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Molecular Targets in Autoimmune Polyendocrine Syndrome Type1 and Their Clinical Implications

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Abstract

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Autoimmune diseases occur when the immune system attacks and destroys healthy body tissue. Autoimmunity is known to cause a wide range of disorders, and is suspected to be responsible for many more. Most autoimmune disorders are chronic and cause severe morbidity for the patients, and are also costly for society. A majority of these disorders are today considered as complex diseases with incompletely known etiology. Hence, model systems for studying the pathogenesis of autoimmunity are important to unravel its causes.

Autoimmune Polyendocrine Syndrome Type 1 (APS-1), (OMIM 240300), is a rare autoimmune disorder. Patients with APS-1 progressively develop multiple organ-specific autoimmune lesions involving both endocrine and non endocrine tissues. Typical autoimmune disease components in APS-1 are hypoparathyroidism, Addison's disease, vitiligo, alopecia and type 1 diabetes. The gene preventing APS-1 has been identified and designated Autoimmune Regulator (AIRE). It has been shown that mutations of AIRE cause loss of tolerance to self-structures, resulting in organ-specific autoimmunity.

Although APS-1 is a rare syndrome occurring mainly in genetically isolated populations, the disease components of APS-1 are, in isolated forms, not unusual in the general population and affect many patients. Hence, APS-1 is an attractive model disease for studies of molecular mechanisms underlying organ-specific autoimmunity.

This thesis concerns investigations in which two novel autoantigens are identified in APS-1 and used in serological diagnosis of the disease. NALP5, is identified as a parathyroid autoantigen - an important finding since autoimmune hypoparathyroidism is one of the cardinal symptoms of APS-1. Additionally, KCNRG is identified as a bronchial autoantigen in APS-1 patients with respiratory symptoms. Finally, studies that compare the immune response in APS-1 patients and the mouse model for APS-1 are presented.

Keywords: autoimmunity, autoantibodies, endocrinology, parathyroid, hypoparathyroidism, Addison's disease, pulmonary symptoms, NALP, NALP5, NLR, KCNRG

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To my Father

Cover image:

Trousseau's sign, vitiligo and nail candidiasis in an APS-1 patient. Trousseau's sign is a clinical test for hypocalcemia described by Armand Trousseau (1801-1867). To provoke the sign, patient's brachial artery is compressed with a blood pressure cuff. If the patient's hand undergoes a painful carpal spasm, manifested as flexion at the wrist and metacarpophalangeal joints, extension of the distal interphalangeal and proximal interphalangeal joints, and adduction of the thumb and fingers, then hypocalcaemia should be suspected. The sign is also known as "*main d'accoucheur*" (French for hand of the obstetrician) because the extended fingers are said to resemble the position of the obstetrician's hand in delivering a baby.

List of Papers

This thesis is based on the following papers which will be referred to by their roman numerals:

- I. Pöntynen N, Miettinen A, Petteri Arstila T, Kämpe O, **Alimohammadi M**, Vaarala O, Peltonen L, Ulmanen I.
AIRE deficient mice do not develop the same profile of tissue-specific autoantibodies as APECED patients.
**Journal of Autoimmunity, 2006; 27(2):96-104.*
- II. **Alimohammadi M**, Björklund P, Hallgren Å, Pöntynen N, Szinnai G, Shikama N, Keller M, Ekwall O, Kinkel S, Husebye E, Gustafsson J, Rorsman F, Peltonen L, Betterle C, Perheentupa J, Åkerström G, Westin G, Scott H, Holländer G, and Kämpe O
Autoimmune Polyendocrine Syndrome Type 1 and NALP5, a Parathyroid Autoantigen.
**The New England Journal of Medicine, 2008; 358(10):1018-28.*
- III. **Alimohammadi M**, Szinnai G, Hubert F-X, Pöntynen N, Shum A, Su M, Hallgren Å, Shikama N, Kinkel S.A, Björklund P, Scott H.S, Anderson M.S, Holländer G, Kämpe O
NALP5 - a Target for Autoantibodies in AIRE Deficient Mice Reflecting the Autoimmune Status
Manuscript
- IV. **Alimohammadi M**, Dubois N, Sköldberg F, Hallgren Å, Tardivel I, Hedstrand H, Haavik J, Husebye E. S, Gustafsson J, Rorsman F, Meloni A, Janson C, Vialettes B, Kajosaari M, Egner W, Sagur R, Amoura Z, Grimfeld A, Pontén F, De Luca F, Betterle C, Perheentupa J, Kämpe O and Carel J-C
Pulmonary Autoimmunity as a Feature of Autoimmune Polyendocrine Syndrome Type 1 and Identification of KCNRG as a Bronchial Autoantigen
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Abbreviations

21OH	21-hydroxylase
Aire	Autoimmune regulator (murine ortholog), this acronym is only used in the articles.
AIRE	Autoimmune regulator (human ortholog)
AOD	Autoimmune ovarian disease
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dysplasia
APS-1	Autoimmune polyendocrine syndrome type 1
ATF	Autoimmune testicular failure
CaSR	Calcium sensing receptor
CLLD4	Chronic lymphocytic leukemia deletion gene 4
COPD	Chronic obstructive pulmonary disease
CY3	Indocarbocyanine, Cy3 (fluorescent dye)
FITC	Fluorescein isothiocyanate (fluorescent dye)
HLA	Human leucocyte antigen
KCNRG	Potassium Channel Regulator
LRR	Leucine rich repeats
MHC	Major histocompatibility complex
mTECs	Medullary thymic epithelial cells
NALP5	NACHT, leucine rich repeat and PYD containing 5
NLR	NOD like receptor
PAMP	Pathogen-associated molecular pattern
POF	Premature ovarian failure
PRM	Pattern recognition molecule
PTH	Parathyroid hormone
PTHrp	Parathyroid hormone related protein
SCC	Side chain cleavage enzyme
SLE	Systemic lupus erythematosus
TCR	T cell receptor

