMC(3)	V(15), PA(13), E, M	c.967-976del13/c.977C>T	Positive	AADC, GAD65, IL-22, PCA, PDILT, TPH1

Patient number, family, sex (M, male; F, female), YoB, clinical manifestations, AIRE mutations and auto-antibodies in APS-1 patients. The age of debut denotes the age at which the first APS-1 main component appeared. The age at diagnosis is written in parentheses.

21OH, 21-hydroxylase; 17OH, 17-α-hydroxylase; AADC, aromatic L-amino acid decarboxylase; AI, alopecia; AS, asplenism; AT, autoimmune thyroiditis; CMC, candidiasis; DM, diabetes mellitus; E, enamel hypoplasia; G, hypogonadism; GAD65, glutamic acid decarboxylase 65-kDA isoform; HP, hypoparathyroidism; IFNω, interferon-omega; IL-17, interleukin-17; IL-22, interleukin-22; K, keratoconjunctivitis; M, malabsorption; MAGEB2, melanoma antigen B2; N, nail hypotrophy; NALP5, NACHT leucine-rich-repeat protein 5; PA, pernicious anemia; PAI, primary adrenocortical insuffiency; PCA, parietal cell antigen; PDILT, protein disulfide isomerase-like testis expressed; SCC, side-cleavage enzyme; SOX10, sex-determining region Y-box 10; TGM4, transglutaminase 4; TIN, nephritis; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; V, vitiligo.

temperature. Finally, another washing step was done before the fixed and stained cells were re-suspended in 0.3 mL buffer. Cells were analyzed on a BD FACS Accuri C6 flow cytometer. Individual populations were gated according to forward scatter (FSC), side scatter (SSC), and specific markers, and the data were subsequently analyzed with FlowJo X software.

Statistical and Bioinformatical Analyses

Paired *t*-tests were used analyzing paired data, and the Mann–Whitney *U* test was used when comparing groups. The level of significance was defined to a P value less than 0.05. Statistical analyses were performed using IBM SPSS Statistics 23 or Prism 7 (Graph Pad Software, Inc., San Diego, CA, USA). Hierarchical cluster analyses were performed using the J-Express (MolMine AS, Bergen, Norway) (23), and the alterations analyzed were standardized after ratio after/before stimulation and log(2) transformed before unsupervised hierarchical clustering with Squared Euclidean distance measure with weighted average linkage was performed (23). Functional annotation and generation of the basic framework of the network displayed in Figure S1 in Supplementary Material were computed using ClueGO 2.3.3 (24) within the Cytoscape 3.4.0 suite (25). Proteins that in APS-1 patients failed to be up-regulated in response to *C. albicans* ($P > 0.05$) while found to be significantly increased in healthy controls upon *C. albicans* exposure ($P < 0.05$) were entered as group 1. Group 2 includes SERPINA1 and GC, which were found significantly down-regulated in APS-1 patients but were unchanged in healthy subjects when responding to *C. albicans* stimulation. These two groups were interrogated regarding their associations with *a priori*-defined biological terms comprised in the complete biological process gene ontology (GO) (GO:0008150; 23.02.2017). The analysis settings were (i) hits

required to map a GO term ≥1, (ii) percentage of a term covered by proteins reliably detected by the MAPs ≥4%, and (iii) GO term fusion and GO term grouping was applied based on a kappa score of 0.4 (similarity measure between the GO terms). For each group of terms, the GO term accommodating the largest number of proteins was selected as its leading term. All other settings were used as per default.

RESULTS

In the TrueCulture assay, a total of 46 single mediators were analyzed in supernatants after the activation period of 48 h. **Table 2** gives an overview of all mediators analyzed, the response to *C. albicans* stimuli in patients and healthy controls, and *P* values for paired analyses within each group.

TABLE 2 | Change of different immune mediators in response to *Candida albicans* in patients and controls.

Mediator	Autoimmune polyendocrine syndrome type 1		Controls	
	Response to *C. albicans*	*P* value	Response to *C. albicans*	*P* value
Interleukins				
IL-1A	↑	0.001	↑	0.001
IL-1B	↑	0.001	↑	<0.001
IL-2	→	**Ns (0.115)**	↑	**0.001**
IL-3	Nd	–	Nd	–
IL-4	↑	<0.001	↑	<0.001
IL-5	Nd	–	Nd	–
IL-6	↑	0.014	↑	0.002
IL-7	↑	0.001	↑	0.037
IL-10	↑	0.008	↑	0.003
IL-12p40	↑	0.020	↑	<0.001
IL-12p70	Nd	–	Nd	–
IL-15	Nd	–	Nd	–
IL-17A	→	**Ns (0.115)**	↑	**<0.001**
IL-18	↑	<0.001	↑	<0.001
IL-22[a]	→	**Ns (0.240)**	↑	**0.002**
IL-23	→	**Ns (0.342)**	↑	**0.035**
Growth factors				
BDNF	→	Ns (0.463)	→	Ns (0.203)
CSF2	↑	0.007	↑	0.002
VEGFA	↑	0.001	↑	<0.001
Chemokines				
CCL2	↑	0.002	↑	<0.001
CCL3	↑	0.001	↑	<0.001
CCL4	↑	<0.001	↑	<0.001
CCL5	→	Ns (0.514)	→	Ns (0.072)
CCL11	↓	0.014	↓	0.004
CXCL8	↑	<0.001	↑	<0.001
Immunomodulatory cytokines				
INFg	↑	0.003	↑	0.014
LTA	↑	<0.001	↑	<0.001
TNF	↑	0.002	↑	<0.001
Adhesion molecules				
ICAM1	→	Ns (0.582)	→	Ns (0.377)
VCAM1	→	Ns (0.401)	→	Ns (0.546)
Matrix metalloproteases				
MMP3	→	Ns (0.740)	→	Ns (0.247)
MMP9	↑	0.003	↑	0.004
Coagulation factors				
C3	→	**Ns (0.661)**	↑	**0.030**
F7	→	Ns (0.060)	→	Ns (0.101)
FGA	→	**Ns (0.450)**	↑	**0.011**
vWF	→	Ns (0.807)	→	Ns (0.464)
Serum proteins				
B2M	→	**Ns (0.637)**	↑	**0.024**
FTL	↑	0.002	↑	<0.001
HP	→	Ns (0.562)	→	Ns (0.967)
Traditional markers				
CRP	→	0.124	→	0.725
Others				
GC	↓	**0.037**	→	**Ns (0.974)**
SERPINA1	↓	**0.028**	→	**Ns (0.683)**
IL1RN	↑	<0.001	↑	<0.001
KITL	↑	0.002	↑	<0.001
TIMP1	↑	0.017	↑	<0.001
TNFRSF1B	↑	<0.001	↑	<0.001

The table gives the mediators analyzed in the TrueCulture system and the response to C. albicans in both patients and healthy controls. T-test for paired data was used for statistical comparison. ↑ indicates a significant up-regulation, ↓ indicates a significant down-regulation, and → indicates no significant change in the mediator concentration. Mediators with a significant different response in patients and controls are marked bold. Nd, not detectable; Ns, not significant.

[a]*IL-22 was not included in the TrueCulture system but analyzed with enzyme-linked immunosorbent assay.*

Most Chemokines, Interleukins, and Immune Regulatory Cytokines Increase in Response to *C. albicans* in Both Patients and Healthy Controls

Both APS-1 patients and healthy controls had a significant increase in levels of several mediators in response to *C. albicans*. A significant up-regulation in APS-1 and controls was found in the analyzed γ-chemokines CCL2, CCL3, and CCL4, the α-chemokine CXCL8, and the interleukins IL-1β, IL-4, IL-6, IL-7, IL-10, IL-12p40, and IL-18 (**Table 2**). Similarly, IL-1RN, the three immune regulatory cytokines LTA, TNF, and IFNγ and the growth factors CSF2 and VEGF were found to be significantly increased in both groups. Notably, when comparing the responses in the two groups, significant differences were found for 16 mediators (Table S1 in Supplementary Material; **Figure 1**). CCL11 was the only mediator analyzed, which significantly decreased in response to *C. albicans* in both groups. Finally, a total of 11 mediators analyzed remained unchanged in both groups after stimulation of whole blood by *C. albicans* (**Table 2**).