Introduction

Autoimmune diseases occur in up to 5% of the general population and except for the suffering for the affected individuals, this disease group constitutes the third most common cause of morbidity and death, placing a considerable cost on the health care system and society^{1,2}. Understanding of the underlying pathophysiology of autoimmunity is therefore important for disease prevention and development of improved therapies.

Autoimmune polyendocrine syndrome type 1 (APS-1) is a rare autoimmune disorder. Affected patients develop spontaneous autoimmune disease components targeting primarily endocrine organs including parathyroid and adrenal glands. Interestingly, the gene that prevents the onset of APS-1 has been discovered and enabled the development of an animal model for the disease^{3,4}. Many of the known disease components of APS-1 such as type 1 diabetes and vitiligo are frequent in the general population. Therefore, APS-1 serves as a good model disease for studies of autoimmunity. Studies on APS-1 contribute to understanding of some crucial molecular mechanisms leading to development of autoimmunity.

This thesis focuses on APS-1 and two of its disease components and includes studies that start to elucidate the pathomechanisms for autoimmunity against the parathyroid glands and lungs. Additionally, it brings together the clinical findings in the human patients with the animal model for APS-1.

The Immune System

Our immune system constitutes a complex defense network, consisting of several cell types, proteins and molecules, organized to protect us from invading microorganisms. The immune system may also have a suppressive effect on neoplastic cells⁵.

As soon as a microorganism invades the body through epithelial barriers in the skin, gastrointestinal, urogenital, or respiratory tract, it encounters phagocytes which engulf and digest the invading microorganisms. This innate immune response is fast but not antigen-specific. The phagocytes also

send signals that induce inflammation and recruit other immune cells to the site, and consequently activate the antigen-specific adaptive immune system. The adaptive immune system is activated by binding of lymphocyte receptors to specific structures on the microorganism, but the activation of this system also depends on cytokine signals from the innate immune system. Hence, an adequate communication between innate and adaptive systems is important for a functional immune response. The innate and adaptive immune systems thus act in synergy to provide protection against pathogenic microorganisms.

Innate Immunity

The innate, first line defense neutralizes an invasion of pathogens fast and simultaneously alerts the adaptive immune system about the invasion. Examples of components which belong to the innate immune system are mucosal barriers, the complement system, phagocytic cells, natural killer cells, and pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs). Many components of the innate immune system such as TLRs are very old in evolutionary terms and can even be found in primitive organisms such as insects⁶.

Adaptive Immunity

The adaptive immune system, which is younger in evolutionary terms than the innate immune system and unique for vertebrates, acts slower but has immunological memory and is antigen-specific. The adaptive immune system mounts its response within 4-5 days after pathogen invasion. Thymus-derived lymphocytes (T cells) specific for a given antigen can be activated by dendritic cells, and this process is characteristic for the adaptive immune system. In turn, bone marrow-derived lymphocytes (B cells) can be activated by T cells. Depending on their environment and in order to communicate with other cells, all these cells secrete different cytokines. B cells produce and secrete proteins called antibodies, which are antigen-specific and usually directed against structures on an invading pathogen.

Lymphocyte Development and Central Tolerance

T cells and B cells develop from a common lymphoid progenitor which divides into high numbers of T and B cells. Each T cell carries T cell receptors (TCR) on its surface that recognize a given antigen and each B cell

carries B cell receptors (BCR) specific for a given antigen. In order to allow recognition of a broad range of antigens, TCRs and BCRs undergo somatic recombination in which large numbers (10^{16}) of TCRs and BCRs with different specificities are generated. However, each T or B cell carries only copies of one of these 10^{16} receptors on its surface.

Thymus is an intra-thoracic organ located in front of the heart. The thymus of infants is large but with increased age this organ degenerates and is difficult to find in adults. Thymus is important for development of T cells. Pre-T cells enter the thymus to undergo positive selection and subsequently negative selection (**Figure 1**). The purpose of these selection steps is to produce T cells that are effective in the defense against invading pathogens but tolerant to self-structures.

As soon as the T cell progenitors enter the thymus, they meet the cortex epithelial cells and receive proliferative cytokine signals that initiate the recombination of TCR. Once a functional TCR is produced, the T cells undergo positive selection. In this step, only those developing T cells whose TCR can recognize antigens presented by body's own major histocompatibility complex (MHC) molecules are allowed to mature in the thymus; all other developing T cells die before reaching maturity.

The cells which continue to develop are at this stage capable of recognizing both foreign antigens and self-antigens on MHC. In order to avoid self-reactivity, the T cells that can bind self-antigens with strong affinity must die, a process called negative selection. At this step a cell population in the thymus, entitled medullary epithelial cells, present self-antigens on their surface MCH molecules (promiscuous antigen expression). T cells with TCRs that can bind to self-antigens with high affinity receive an apoptotic signal and die, a process called clonal deletion. The autoimmune regulator gene (AIRE) is believed to have an important function during the negative selection, through regulating the expression of self-antigens on the surface of the thymic medullary epithelial cells during negative selection. Often, low affinity self-reactive T cells escape from the clonal deletion in the thymus and enter the circulation. Peripheral tolerance prevents these cells from becoming self-reactive. The surviving, naïve T cells leave the thymus and re-circulate in the peripheral lymphoid tissues and blood. Naïve T cells become activated after encounter of specific antigen in context of MHC presented on an antigen presenting cell (APC).

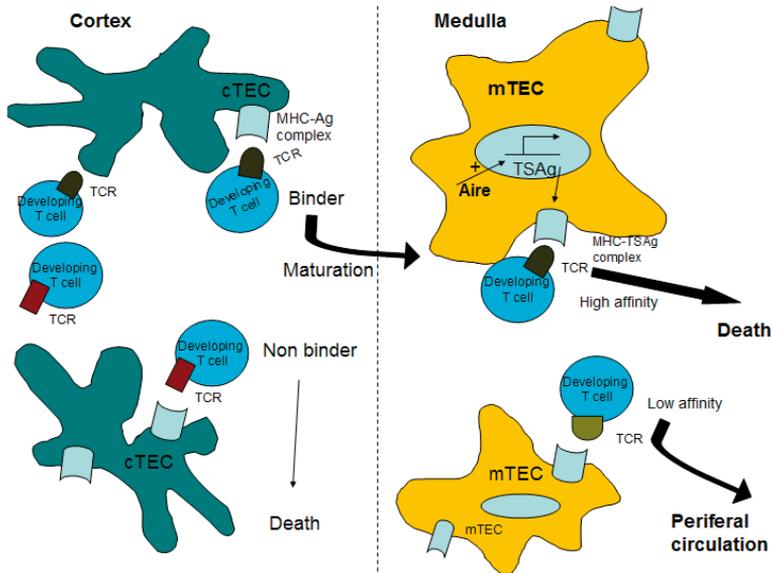


Figure 1. Illustration of the positive and negative selection steps in the thymus and the role of AIRE in the expression of tissue self antigens (TSAg). In the absence of AIRE, TSAg are not displayed on mTECs and consequently, T cells with high affinity for TSAg escape from the negative selection.

T cell Activation and Tolerance

Cytotoxic T cells recognize MHC I-peptide complex and have a CD8 receptor. They are T cells that can kill the cell that presents specific antigen by releasing effector molecules. T helper cells recognize MHC II and express CD4. They develop into either T helper 1 (Th1) cells, which participate in cell mediated immunity, or Th2 cells, which participate in antibody production. The balance between the different types of T helper cells present at an infection site determines the subsequent type of immune response against an infection. For example, excess of Th1 cells is known to shift the immune response towards cell-mediated immunity and is also believed to provoke development of autoimmunity.

A CD4-expressing population called Th17 are T helper cells which are positioned near epithelial surfaces and induce inflammation upon invasion of microorganisms⁷. These cells are also believed to be important in development of autoimmunity. Another subset of CD4-expressing T cells has regulatory functions, and hence are called regulatory T cells (T-regs). This cell type binds to self-antigen and can release cytokines that suppress other T cells and inhibit cellular immune responses⁸.

Table 1. Summary of the different subtypes of T helper cells and their role in health and disease.

Type	Effector Cytokine(s)	Suggested role in immunity	Suggested role in pathologic conditions
Th1	IFN- γ	Intracellular pathogens	Autoimmunity; cell-mediated allergies
Th2	IL-4	Extracellular pathogens	Asthma and IgE-mediated allergies
Th17	IL-17 & IL-22	Extracellular bacteria; mediates inflammation	Autoimmune diseases
T-reg	TGF- β	Immunosuppression; anti-inflammatory	Prevention of autoimmunity

B cell Activation and Tolerance

The binding of an antigen to the BCR leads to signaling in the B cell. If this signaling occurs in the presence of cytokines provided by T helper cells, the B cell shifts to clonal expansion, which renders a clone of B cells with the same receptor specificity. Most of these B cells develop into plasma cells which are the effector cells acting through production of large number of antibodies. Some cells develop into memory B cells which recognize the antigen and lead to a faster immune response on a second encounter of antigen.

In the bone marrow, the BCR on the immature B cell receptor is rearranged in a complex process called somatic recombination. If a B cell is unable to produce a functional receptor after rearrangement, it dies by apoptosis. If it expresses a functional receptor, the immature B cell leaves the bone marrow and enters the circulation and then peripheral lymphoid tissues where it undergoes positive and negative selection. Naïve B cells recirculate in the peripheral lymphoid tissues and blood to encounter their specific antigen and become activated. When B cells bind to specific antigen in the presence of signals from Th2 cells, they become activated and start to proliferate. The B cells also undergo somatic hypermutation, which means that the binding sites of their BCRs become subject to point mutations at a high rate. The cells with mutant receptors that no longer recognize the antigen after the mutations undergo apoptosis, whereas the receptors that have increased their binding affinity of antigen survive. Several cycles of mutations can increase the binding affinity - a process called “affinity maturation”⁹.