APS-1 Patients Have an Altered Th17 Cytokine Response to *C. albicans* Compared to Healthy Controls

A subset of related mediators failed to increase in response to *C. albicans* in APS-1 patients compared to healthy controls. Interestingly, patients fail to increase the essential interleukins

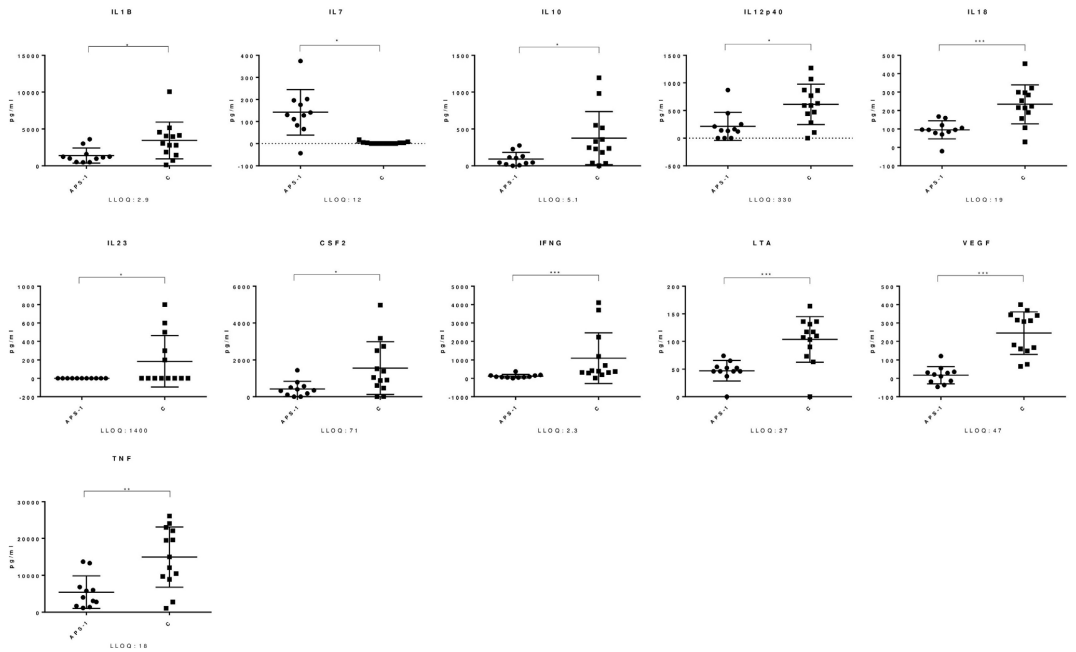

FIGURE 1 | Mediators with significant different response in Autoimmune polyendocrine syndrome type 1 (APS-1) and controls. The figure shows selected mediators from the TrueCulture assay and significant differences were found comparing APS-1 and controls (C). The lines indicate the mean with standard deviation. Concentrations are given at the *y*-axis. LLOQ, lower limit of quantitation. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

IL-17A, IL-22, and IL-23 (**Table 2**). IL-22 was not included in the TrueCulture assay and, therefore, analyzed with standard ELISA on the TrueCulture supernatants, and IL-17A and IL-23 were analyzed in both assays with comparable results. In controls, the strength of IL-22 correlated with IL-17A (correlation 0.869, $P = 0.001$) and IL-23 correlated with IL-17F (correlation 0.612, $P = 0.026$). These correlations could not be observed in patients. No APS-1 patients or controls had auto-antibodies against IL-23 assayed by sandwich ELISA. The other mediators revealing a different response in patients were β2-microglobulin (B2M), C3, and FGA, whereas GC and SERPINA1 remained unchanged in healthy controls and decreased significantly in APS-1 patients. All mediators with a significant different response comparing patients and controls are highlighted in **Table 2**.

Unsupervised Hierarchical Clustering Based on Changes in Immune Mediators and Functional Annotation of the Protein Profile Characterizing the Altered Immune Response in APS-1 Patients

We used the relative values of the measured levels of all mediators analyzed in the TrueCulture assay in an unsupervised hierarchical clustering analysis (**Figure 2**). Patients and controls were divided into two main clusters/subsets, and most patients were included in the left cluster (7 of 11) and most controls in the right cluster (9 of 13). However, no significant difference was found when comparing expected and observed frequencies using the Chi-square test ($P = 0.107$). Figure S1 in Supplementary Material shows a protein:gene ontology-(GO) term network, which identified four clusters of biological processes, each with its leading term, that most efficiently interconnect the nine proteins showing significantly different responses to *C. albicans* among patients and controls. Any regulatory relationships between the proteins, as per CluePedia v10.0, are displayed as well.

Monocytes from APS-1 Patients Have an Impaired IL-23p19 Response When Stimulated with Curdlan

We found comparable numbers of monocytes in unstimulated PBMCs from patients and controls (**Table 3**), and no significant difference in frequencies of IL-23p19^{+} cells was found comparing unstimulated cultures. Although not significant, patients seem to have a greater variability in both numbers of monocytes when gating on FSC/SSC and CD14^{+} monocytes (**Table 3**; Figure S2 in Supplementary Material). However, when comparing

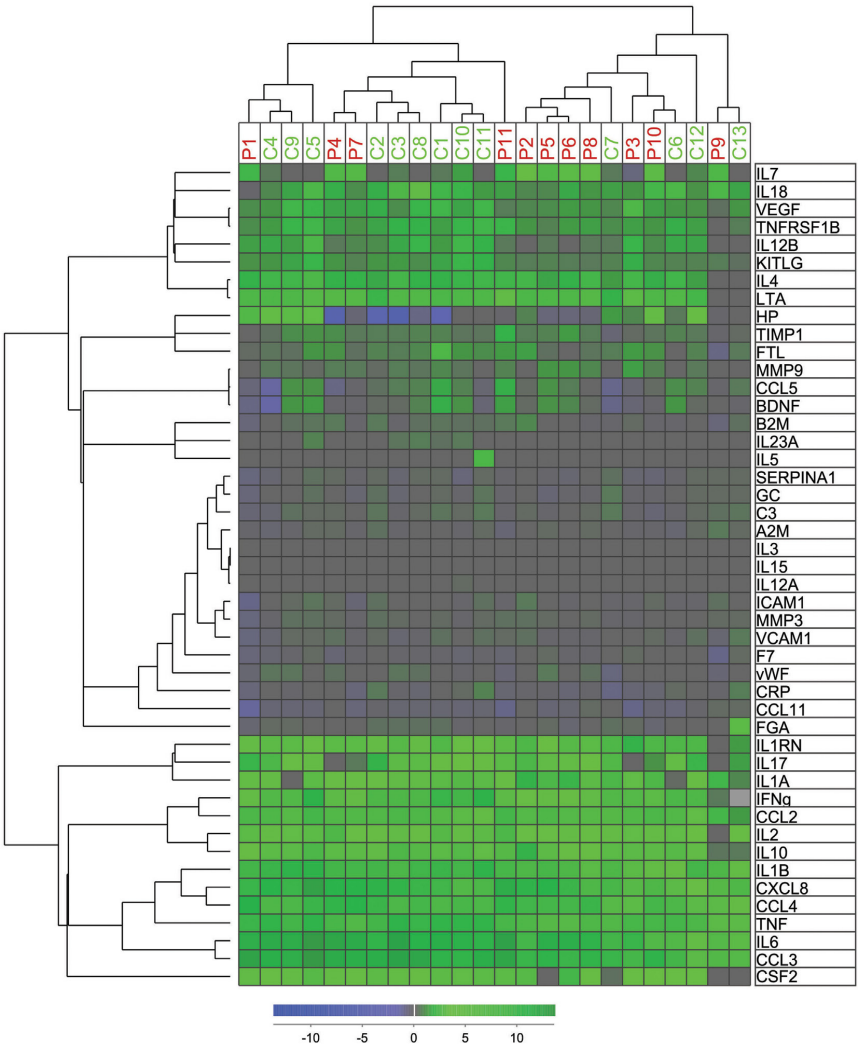

FIGURE 2 | Hierarchical cluster analyses of mediator alteration in control and patients. Unsupervised hierarchical cluster analyses and distance matrix analyses for 11 patients (red P1–P11) and 13 controls (green C1–C13) were performed. Concentrations of mediators were measured before and after intervention as previously described. The alterations were standardized after ratio after/before intervention and log(2) transformed before unsupervised hierarchical clustering with Squared Euclidean distance measure with weighted average linkage was performed, resulting in a heat map for visualization and interpretation. The mediator alteration profiles identified to main patients/control clusters and two main cytokine clusters.

unstimulated and stimulated monocytes, the levels of IL-23p19 were significantly increasing in controls, whereas this was not found in APS-1 (**Figures 3A,B**; Figure S2 in Supplementary Material). Moreover, healthy controls had a significant greater increase in total IL-23p19^{+} monocytes upon stimulation with curdlan (**Figure 3C**).