Antibodies

Antibodies, also called immunoglobulins (Ig), are very similar to the BCR but they lack the transmembrane part. Different forms of antibodies named after their constant region segment exist i.e. IgA, IgG, IgM and IgE (this thesis concerns studies on only IgG antibodies). Antibodies bind soluble antigens and protect against the pathogens in three different ways: i) The binding of antibodies to the pathogenic microorganism can block its binding site to the epithelium and in this way neutralize the pathogen from invading and establishing an infection. ii) An antibody bound to an antigen can “tag” the microorganism and enable its recognition by a phagocytic cell that can phagocytize and digest the tagged microorganism (opsonisation). iii) Antibodies bound to their antigen can activate the complement cascade, which leads to recruitment of immune cells and direct action of the complement system creating a pore in the cell membrane and destruction of the pathogen.

Autoimmunity

Although the central tolerance mechanisms described above serve to prevent self-reactivity by the adaptive immune system, reactions to self-molecules still occur and cause autoimmunity in some individuals. The autoimmune reaction is due to the escape of some self-reactive lymphocytes (T cells with self-specific TCR) from the negative selection in the thymus. An autoimmune reaction is a T cell-mediated and/or antibody-mediated reaction in which self-structures are recognized, targeted and attacked by the immune system.

To prevent the activation of these lymphocytes, several peripheral tolerogenic mechanisms occur: i) anergy is induced in self-reactive lymphocytes (T cells with self-specific TCR) when co-stimulatory signals are lacking from the innate immune system. ii) Chronic and continuous signaling through the TCR or BCR, without appropriate activation signals from the innate immune system, leads to activation induced cell-death or anergy. Still, self-reactive lymphocytes with low affinity to their specific self-antigen, may escape from peripheral tolerance.

There are several ways in which anergic self-reactive lymphocytes can become activated: i) Strong co-stimulatory signals caused by infection or inflammation may activate anergic lymphocytes⁹. ii) Increased availability of the antigen can trigger activation of low affinity lymphocytes, for example when intracellular antigens or antigens from behind a tissue barrier are released due to tissue damage. iii) Another cause of activation of low affinity self-B cells is hypermutation which results in high affinity binding to the self-antigen. iv) Molecular mimicry occurs when a microorganism presents an antigen that is very similar to a self-protein - perhaps as an immune escape mechanism. Lymphocytes recognizing this antigen may subsequently recognize self-antigen and mediate tissue damage.

The activated self-reactive lymphocytes do not necessarily cause autoimmunity; they can be suppressed by T-regs. Nevertheless, in some individuals self-reactive, activated lymphocytes may escape all tolerogenic mechanisms and cause autoimmunity. The classical definition of an autoimmune disease includes four criteria¹⁰.

1. Existence of autoantibodies or autoreactive T cells.
2. Existence of a corresponding autoantigen.
3. Induction of disease in experimental animals by immunization with the autoantigen.
4. Transfer of T cells, B cells or autoantibodies from an individual with autoimmune disease induce disease in healthy individuals.

Although autoimmune disorders affect up to 5% of the population¹, the underlying mechanisms of these disorders is still incompletely understood. By studying the risk for development of autoimmunity in relatives of autoimmune patients (including twin studies), it has been found that many autoimmune diseases show familial clustering, suggesting involvement of genetic factors. Individuals with one autoimmune disease are predisposed to develop additional autoimmune diseases. Different HLA (MHC) alleles have been shown to be either protective or to increase the susceptibility for different autoimmune diseases¹¹.

The autoimmune diseases can be divided into organ-specific i.e. type 1 diabetes mellitus in which pancreatic beta cells are selectively attacked by the immune system, and systemic autoimmune diseases, i.e. Systemic Lupus Erythematosus (SLE or lupus) in which several different organs such as joints, heart, skin, lungs, blood vessels, liver, kidneys and nervous system may be affected.

Autoantibodies

Antibodies that recognize self-structures and are present in patients with autoimmune diseases are called autoantibodies. Whether the presence of autoantibodies is a primary cause of autoimmune diseases or secondary to tissue destruction is unknown. In autoimmune diseases such as primary biliary cirrhosis, the epitopes for both the autoantibodies and the autoreactive T cells are found in the same region of the antigen, which suggests involvement of both T and B cells in the autoimmune pathology.

Assessment of autoantibodies is of diagnostic value in several autoimmune disorders. The occurrence of autoantibodies may precede the clinical onset of the disease^{12, 13}, and can thus be used to screen individuals at risk of developing the disease. The titers of some antibodies are also correlated to disease activity, and can be used to monitor the patients e.g. anti-dsDNA antibodies in SLE¹⁴, while other autoantibodies are of less value for monitoring of the disease.

The Role of Autoantibodies in Disease Development

Whether circulating autoantibodies in different autoimmune disorders cause the disease or whether they are only a secondary disease marker is an interesting and intriguing subject for discussion. To elucidate this discussion, it is important to distinguish between autoantibodies directed against intracellular autoantigens in destructive autoimmune disorders, and stimulatory autoantibodies directed against extracellular autoantigens.

The role of circulating autoantibodies in destructive autoimmunity such as type 1 diabetes still remains uncertain. However, the strict specificity points to an important function of autoantibodies in the pathogenesis, possibly by providing efficient presentation for T cells. In organ-specific autoimmunity, there is no proven case when autoantibodies against intracellular autoantigens have a direct pathogenic effect, as illustrated by the lack of any effect with transplacental transport to the fetus during pregnancy. This is in contrast to non-destructive autoimmune diseases with stimulatory or inhibitory autoantibodies against cell surface receptors, which are usually associated with thymomas, such as Graves' disease and myasthenia gravis, where autoantibodies are directed against cell surface receptors. In these latter cases transplacental transport during pregnancy regularly causes a transient and sometimes severe disease in the fetus and the newborn child¹⁵.

Another interesting disease, worth mentioning in this context, is neonatal SLE or Sjögren's syndrome with congenital atrioventricular heart block and subsequent cardiomyopathy. In this disease, autoantibodies directed against the intracellular/nuclear autoantigen SSA/Ro are transplacentally transferred to the fetus. It has been shown that these autoantibodies bind to the heart's conduction tissues and cause congenital heart block presenting with slow heart rate (bradycardia) in the fetus, a frightening condition, which in some cases extends to heart failure (dilated cardiomyopathy)¹⁶. SLE associated congenital heart block can be treated by plasmapheresis, removing the pathogenic autoantibodies from the fetus circulation. Most of the infants undergoing this treatment recover from their heart block. Nevertheless, a small percentage of the treated children will subsequently after a few years develop cardiomyopathy. This disease can therefore exemplify another situation in which autoantibodies mediate disease.

This thesis concerns studies on APS-1. The known antigens in APS-1 have a restricted tissue expression and are usually intracellular proteins with key functions of the tissue that is targeted by the immune system¹⁷. Their exact function is similar to autoantibodies such as GAD-65 - they serve as excellent markers for disease, but are not pathogenic per se as illustrated e.g. by transplacental transfer during pregnancy. Hence, the exact role of the

APS-1 associated autoantibodies remains to be established. Meanwhile, one could speculate that these autoantibodies may be involved in the presentation of autoantigens to T cells and perhaps initiate a vicious circle leading to autoimmunity.

Autoimmune Polyendocrine Syndrome Type 1

History

During the last century, sporadic patient case reports on patients with familial forms of adrenal insufficiency and hypoparathyroidism appeared in the literature¹⁸⁻²¹. It is believed that these reports describe symptoms of a polyglandular syndrome, most likely Autoimmune Polyendocrine Syndrome type I (APS-1) (OMIM 240300). The first extensive clinical report on a large APS-1 patient material (n=106) was published in 1980 by Neufeld et al²². In 1990, a detailed clinical report and follow-up study of 68 Finnish APS-1 patients was presented²³ and in 1994, linkage analysis on Finnish APS-1 families could be used to map the defective gene to the locus 21q22.3³.

Etiology

APS-1 is a rare autosomal recessive inherited disease due to mutations in the autoimmune regulator gene AIRE, localized on chromosome 21, and encoding the 54-kDa AIRE protein^{24, 25}. The mutations in AIRE are enriched in genetically isolated populations such as Finns, Sardinians, and Iranian Jews.

The exact function of AIRE is not completely known. However, it has been shown that AIRE is expressed primarily in the thymus, and to a minor extent in lymph nodes, spleen, and dendritic cells²⁶⁻²⁸. In the thymus, AIRE expression has been proposed to be restricted to a subset of medullary thymic epithelial cells (mTECs)²⁶ and it has been demonstrated that AIRE enhances the presentation of self-proteins in context of MHC on the surface of mTECs during negative selection²⁹ (Figure 1). In this way, it is implied that AIRE has an important role in the process in which the autoreactive T cells are deleted^{29, 30}. With respect to the precise molecular function of AIRE, it has been proposed that one of the PHD domain may function as an ubiquitin ligase³¹. However these data contradict those from later reports³².

Clinical Picture

APS-1 is characterized by development of spontaneous organ-specific autoimmune attacks on several ectoderm- and endoderm-derived tissues (primarily endocrine organs) including the parathyroid glands, adrenal cortex, liver, pancreatic beta cells, gonads, melanocytes of skin and hair follicles.

The onset of the disease usually takes place in early childhood when most of the patients present with recurrent and hard-treated mucocutane candida albicans infection. Approximately 80% of the patients develop hypoparathyroidism as their first endocrinopathy³³. During their lifetime, these patients continue to accumulate a broad spectrum of organ-specific autoimmune disorders, particularly those affecting the endocrine organs (Table 2). Among these, Addison's disease (autoimmune adrenal cortex insufficiency) is most extensively studied^{34, 35}. The earlier the first components of APS-1 appear, the more likely it is that multiple disease components develop. Conversely, patients who have late manifestations of the disease are likely to have fewer disease components³³.

According to the most recent consensus, the clinical diagnosis of APS-1 should be based on three cardinal symptoms namely, mucocutaneous candidiasis, hypoparathyroidism and Addison's disease. At least two of these three disease components are required to establish the clinical diagnosis of APS-1³³.

Table 2. Disease components in APS-1 and their frequency among the patients according to references (Neufeld 1980; Ahonen 1990; Zlotogra 1992; Betterle 1998; Perheentupa 2006)^{22, 23, 33, 36-38} *The frequency of candidiasis in Iranian-Jewish APS-1 patients is about 17%. **Considered as a rare disease component.