DISCUSSION

Chronic mucocutaneous candidiasis as a major clinical disease manifestation in APS-1 suggests immune defects in mechanisms crucial in fungal defense. The aims of the present study were to characterize APS-1 patients' immune activation in response to

C. albicans and our findings clearly state that APS-1 patients have an altered immune activation compared to healthy controls. IL-23p19, which is required for the formation of Th17 cells, was found dysregulated both in whole blood and monocytes upon stimulation with *C. albicans* indicating its contribution to CMC in patients.

The overall differences detected in the immune mediators in APS-1 patients have relatively broad implications on the quality of the immune response against *C. albicans* (Figure S1 in Supplementary Material). Importantly, with regard to immune responses against *Candida*, biological processes involving positive regulation of IL-17- and IL-23 production, as well as positive regulation of lymphocyte mediated immunity, were clearly impaired in patients. The significance of the other enriched biological processes impaired in patients, positive regulation of T-cell-mediated cytotoxicity, and immunoglobulin-mediated immune responses, respectively, is less obvious. However, a severely impaired general $CD8^+$ T-cell homeostasis has been previously reported in APS-1 patients (26). The importance of humoral immunity against *Candida* is suggested to be relatively modest compared to cellular defense mechanisms (27). Still, the most important mediator in the biological process involving immunoglobulin mediated immune responses was the complement factor C3, which APS-1 patients failed to up-regulate in response to *C. albicans*. C3 plays several important roles during the early innate responses against *Candida*, through opsonization and subsequently recognition and phagocytosis by neutrophils and monocytes (28). Moreover, comparing the increase in immune mediators in response to *C. albicans*, significant differences between patients and controls were found for several mediators (IL-1β, IL-10, IL-12p40, IL-18, and IL-23) involved in the crosstalk between innate immune cells and subsets of T cells. This indicates that several cellular mechanisms may be involved in altering the immune response in patients. NOD-like receptors are cytosolic proteins that are implicated in sensing fungi and, once activated, produce IL-1β and IL-18 through the formation of inflammasomes (29), both of which were less up-regulated in patients compared to controls in response to *Candida* stimulation. IL-1β is crucial for the differentiation of Th17 cells and for the activation and recruitment of neutrophil granulocytes, while IL-18 is important for the induction of IFNγ producing T helper 1 (Th1) cells. IL-10 is produced by almost all immune cells and its major role is to limit the extent of immune activation and retain homeostasis (30). IL-12 is a pro-inflammatory molecule primarily produced by professional antigen-presenting cells (31) and it activates natural killer cells and induces the differentiation of naïve CD4+ T lymphocytes to become Th1 cells (31). Notably, the IL-12p40 chain of IL-12 can also form a dimer with p19 giving rise to IL-23 (32), which is required for Th17 differentiation, function and maintenance. Patients failed to increase IL-17A, IL-22, and IL-23, which are found critical for optimal host defense against cutaneous candidiasis (18) and fungal infections (19, 20). Previous studies have reported conflicting results about IL-17 production in APS-1 (20, 33),

TABLE 3 | Monocyte counts in autoimmune polyendocrine syndrome type 1 (APS-1) and controls.

Cells	APS-1	Controls	*P* value
Monocytes [forward scatter (FSC)/side scatter (SCC)]	8.810 (1.98–16.6)	11.70 (8.32–16.00)	Ns (0.0978)
$CD14^+$ monocytes	64.9 (29.0–86.90)	79.45 (67.10–87.80)	Ns (0.3355)
Interleukin (IL)-$23p19^+$ monocytes – 0	0.33 (0.00–1.69)	1.00 (0.078–1.73)	Ns (0.4796)
IL-$23p19^+$ monocytes – curdlan	0.75 (0.00–2.32)	1.655 (0.28–3.33)	Ns (0.0831)

The table gives a comparison of percent monocytes in APS-1 and controls both when gated on FSC/SSC and the number of $CD14^+$ monocytes in the current monocyte gate. Numbers are given as median (and range). The Mann–Whitney U test was used for the statistical comparison.
0, baseline/unstimulated. Ns, not significant.

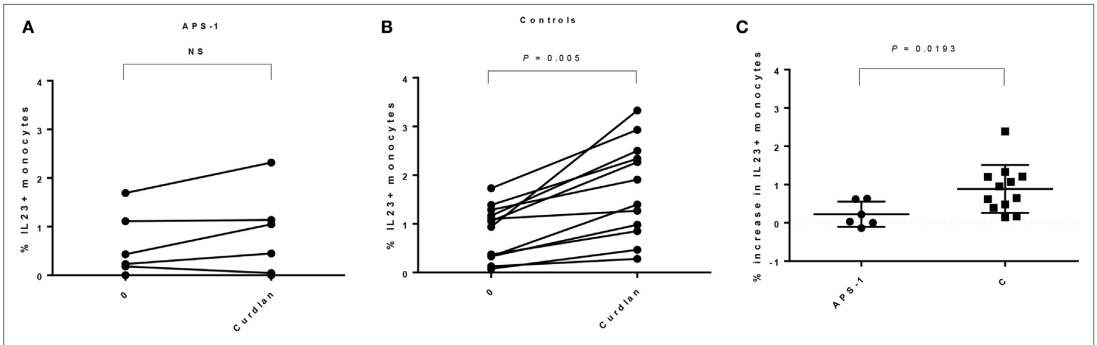

FIGURE 3 | Interleukin (IL)-23p19 responses found in monocytes in autoimmune polyendocrine syndrome type 1 (APS-1) patients and controls. The figure gives an overview of the IL-23p19 response found in monocytes in patients versus controls. **(A)** APS-1 patients; no significant increase in IL-23p19 was detected in monocytes. Numbers at the *Y*-axis gives the percentages of IL-$23p19^+$ monocytes after stimulation with curdlan. **(B)** Controls; a significant increase in IL-23p19 was detected in monocytes. The numbers at the *Y*-axis give the percentages of IL-$23p19^+$ monocytes after stimulation with curdlan. **(C)** Comparing the increase in IL-$23p19^+$ monocytes found in APS-1 and controls at baseline and after curdlan stimulation. NS, not significant.

but the finding of reduced IL-22 producing cells in APS-1 seems more consistent (20, 33, 34). Finally, we also confirm a dysregulation of IL-7, which is previously described in a Finnish APS-1 cohort (26). Although a more comprehensive study of the system biology would generate an even better overview of the immune activation and biological pathways involved in the response to *Candida* in APS-1, our findings indicate an altered immune activation in APS-1 patients, which includes several immune mediators that play important roles in immune homeostasis and particularly in the host defense against fungi (Figure S1 in Supplementary Material).