Disease Component	Frequency	Usual Age of Onset
Candidiasis	75 – 100%*	Infancy-Childhood
Hypoparathyroidism	82 – 96%	Infancy-Childhood
Adrenal failure	60 – 73%	Childhood
Enamel hypoplasia	70-80%	Childhood-Puberty
Gonadal failure (AOD or testicular failure)	12 – 43%	Puberty-Adulthood
Alopecia	13–37%	Puberty- Adulthood
Vitiligo	4–15%	Puberty- Adulthood
Intestinal Symptoms (obstipation; diarrhea; malabsorption)	20%	Childhood-Adulthood
Hepatitis	10 – 15%	Puberty-Young adulthood
Pernicious anemia	9 – 16%	Young adulthood
Ophthalmic symptoms (Keratoconjunctivitis; Chronic iridocilitis; Dry eye)	15%	Childhood-Puberty
Squamous cell carcinoma of mouth or esophagus	10%	Adulthood
Rash with fever**	10%	Childhood
Asplenia **	9%	Congenital?
Tubulointerstitial nephritis**	9%	Indistinct
Pulmonary symptoms (Asthma; Bronchitis obliterans organizing pneumonia)	<10%	Indistinct
Exocrine pancreatic insufficiency**	<5%	Indistinct
Autoimmune Hemolytic anemia**	<5%	Indistinct
Neurological symptoms	Unclear	Indistinct
Pituitary disease	Unclear, 23 cases	Indistinct
Achalasia of esophagus, Alacrimia, osteification of tympanic membrane	Unclear	Indistinct

Autoantibodies in APS-1

APS-1 patients have a deficiency in the development of self tolerance. As a consequence, their B cells produce antibodies directed against self-structures. These autoantibodies are characteristic of APS-1 (Table 3) and their appearance may precede the clinical manifestation of the disease components, predicting the disease^{12, 39}. In APS-1 patients, autoantibodies remain present even after the destruction of the target tissue, but the titers have been observed to decrease after long disease duration⁴⁰. In some thymoma associated autoimmune disorders such as Graves disease and Myasthenia Gravis, the tissue specific autoantibodies are directed against cell surface receptors with either an inhibitory or a stimulatory effect^{41, 42}. In other autoimmune disorders such as autoimmune hemolytic anemia, the autoantibodies can bind to a surface antigen and activate the complement system leading to a tissue destruction⁴³. In these diseases, the pathogenic role of the autoantibodies is clear and the disease can be transmitted to another individual by transfer of these autoantibodies, as exemplified by the trans-placental transport of autoantibodies in a patient with Grave's disease.

Table 3. Autoantibodies in APS-1 and their correlation to different clinical disease components^{17, 44, 45}.

Disease Component	Related Autoantigen	Sensitivity (%)	Specificity (%)
Adrenal failure	Cytochrome P450 21	75	63
	Cytochrome P450 17	65	68
	Cytochrome P450 11A	68	74
Gonadal failure	Cytochrome P450 11a	75	76
Diabetes	GAD65	56	63
	IA-2	33	87
Intestinal Symptoms	Tryptophan hydroxylase	80	64
Hepatitis	Cytochrome P450 1A2	71	76
	Aromatic acid decarboxylase	79	75
Alopecia	Tyrosine hydroxylase		
Vitiligo	SOX9, SOX10?		
????	Interferon alpha	100	???

In APS-1, however, the tissue-specific autoantibodies are usually directed against intracellular antigens (Table 3). Unlike for autoantibodies in thymoma-associated autoimmune disorders, transfer of APS-1 associated autoantibodies to healthy individuals does not lead to development of autoimmune disease. Hence the pathogenic role of these autoantibodies is uncertain and the underlying immunological mechanisms leading to development of these autoantibodies in APS-1 remains to be elucidated.

Nevertheless, these autoantibodies are important for the timely diagnosis of the disease.

Clinical Aspects of APS-1^{46, 47}

Hypocalcemia is the most challenging disease component to manage in APS-1. Calcium is essential for many different physiologic processes, among those neuro-muscular signaling. In a healthy person the concentration of free ionized plasma calcium varies between 1.15-1.30 mmol/l.

Plasma calcium concentration can be regulated by parathyroid hormone (PTH) which increases calcium concentration through increased bone resorption and decreased urinary excretion of calcium (Figure 4). PTH has an inverse effect on plasma phosphate concentration.

Autoimmune destruction of parathyroid glands leads to hypoparathyroidism and subsequently, decreased the plasma calcium concentration. At the same time, the patient develops a hyperphosphatemia. These changes may affect the neuromuscular signaling. Clinical symptoms of hypocalcemia vary from tiredness, headache, numbness of fingertips, paresis/stiffness/twitches or cramps of both skeletal and smooth muscles. There are some clinical tests for confirming hypocalcemia e.g. Chvostek's test or Trousseau's sign as shown on the cover picture.

Recombinant parathyroid hormone has recently been introduced to the market, but not yet evaluated in APS-1 patients⁴⁶. Since vitamin D has an enhancing effect on the calcium uptake from intestines, vitamin D derivatives such as alphacalcidol (Etalpha[®]) or calcitriol (Rocaltrol[®]) together with calcium supplements (Calcium Sandoz[®]) can be used to increase the plasma calcium concentration. However, Vitamin D is not as effective as PTH in decreasing plasma phosphate concentration. This may lead to precipitation of calcium phosphate with the risk for development of kidney stones and kidney damage. In order to minimize the risk for calcium precipitation, it is important that the patient avoids dehydration and excessive intake of dietary phosphate and calcium.

In a patient with hypoparathyroidism, the plasma calcium concentration may be very unpredictable and factors such as dietary calcium intake, sun exposure and physical activity can rapidly change the normocalcemic level. Therefore, laboratory analysis is needed to monitor the patient's plasma calcium concentration regularly. The intervals for controls may vary (from weekly to once every other month) depending on the individual patient and

time. It is also desirable that the plasma calcium concentration is controlled when patient feels symptoms of hypo/hypercalcemia.

Candida albicans infections affecting the mucous membranes and skin are one of the first symptoms of APS-1 with still unknown etiology. The severity of candidiasis varies in different individuals. Mild forms of the infection are localized to mucous membranes and nails. In severe forms, the infection can spread to the gastrointestinal tract. Long-lasting infection due to candidiasis is believed to predispose development of mouth or esophagus cancer.

This highlights the need for prevention and treatment of candidiasis. As efficient therapies for candida infections are absent today, proper dental care is important. Smoking should be avoided because it strongly predisposes for candida infection. Appearance of oral ulcers should be investigated further to exclude malignancy.

Mouth inflammation is an indication for och mycostatic therapy e.g. Nystatine (Mycostatin[®]) or Amphotericin-B (Fungizone[®]). The anti-fungal therapy may be life long. Fungal culture should be performed regularly to determine if anti-fungal resistance is emerging. All patients should have ear, nose and throat examination performed at least annually. Nail candida infections are always very difficult to treat.

Addison's disease, or adrenal cortex deficiency, is another life threatening condition if not treated. Cortisol and aldosterone both produced in the adrenal cortex are essential for health. Cortisol is important for regulating the metabolism, and lack of cortisol leads to severe fatigue, weakness, anorexia and inability to respond to physical and psychological stress. Aldosterone maintains the blood volume and blood pressure through inhibition of urinary sodium excretion. Lack of Aldosterone can therefore lead to waste of sodium resulting in hypotension and death. Other symptoms of adrenal cortex insufficiency are hyperpigmentation of skin and mucus membranes due to an increased ACTH secretion from the pituitary gland, depression, nausea, vomiting, and weight loss.

Adrenal cortex insufficiency can be confirmed by measurement of S-cortisol; however, due to circadian secretion of cortisol, it is difficult to assess the pathologic cortisol level. Therefore a more accurate diagnosis is made by an ACTH test, in which an intravenous dose of recombinant ACTH is administered to the patient and the patient's adrenal cortex response by production of cortisol is measured. When the cortisol response is inadequate to the given ACTH dose, the diagnosis of adrenal cortex insufficiency can be

confirmed. 21-hydroxylase autoantibodies serve as a very good markers for the risk of developing Addison's disease¹².

Cortisol deficiency can be substituted by hydrocortisone or prednisone. The total daily dose can be spread over the day in order to mimic the natural circadian circulation. Since excessive cortisol therapy may lead to side-effects such as Cushing's syndrome and osteoporosis, the lowest necessary dose to reduce patient's symptoms is given.

In periods with physical stress such as fever, trauma or infections, the cortisol dose should be increased. In example, for each centigrade degree of fever, the substitution dose of cortisol should be doubled. Aldosterone deficiency can be replaced by Fluorocortone (Florinef[®]).

Tooth enamel deficiency/hypoplasia is one of the major ectodermal manifestations of APS-1, affecting more than 70% of the patients. The underlying pathomechanism to this disease component is still unknown. Proper oral hygiene and regular controls by dentist with special interest in enamel deficiencies is recommended. To prevent further stress of the defect enamel, sour (acidic) and spicy foods should be avoided. Moreover, patients should choose mild toothpastes.

Gonadal Insufficiency appears in both male and female APS-1 patients, but is rare in males. This is probably due to the blood-testis barrier. In females, the autoimmune destruction of the ovaries usually often appears before puberty leading to a delayed puberty. If developed later, females will develop premature ovarian failure (POF). These patients are treated with gradually increasing estradiol doses, and periods with addition of progesterone. If the ovarian destruction occurs after the puberty, the women become menopausal. The treatment in these patients should focus on prevention of osteoporosis and cardiovascular disorders.

Testicular deficiency (ATF) is treated by replacement of testosterone either by intramuscular depo-injections (Testoviron[®]-Depot) or gel (Androgel[®]). This treatment improves the strength and virility of patients.

Infertility in APS-1 patients, due to lack of mature oocytes or sperms, is not curable and fertility should be promoted by egg or sperm donations.

Type 1 Diabetes in APS-1 patients occurs due to destruction of pancreatic beta-cells and is treated by conventional diabetic care and insulin replacement. Development of type 1 diabetes usually occur later in life³⁶.

Hepatitis is usually asymptomatic in early stages, fatigue and icterus appear at later stages. Hence, for early diagnosis, the concentration of liver

enzymes (ASAT/ALAT) in peripheral blood should be monitored regularly to detect liver cell damage. Onset of hepatitis should be treated by long-term immunosuppressive medication, usually Azathioprin (Imurel®) and/or Prednisolone.

Intestinal Symptoms may appear as either severe malabsorption or pernicious anemia. A good nutritional status, with monitoring of the vitamin B status is important. When diarrhea or constipation appears, hypo/hypercalcemia should be excluded. Severe malabsorption is caused by an autoimmune destruction of the enterochromaffin serotonin producing cells of the intestine. Treatment is usually only symptomatic, but in severe cases Cyclosporin A (Sandimmun®) may be tried⁴⁸.

Keratoconjunctivitis is considered as a serious APECED component, since untreated keratoconjunctivitis may lead to permanent impairment of the vision. The condition is treated with local steroid therapy. Dryness of eyes may predispose eyes to infection and should be symptomatically treated by artificial tears of different viscosity.