Interleukin-23 and Th17 cytokines have been implicated in the pathogenesis of many autoimmune diseases (35–37), and we therefore found the total lack of IL-23 in APS-1 patients particularly interesting. IL-23 is secreted by activated monocytes and dendritic cells (38) and induces the differentiation of naïve T cells into Th17 cells and can promote the further expansion and maintenance of Th17 cells, and the production of Th17 cytokines via IL-23 receptor and STAT3 (38). In the literature, there is already conflicting information about IL-23 signaling in APS-1. Ryan and collaborators described monocyte-derived DCs from APS-1 patients to over-produce IL-2, INFγ, TNF-α, and IL-13 and demonstrated both impairment in maturation and hyper activation in response to *C. albicans* (39). However, the IL-23 response was comparable to controls (39). Another study assessed Th17 responses of PBMCs to *Candida* and non-*Candida* species stimuli finding that PBMCs from APS-1 patients had a normal or increased IL-17 production and Th17 cell proliferation, although only in the absence of their own plasma, which had an inhibitory effect on both IL-17 production and Th17 cell proliferation (34). This study reported normal IL-6 and IL-23 responses in APS-1 patients. Furthermore, expression levels of all pattern recognition receptors (PRRs) involved in anti-candida responses, levels of plasmacytoid and myeloid DCs, and monocyte toll-like receptor (TLR)-2/TLR-6 expression are described similar in APS-1 and controls (40). Finally, we have previously reported reduced numbers of $CCR6^+CXCR3^+$ T helper cells, $CD16^+$ monocytes, and Tregs in patients with APS-1 (41). These previous studies have investigated IL-23 from DCs and PBMCs in APS-1 (34, 39, 40), but less information about monocytes exists. We report comparable numbers of monocytes in both patients and controls and similar levels of IL-23p19 in unstimulated monocytes. Interestingly, monocytes from patients fail to increase IL-23p19 production both when comparing the steady-state levels and stimulated levels within each group and when comparing the total increase between the groups. The reason for the discrepancy in IL-23 production in the studies mention above and our current findings could be due to differences in cell types studied (PBMCs, DCs, monocytes, and whole blood), or that PRRs activate differently to particular *Candida* strains. These important aspects need to be further investigated.

Furthermore, there are conflicting information about the clinical relevance of monocyte-specific IL-23 signaling and CMC. Patients with autosomal recessive IL-12RB1 or IL-12p40 deficiency suffer from CMC and, therefore, indicate that impairment of IL-23 signaling can be the molecular pathogenesis of CMC (42, 43). On the other hand, CMC is not well described in patients with GATA2 deficiency, which severely impairs monocytes (44). Our findings suggest a monocyte-specific IL-23 deficiency in APS-1 patients, which resonates well with a previously published study reporting an extrathymic role of AIRE in monocytes (45). Specifically, that study described how AIRE interacts with CARD9, SYK, and Dectin-1 in healthy monocytes and thus plays a major role in activation of the Dectin-1 pathway by stimulation with curdlan. Consequently, PBMCs from AIRE-deficient APS-1 patients produced significantly less TNF in response to curdlan stimulation compared to healthy controls. Noteworthy, the expression and biological role of the AIRE protein in peripheral blood cells are controversial (46, 47), and the interpretation of our data in this context should be approached with caution until the expression of AIRE in monocytes has been further verified. Based on the above, a general impairment in immune activation and an altered monocyte response probably contribute to CMC found in APS-1 and is likely to involve the Dectin-1 pathway.

Auto-antibodies play a key role in CMC in APS-1, and auto-antibodies against IL-6, IL-17A, IL-17F, IL-22, and IFNω are previously described (19, 20, 48). We searched for auto-antibodies against IL-23 in all patients without any positive findings, confirming a previous report (48). All patients included in our study present auto-antibodies against INFω, and about 60% have auto-antibodies against IL-22. To speculate, these auto-antibodies may influence the difference in immune response found in whole blood by interacting in autocrine and paracrine signaling loops and thereby disturb cellular responses. These speculations are supported by the finding that APS-1 patients failed to up-regulate the interferon regulated serum inflammation marker B2M after *C. albicans* stimulation. The inhibition of interferon regulated genes by APS-1 patient auto-antibodies is well established (49), and the importance of type I interferons in immunity against *Candida* was recently demonstrated (50).

Our study has some limitations. First, the rarity of APS-1 makes biological samples limited. We therefore had to strictly prioritize our samples and chose to focus on stimulation assays in the search of differences in the protein expression using flow cytometry, rather than for example detecting RNA transcripts using quantitative polymerase chain reactions. Our cell assays were also more robust compared to pilot experiments of culturing PBMCs or whole blood with *C. albicans*, and analyzing supernatants for signaling molecules using ELISA. Second, in our cell assay, few monocytes produced IL-23p19 when stimulated with curdlan and some cells were border line positive for IL-23p19 after stimulation. However, we carefully optimized our assay regarding incubation time, concentration of Dectin, and Brefeldin A stimulation. Moreover, samples from two patients and two controls were always stimulated and analyzed together, we used a consistent gating strategy when analyzing data, and our findings regarding IL-23 were comparable in both the cell assay and the TrueCulture system. In general, whole blood assays probably mirror the *in vivo* conditions of inflammation more precisely than PBMC assays regarding interplay between subsets of immune cells and mediators.

We found significantly altered IL-23p19/IL-23 signaling in both our assays making the findings reliable.

In summary, in order to gain further insights into the molecular mechanisms of CMC in APS-1, we used different approaches to investigate the immune activation in APS-1 in response to *C. albicans*. Our findings indicate that patients have a significant altered immune activation with broad implications on the quality of the immune response to *C. albicans* and that monocytes contribute to a dysregulation of the IL-23/Th17 axis, which is crucial for proper immunity to fungal infections.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of The Regional Committee for Medical and Health Research Ethics with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by The Regional Committee for Medical and Health Research Ethics.

AUTHOR CONTRIBUTIONS

ØB, EB, AW, and BO collected samples and clinical information. ØB, HR, and ND did the statistical and bioinformatical analyses. ØB, EB, and AH executed the cell experiments. All authors contributed in writing the manuscript and have approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01074/full#supplementary-material.

FIGURE S1 | Functional annotation of the protein profile characterizing the altered immune response to *C. albicans* in APS-1 patients versus healthy individuals. This protein:gene-ontology-(GO) term network identified four clusters of biological processes, each with its leading term, that most efficiently interconnect the nine proteins here relevant. Any regulatory relationships between the proteins, as per CluePedia v10.0 are displayed as well.

FIGURE S2 | Gating strategy for flow cytometric analyses.

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ORIGINAL ARTICLE

Oral microbiota in autoimmune polyendocrine syndrome type 1

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ABSTRACT

Background: Autoimmune polyendocrine syndrome type-1 (APS-1) is a rare, childhood onset disease caused by mutations in the *Autoimmune Regulator* gene. The phenotypic expression is highly variable and includes disease manifestations in the oral cavity, including mucocutaneous candidiasis. Increasing evidence suggests a potential role of the skin, oral and gut microbiotas in the pathogenesis of autoimmunity. To date, no information exists regarding the oral microbiota in APS-1.

Objective: To assess the bacterial microbiota of whole saliva in APS-1 patients by using high throughput sequencing.

Design: Whole unstimulated saliva was collected from 10 APS-1 patients and 17 healthy controls and examined by high throughput sequencing of the hypervariable region V1-V2 of 16S rRNA using the 454 GS Junior system. Metastats (http://cbcb.umd.edu/software/metastats) was used to analyse the pyrosequencing reads.

Results: A reduction in the total number of bacterial genera and species was detected in APS-1 compared to healthy controls. The proportion of the major phyla Firmicutes was higher (60% vs 41%, $p = 0.002$) and Bacteroidetes lower (15% vs 28%, $p = 0.007$) in APS-1 compared to healthy controls. On the genus level, *Streptococcus* and *Gemella* were prevalent in APS-1.

Conclusion: Our findings indicate a significantly altered oral microbiota in APS-1.

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Autoimmune polyendocrine syndrome type-1 (APS-1) or autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (OMIM 240300) is a rare, monogenic, childhood onset disorder which is clinically defined by the presence of two of the three major disease components: primary adrenal insufficiency, hypoparathyroidism, and chronic mucocutaneous candidiasis (CMC) [1]. However, the clinical picture is highly variable and includes many minor disease components [2]. The *Autoimmune Regulator* (*AIRE*) gene is the disease-causing gene [3,4]. *AIRE* acts as a transcriptional regulator and is almost exclusively expressed in the thymus [5] where it orchestrates the process of negative selection of self-reactive T cells and contributes to the development of regulatory T cells (Tregs) [6,7]. All patients present autoantibodies against autoantigens expressed in the affected tissue [8] and/or against immune mediators such as interferon-omega (ω) and interleukin (IL)-22 [9,10]. Interestingly, circulating autoantibodies and *AIRE*-mutations can be found before development of clinical APS-1 [11,12] making the role of environmental triggers particularly relevant in the pathogenesis and phenotypic expression.

Increasing evidence indicates that the environment shapes the human immune system and accounts for its heterogeneity among individuals [13]. The oral, gut, and skin microbiotas could play a key role in the pathogenesis of systemic and organ-specific autoimmune diseases [14]. Most APS-1 patients develop disease components affecting the oral cavity; enamel hypoplasia and CMC are both common manifestations [9,15]. Also a Sjögren's-like syndrome without extractable nuclear antigen autoantibodies has recently been described [15,16]. These oral manifestations probably interfere with the homeostasis of the oral microbiota. Furthermore, autoimmunity against defensins and other antimicrobial substances as observed in APS-1 could change the microbiota [17]. Reduced salivary flow rate changes the oral microbiota [18] and in a study of patients with severe Sjögren's syndrome *Streptococcus salivarius, Neisseria pharyngis, Veillonella* species, and *Micrococcus mucilaginosus* were reduced and the number of *Staphylococcus aureus* and *Candida* species were increased compared to

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Supplemental data for this article can be accessed here.

healthy controls [19]. Another recent study of primary Sjögren's syndrome patients with normal salivary flow rate found that the number of bacterial genera and species was lower in patients, and concluded that saliva dysbiosis is a key characteristic of primary Sjögren's syndrome [20]. Moreover, changes in the oral microbiota are found to be associated with several other diseases including squamous cell carcinoma, atherosclerosis, bacteraemia, and rheumatoid arthritis [21].

In APS-1, no information exists regarding the oral microbiota and only a few studies of the gut microbiota have been reported [17,22,23]. In this study, we characterized the bacterial profile in whole unstimulated saliva of patients by high throughput sequencing, a technique which recovers both cultivated and not-yet-cultivated bacteria, thus giving an in-depth overview of bacteria present.

Materials and methods

Patients and clinical data

A total of 10 APS-1 patients from 6 different families were included. All patients fulfilled the diagnostic criteria of APS-1. They were previously described in the Norwegian cohort [9,24,25] and included in our National Registry of Autoimmune Diseases. Three patients were excluded after an initial quality control. A detailed characterization of the seven remaining patients (five females and two males) is given in Table 1. The mean age was 32.3 years (range 10–64). All participants had their disease onset before the age of 8 years. Three patients presented the three major disease components and the mean number of disease components was five (range 4–7). Enamel hypoplasia was found in all patients. CMC was previously diagnosed in five patients and three had oral candidiasis at the time of sampling (Table 1). Disease-causing *AIRE*-mutations and autoantibodies against interferon-ω were present in all. All participants gave informed and written consent and the study was approved by The Regional Committee for Medical and Health Research Ethics for Western Norway.

Saliva sampling and sample processing

Whole unstimulated saliva was collected from patients ($n = 10$) and healthy controls ($n = 17$) with the same age and sex distributions as patients. Saliva samples were collected between 9 a.m. and 11 a.m., and participants were asked not to brush their teeth, eat, or drink for at least 2 h before sampling. No participants were regular smokers or had received antibiotics within the last month before sampling. Saliva pH was measured using a strip

Table 1. Characteristics of the APS-1 patients. The age at diagnosis for each disease component is written in parentheses. The age of onset denotes the age at which the first APS-1 main component appeared.

Pat. no.	Family no.	Sex	Age	Age of onset	Classic triad	Other manifestations	*AIRE*-mutations	Autoantibodies	Unstimulated saliva flow rate, saliva pH	Fungal load*	Other oral manifestations
1	I	M	26	4	HP(4), CMC (9), AI(11)	E(11)	c.967_979del13/ c.967_979del13	21OH, IL17, IL22, INF-ω, MAGEB2, PDILT, SCC, TGM4, TH, TPH1	Pathological (<0.1 ml/min) pH 5.0	Positive. Cheilitis angularis.	Caries.
2	I	F	36	4	HP(4), CMC	E(14), AT(20), V(25)	c.967_979del13/ c.967_979del13	21OH, 17OH, AADC, GAD65, IL22, INF-ω, MAGEB2, NALP5, PDILT, SCC, SOX10, TH, TPH1	Normal (>0.1 ml/min) pH nd.	Negative.	–
3	II	F	10	7	PAI(7), HP (10), CMC	E, M	c.967_979del13/ c.967_979del13	21OH, 17OH, AADC, GAD65, IL22, INF-ω, MAGEB2, NALP5, SCC, TH, TPH1	Normal (>0.1 ml/min) pH 7.0	Negative.	–
4	III	M	64	7	CMC(7), HP (9), PAI (16)	V(17), AI(21), B12(63), E	c.769C>T/ c.769C>T	21OH, AADC, IL17, IL22, INF-ω, MAGEB2, SCC, SOX10, TGM4	Normal (>0.1 ml/min) pH 5.5	Positive. Cheilitis angularis.	Gingivitis. Caries.
5	IV	F	24	3	HP(3)	AT(24), E, M	c.967_979del13/ c.967_979del13	INF-ω, NALP5	Normal (>0.1 ml/min) pH 7.0	Negative.	Gingivitis.
6	V	F	25	2	CMC(2), HP (15)	E(24), AI, E	c.1163_1164insA/ c.1249_1950dupC	21OH, AADC, IL17, IL22, INF-ω, MAGEB2, NALP5, SOX10	Normal (>0.1 ml/min) pH 6.0	Positive. Cheilitis angularis.	Mucosal lesions.
7	VI	F	41	5	HP(5)	G(19), B12(35), M(39), E	c.934G>A (dominant) mutation)	AADC, GAD65, INF-ω, NALP5, PCA	Pathological (<0.1 ml/min) pH nd.	Negative.	Caries.

21OH, 21-hydroxylase; 17OH, 17-α-hydroxylase; AADC, aromatic L-amino acid decarboxylase; AI, alopecia; AT, autoimmune thyroiditis; B12, vitamin-B12 deficiency; CMC, candidiasis; E, enamel hypoplasia; G, hypogonadism; GAD65, glutamic acid decarboxylase 65-kDA isoform; HP, hypoparathyroidism; IL17, interleukin-17; IL22, interleukin-22; INF-ω, interferon-omega; M, malabsorption; MAGEB2, melanoma antigen B2; NALP5, NACHT leucine-rich-repeat protein 5; PAI, primary adrenocortical insufficiency; PCA, parietal cell autoantibodies; PDILT, protein disulphide isomerase-like testis expressed; SCC, side-chain-cleavage enzyme; SOX10, sex determining region Y-box 10; TGM4, transglutaminase 4; TPH1, tryptophan hydroxylase 1; TH, tyrosine hydroxylase; V, vitiligo; YoB, year of birth, nd; not done.

*Standard methods were used for culturing *Candida albicans*.

test (mColorpHast™ pH Test Strips, MilliporeSigma) and standard methods were used for culturing *Candida albicans*. Unstimulated saliva flow rate (ml/min) was measured based on a collection period of 15 min (Table 1). Samples were immediately stored at −80°C until analysis.

Sample processing was done as previously described [20,26]. In summary, DNA was extracted from a 250 µl sample volume using the MasterPure™ DNA Purification kit (Epicentre, Illumina Company, Madison, WI) and was dissolved in 45 µl 1 × TE buffer. The 16S rRNA hypervariable region V1-V2 was amplified in three parallel PCRs and sequenced on a 454 GS Junior System (Roche, Branford, CT). Primer sequences and amplifications reactions are given previously [20]. DNA quality and concentrations were assessed with Bioanalyzer 2100 (Agilent, Santa Clara, CA) and Nanodrop 3300 Flurospectrometer (Thermo Scientific, Wilmington, DE), all within the range to perform high throughput sequencing.

Bioinformatics analysis of sequence reads

Bioinformatics analysis of sequence reads was executed as described previously [20]. In brief, raw sequence reads were subjected to a species-level, reference-based taxonomy assignment especially designed for studying the human oral microbial community [20]. The set of 16S rRNA reference sequences previously published by Al-Hebshi et al. [27] and the NCBI 16S rRNA reference sequence set (ftp://ftp.ncbi.nlm.nih.gov/blast/db/16SMicrobial.tar.gz) were combined giving reference sequences representing a total of 1,151 oral and 12,013 non-oral species that were BLASTN-searched for each of the sequence reads. Unassigned reads were then screened for high-quality non-chimeras and subjected to *de novo* species-level operational taxonomy unit calling for potential novel species. The quantitative insights into microbial ecology pipeline software package version 1.9.1 [28] was used for down-stream analyses, including alpha and beta diversities. A statistical method introduced in Metastats (http://cbcb.umd.edu/software/metastats) was used to reveal significant differences between the microbiota of control saliva and APS-1 saliva. This method employs a false discovery rate to improve specificity in high complexity environments, and handles sparsely sampled features using Fisher's exact test [29]. p-values ≤ 0.05 were considered significant and Bonferonni Correction for multiple testing was included.

Results

Sequence data

An overview of the sequence read counts in each analysis step is given in Supplemental Table 1.

Composition of the salivary microbiota of APS-1 patients

Five different phyla were detected in APS-1 patients with DNA sequences predominately assigned to the phyla Firmicutes (60%), Bacteroidetes (15%), Proteobacteria (10%), Fusobacteria (8%), and Actinobacteria (6%). Table 2 gives an overview of the significantly different abundances of taxa from control saliva and APS-1 saliva by phyla, genera and species, respectively. In Figure 1, a comparison of the bacterial content of saliva in APS-1 and controls based on the sequencing of the hypervariable 16S rDNA region V1-V2 is presented. Saliva from APS-1 displayed a significantly increased relative abundance of Firmicutes (p = 0.002) and a lower frequency of Bacteroidetes (p = 0.007) compared with controls (Figure 2).

A total of 64 bacterial genera were detected in APS-1 and 90 in healthy controls. Figure 3 gives the relative abundances of the 18 major genera found in APS-1 samples and controls. Moreover, a total of 18 genera showed significant differences in abundances (Table 2). Metastats analyses showed that the genera *Streptococcus* and *Gemella* were significantly higher in patients than controls (p = 0.0001 and 0.036, respectively; Figure 4) whereas *Prevotella* and *Veillonella* were significantly higher in controls (p = 0.001 and <0.001, respectively; Figure 4). Among the genera with significant difference in abundance, eight genera were absent from whole saliva of APS-1 patients (Table 2).

In APS-1 patients, the sequencing data revealed the most abundant species to be *Streptococcus* sp. str. C300, *Streptococcus* multispecies spp.24_2, *Streptococcus mitis, Streptococcus infantis*, and *Haemophilus parainfluenzae*. Among these, only *Streptococcus* sp. str. C300 and *Streptococcus* multispecies spp.24_2 were increased compared to controls (p = 0.001, 0.032, respectively). The other species found more abundant in APS-1 patients were *Fusobacterium nucleatum* subsp. *animalis, Gemella haemolysans, Ruminococcaceae* [G-3] sp. Oral taxon 366, and *Capnocytophaga ochracea* (p = 0.046, 0.047, <0.001, 0.002, respectively). The *Veillonella parvula* group and *Prevotella melaninogenica* were most abundant in controls. Table 2 shows all species with significant differences in abundance compared to control saliva and APS-1 saliva as estimated by Metastats.

Species richness and diversity

Samples from healthy controls had a higher species richness and diversity than APS-1 samples. Figure 5 (a) illustrates that the average species richness is

Table 2. Differences in abundances of taxa in APS-1 and control saliva. Significant ($p \leq 0.05$) differences in relative abundance of taxa from control saliva and APS-1 saliva as estimated by Metastats http://cbcb.umd.edu/software/metastats. Differences significant after Bonferonni Correction for multiple testing are marked with *.

Taxon	APS-1 saliva (n = 7)	Control saliva (n = 17)	Metastat p-value
Phyla			
Firmicutes	0.65118 ± 0.02551	0.41118 ± 0.02572	0.002
Bacteroidetes	0.11271 ± 0.03162	0.26009 ± 0.03845	0.007
Genera			
Streptococcus	0.53448 ± 0.08523	0.18016 ± 0.01963	0.0001*
Gemella	0.01467 ± 0.00023	0.00253 ± 0.00062	0.036
Prevotella	0.05995 ± 0.01021	0.17357 ± 0.02970	0.001
Veillonella	0.04049 ± 0.00925	0.16254 ± 0.02760	0.0001*
Neissera	0.02065 ± 0.00965	0.05508 ± 0.01269	0.029
Actinomyces	0.00148 ± 0.00055	0.00673 ± 0.00159	0.002
Megashaera	0.00000	0.00631 ± 0.00287	0.027
*Lachnospiraceae_* [G-3]	0.00059 ± 0.00033	0.00417 ± 0.00097	0.001
Lachnoanaerobaculum	0.00024 ± 0.00019	0.00123 ± 0.00033	0.008
*Eubacterium_*[XIVa][G-1]	0.00025 ± 0.00018	0.00109 ± 0.00031	0.019
*Ruminococcaceae_*[G-3]	0.00002 ± 0.00776	0.00119 ± 0.00448	0.016
Bacteroides	0.00000	0.00049 ± 0.00019	0.012
Veillonella	0.00000	0.00053 ± 0.00025	0.031
*Bacteroidetes_*[G-3]	0.00000	0.00032 ± 0.00010	0.002
*Peptostreptococcaceae_*[XI][G-4]	0.00000	0.00023 ± 0.00011	0.034
Mitsuokella	0.00000	0.00033 ± 0.00016	0.038
Desulfovibrio	0.00000	0.00405 ± 0.00040	0.018
Catonella	0.00000	0.00015 ± 0.00013	0.048
Species			
Streptococcus sp._str._C300	0.12958 ± 0.03003	0.03065 ± 0.00618	0.001
Streptococcus multispecies_spp24_2	0.11555 ± 0.05022	0.00877 ± 0.00349	0.031
Fusobacterium nucleatum_ss_animalis	0.02401 ± 0.01178	0.00083 ± 0.00038	0.046
Gemella haemolysans	0.01308 ± 0.00599	0.00132 ± 0.00047	0.047
Capnocytophaga ochracea	0.00112 ± 0.00035	0.00004 ± 0.00002	0.023
*Veillonella parvula_*group	0.03273 ± 0.00653	0.10745 ± 0.01904	0.0003*
Prevotella melaninogenica	0.02205 ± 0.00876	0.09752 ± 0.01762	0.0002*
Veillonella atypica	0.00776 ± 0.00389	0.05509 ± 0.01369	0.001
Neisseria flavescens/subflava	0.01723 ± 0.00873	0.04995 ± 0.01204	0.023
Porphyromonas gingivalis	0.00000	0.02849 ± 0.01465	0.048
Prevotella pallens	0.00059 ± 0.00036	0.02308 ± 0.00617	0.0004*
Campylobacter concisus	0.00133 ± 0.00083	0.01390 ± 0.00382	0.001
Prevotella salivae	0.00054 ± 0.00024	0.00848 ± 0.00293	0.006
*Prevotella veroralis_*nov_95.28%	0.00000	0.00723 ± 0.00287	0.010
Solobacterium moorei	0.00251 ± 0.00121	0.00638 ± 0.00154	0.045
Megasphare micronuciformis	0.00000	0.00631 ± 0.00287	0.023
Atopobium parvulum	0.00099 ± 0.00026	0.00579 ± 0.00223	0.028
*Lachnospiraceae_*[G-3] sp._oral_taxon_100_nov_83.29%	0.00049 ± 0.00032	0.00342 ± 0.00082	0.001
Actinomyces sp._Oral_Taxon_180	0.00043 ± 0.00022	0.00328 ± 0.00103	0.006
Actinomyces odontolyticus	0.00047 ± 0.00015	0.00247 ± 0.00062	0.001
Prevotella multispecies_sppn1_2_nov_82.34%	0.00027 ± 0.00019	0.00158 ± 0.00579	0.026
Lachnoanaerobaculum orale	0.00024 ± 0.00019	0.00123 ± 0.00332	0.008
*Eubacterium_*[XIVa][G-1] *saburreum*	0.00025 ± 0.00018	0.00109 ± 0.00313	0.016
*Tannerella forsythia_*nov_96.52%	0.00000	0.00095 ± 0.00311	0.002
Aggregatibacter aphrophilus	0.00000	0.00080 ± 0.00035	0.018
*Porphyromonas gingivalis_*nov_96.46%	0.00000	0.00079 ± 0.00037	0.026
Leptotrichia sp._oral_taxon_219_nov_83.33%	0.00021 ± 0.00019	0.00077 ± 0.00020	0.039
Fretibacterium fastidiosum	0.00000	0.00074 ± 0.00033	0.019
*Ruminococcaceae_*[G-3] sp._oral_taxon_366_nov_78.83%	0.00002 ± 0.00002	0.00054 ± 0.00015	0.001
Veillonella sp._oral_taxon_780_nov_82.14%	0.00000	0.00053 ± 0.00025	0.029
Mogibacterium neglectum	0.00010 ± 0.00008	0.00051 ± 0.00190	0.044
*Haemophilus parainfluenzae_*nov_96.89%	0.00000	0.00043 ± 0.00026	0.018
Desulfovibrio sp._oral_taxon_040	0.00000	0.00040 ± 0.00040	0.018
Mitsuokella sp._Oral_Taxon_H31_nov_78.38%	0.00000	0.00033 ± 0.00016	0.035
*Streptococcus constellatus_*nov_80.22%	0.00000	0.00032 ± 0.00013	0.008
*Porphyromonas gingivalis_*nov_91.05%	0.00004 ± 0.00004	0.00032 ± 0.00011	0.019
*Bacteroidetes_*[G-3] sp._oral_taxon_280_nov_92.66%	0.00000	0.00031 ± 0.00010	0.015
*Alloprevotella rava_*nov_86.47%	0.00000	0.00029 ± 0.00009	0.002
Fretibacterium sp._oral_taxon_359	0.00000	0.00025 ± 0.00016	0.029
Prevotella sp._oral_taxon_515_nov_80.56%	0.00000	0.00024 ± 0.00013	0.018
Prevotella sp._oral_taxon_515_nov_78.25%	0.00000	0.00023 ± 0.00016	0.029
*Peptostreptococcaceae_*[XI][G-4] sp._oral_taxon_369	0.00000	0.00022 ± 0.00011	0.031
Catonella sp._oral_taxon_164_nov_95.73%	0.00000	0.00015 ± 0.00014	0.048

higher in healthy subjects based on the observed species rarefaction curves Figure 5(b,c) gives the estimated species richness evaluated on the Chao1 matrix and the rarefaction curves based on the Shannon index, respectively showing an overall difference in alpha diversity, although the latter shows no difference between health and APS-1. However, the Shannon index measures both richness and abundance, hence the evenness of the community. Figure 5(c) therefore indicates that there is no significant difference between APS-1 and controls in terms of species evenness. Finally, Figure 5(d) gives

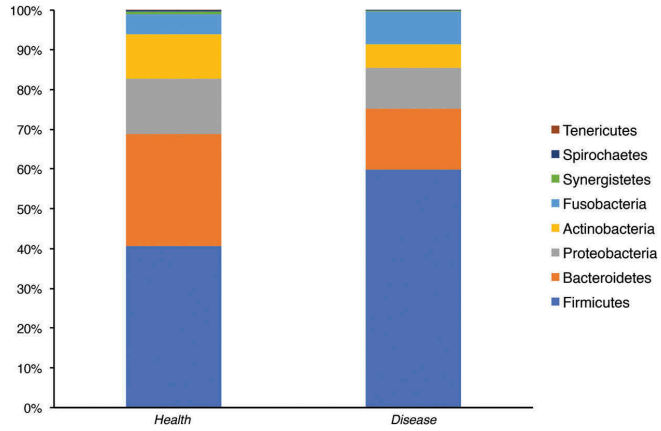

Figure 1. Bacterial phyla detected in APS-1 patients (n = 7) and control saliva (n = 17). Comparison of microbiota in APS-1 and healthy saliva determined by sequencing the hypervariable *16S rDNA* region V1-V2. Relative abundance of the different phyla in control and APS-1 samples is shown.

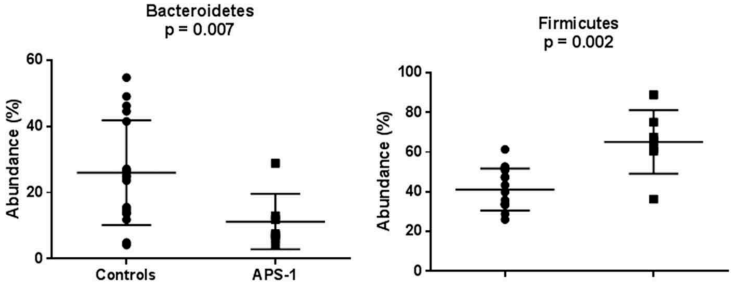

Figure 2. The relative abundances of the phyla Bacteroidetes and Firmicutes in saliva of healthy controls (n = 17) and APS-1 patients (n = 7) illustrated with boxplots. The lines indicate means and standard deviations.

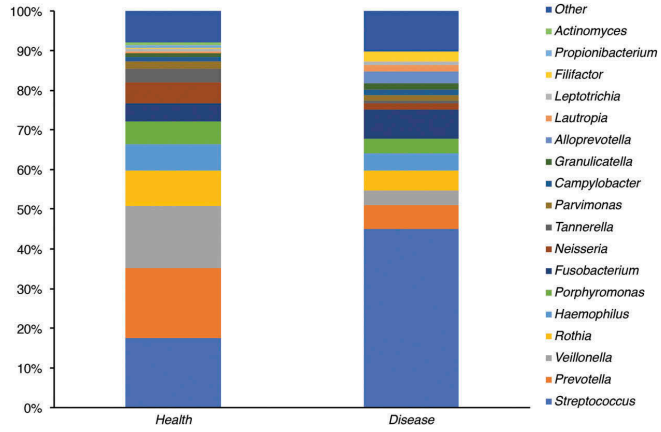

Figure 3. Bacterial genera detected in saliva from APS-1 patients (n = 7) and controls (n = 17). Groups designated as 'Other' represents minor groups classified. The *Y*-axis represents relative abundance. An increase in the genus *Streptococcus* in APS-1 saliva relative to control saliva is demonstrated.

a PCoA 3D plot of all samples with the distances calculated using weighed normalized UniFrac matrix, and clearly indicates that the two groups have distinct beta diversity. Taken together, these results show a richer diversity in controls compared to APS-1.

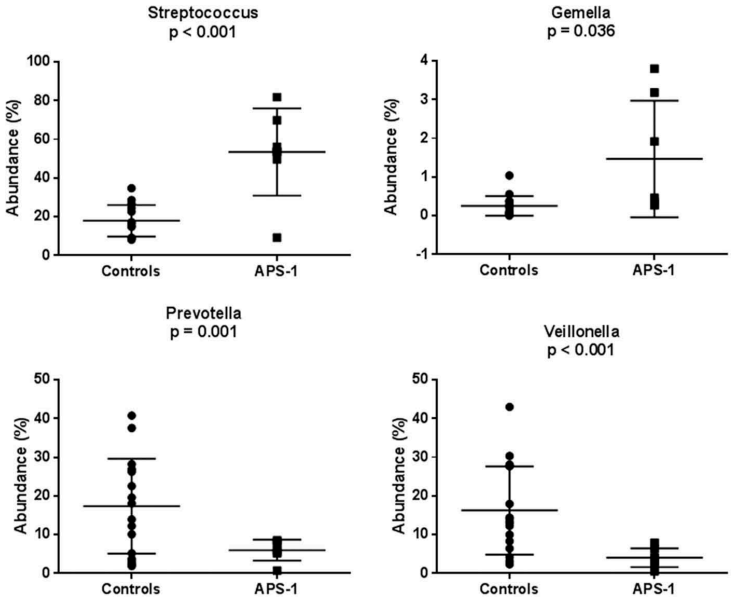

Figure 4. The relative abundances of the genera *Streptococcus, Gemella, Prevotella*, and *Veillonella* in saliva of healthy controls (n = 17) and APS-1 patients (n = 7) illustrated with boxplots. The lines indicate means and standard deviations.

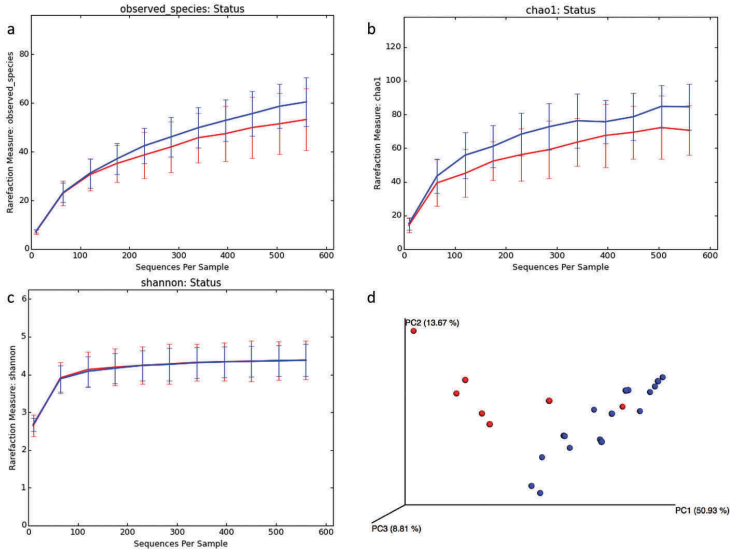

Figure 5. Comparison of microbial diversity in saliva samples of APS-1 patients (n = 7) and health samples (n = 17). Average rarefaction curves of APS-1 and health samples reported as (a) observed species, (b) Chao1 estimator, (c) Shannon index, (d) PCoA 3D plots of all samples with distances calculated using weighed normalized UniFrac matrix. Blue curves and dots represent health samples; and red represents APS-1.

Discussion

Increasing evidence suggests a potential role of the skin, oral, and gut microbiotas in the pathogenesis of autoimmunity [14]. Using high throughput sequencing and a comprehensive bioinformatics approach to analyse next generation 16S rDNA pyrosequencing reads, we here present hitherto undescribed significant differences in the composition of the salivary microbiota comparing APS-1 patients and healthy controls, indicating a possible contribution in pathogenesis and clinical expression.

The most striking differences were a higher portion of Firmicutes and a reduction of Bacteroidetes in APS-1 patients. Similar findings were recently described in primary Sjögrens syndrome [20] and a reduced species diversity and an altered ratio between Firmicutes and Bacteroidetes were described in the intestinal microbiota in several autoimmune diseases [30–33]. However, Firmicutes represents a phylum where most bacteria have a Gram-positive cell wall including the oral genera *Streptococcus, Lactobacillus, Selenomonas, Clostridium*, and *Eubacterium*. The phylum Bacteroidetes is composed of Gram-negative bacteria. On a genus level, *Streptococcus* and *Gemella* were increased in APS-1. However, a reduction in the total number of bacterial genera was seen. Overall, these novel findings indicate a significant altered oral microbiota in APS-1.

This is the first report on the oral microbiota in APS-1, and only a few studies have investigated the gut microbiota in patients [17,22,23]. A comparative analysis of the intestinal microbiota of APS-1 patients with gastrointestinal manifestations showed significant enrichment of segmented filamentous bacteria [17], which are Gram-positive commensal bacteria with the potential to adhere to epithelial cells and induce T helper (Th) 17 responses. Another study reported that APS-1 patients develop early and sustained responses to gut microbial antigens reminiscent to Crohn's disease [23] linked to defects in Tregs. Finally, products from commensal bacteria have the potential to indirectly regulate thymic *Aire* expression in mice [22]. Based on the above, this indicates that the microbiota contributes in shaping immunity in APS-1 although the molecular mechanisms are incompletely characterized.

Factors known to directly affect the immune system, and consequently, the risk of autoimmunity, such as genetics, gender, and diet may also exert their effects by modulating microbiota profiles and functions. Using animal models of experimental colitis [34] and arthritis [35] it was shown that Gram-negative bacteria, possibly through the TLR2/IL-10 axis, reduced inflammation [34], whereas Gram-positive bacteria contributed to a more severe disease [35]. In a mouse model of Sjögren's disease, depletion of the intestinal microbiome worsened the ocular response to desiccation, while the overall severity of disease correlated with intestinal microbiome diversity [36]. Two recent characterizations of the oral microbiota in primary Sjögren's syndrome demonstrated a significant shift in the oral microbiota of patients and reduced numbers of genera [20,37], suggesting a role of the oral microbiota in the pathogenesis. Interestingly, these findings are comparable to what we currently describe in APS-1.

CMC caused by *C. albicans* is the most common and earliest manifestation of APS-1 [2]. The clinical course varies from periodical to chronic and usually affects the oral mucosa [1,2,9]. To date, neutralizing autoantibodies against the Th17 cytokines IL-17A, IL-17F, and IL-22 are suggested to explain the impairment in mucosal immunity in APS-1 patients [10,38]. However, an important line of defence against oral CMC is the oral microbiota that prevents infections by their interplay with immune cells, nutrients, metabolic products and by secreting antagonistic molecules, which together balance local inflammatory responses [39]. *C. albicans* can form biofilms with many oral bacteria, including streptococci [40], which have synergistic or antagonistic influences on *C. albicans*. Noteworthy, recent work has highlighted the critical role of metabolic products from specific gut microbiota such as lactobacilli in priming IL-22 dependent mucosal immune responses by innate lymphoid cells via the aryl hydrocarbon receptor, which is fundamental for protection against uncontrolled local *Candida* expansion [41]. Further, a shift in salivary microbiota has been linked to the risk of oral cancer in selected groups of patients [42]. We found significantly increased abundance of streptococci in APS-1 saliva compared to healthy controls and several streptococci were among the most abundant species in APS-1 saliva. To speculate, an altered microbiota may change the profile of immune regulatory metabolic products, and thus, contribute in altering immunity in APS-1 patients. Still it remains unclear whether an altered microbiota causes disease manifestations or the altered microbiota is an effect of disease components.

The obvious drawbacks of this study are the low number of patients included and the heterogeneity within this group. Initially 10 patients were included but technical issues with the sequencing made us exclude 3 patients. We plan to include more patients trying to correlate the different taxa to clinical manifestations of APS-1 and calculate absolute microbial abundances using a quantitative polymerase chain reactions assay. The same approach could be used to characterize the skin and the gut microbiotas in APS-1.

In conclusion, the oral microbiota in APS-1 patients is altered compared to healthy controls and seems to be a characteristic of the syndrome. In general, the skin, oral, and intestinal microbiotas in APS-1 patients should be further investigated to reveal their potentially contribution in pathogenesis and phenotypic expression of the syndrome.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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