Hypertension appears in APS-1 patients with unclear/multi-factorial etiology and therefore their blood pressure should be monitored regularly.

Asplenia leads to susceptibility to infections by meningococcal and pneumococcal infections; therefore, vaccination against these pathogens is necessary in the subset of patients with asplenia. Whether APS-1 associated asplenia is congenital or acquired remains to be studied.

Alopecia results from autoimmune destruction of the hair follicles. It may occur localized as alopecia areata or affect a larger area such as the whole scalp in alopecia totalis. Until today the only way to solve this problem is to use a wig.

Vitiligo is caused by an autoimmune destruction of the melanocytes. There is still no effective treatment for this component. However if the patches are localized, ultraviolet light B phototherapy or topical treatment with steroids or tacrolimus (Prograf®) may be tried.

Parathyroid Glands

Parathyroid glands are believed to be the latest discovered anatomical organs and were identified in the second half of the nineteenth century in Uppsala by Ivar Sandström (1852-1889)^{49, 50}. Humans usually have two pairs of parathyroid glands and these glands are normally located in the posterior part of the thyroid gland. During embryogenesis, parathyroid glands develop from the third and fourth pharyngeal pouch⁵¹. The fourth pharyngeal pouch gives rise to the upper pair of the parathyroid glands and the thyroid gland. The third pharyngeal pouch gives rise to the lower pair of the parathyroid glands and the thymus. Hence, the parathyroid glands have embryological relation to both thymus and the thyroid gland (Figure 2).

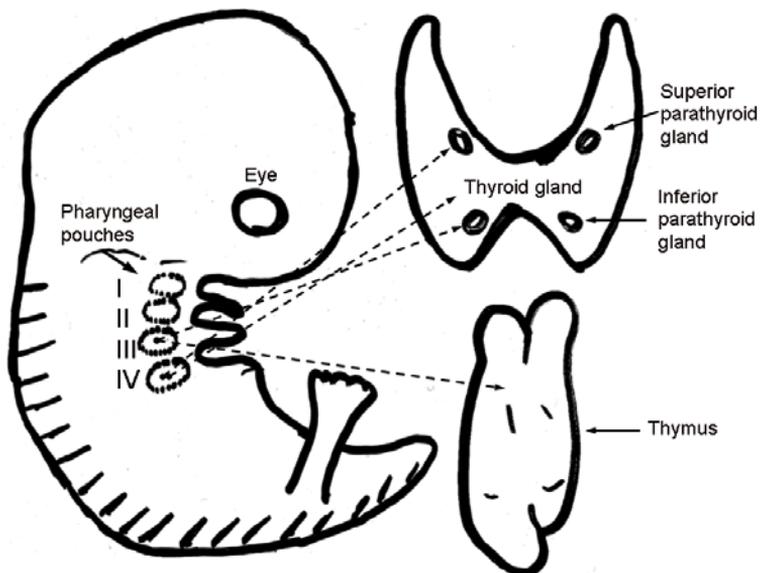


Figure 2. Embryonic development of parathyroid glands and their embryonic relationship to the thymus and thyroid gland.

Parathyroid glands constitute three cell types (Figure 3), the chief cells which are small cells with a large cytoplasm that synthesize and secrete parathyroid hormone (PTH), the oxyphilic cells which are large mitochondria-rich cells with still unknown function, and abundant fat cells also with unknown function⁵².

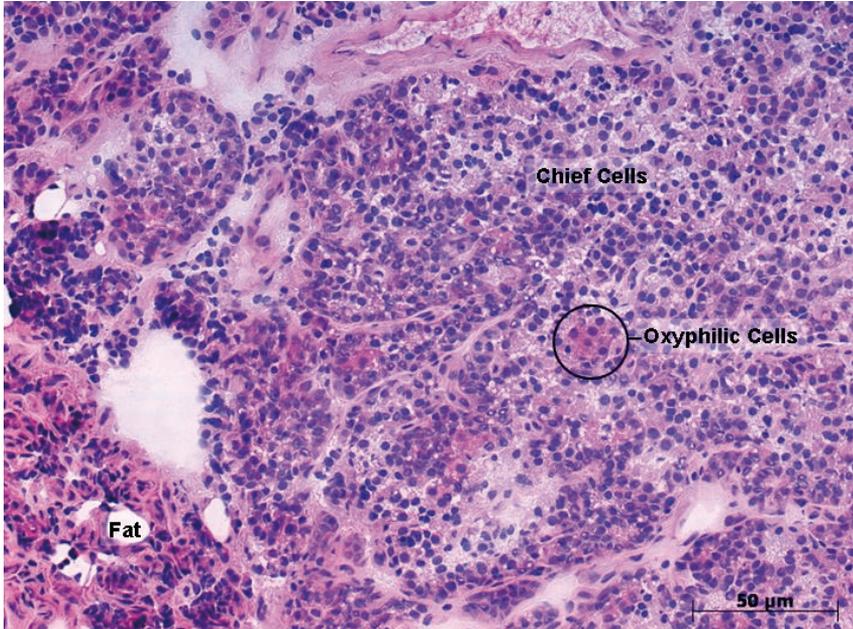


Figure 3. Hematoxylin-eosin stained section of a normal human parathyroid gland demonstrating the three different cell types of the gland.

The function of the parathyroid glands includes homeostasis of calcium and phosphate concentrations. This function is maintained by parathyroid hormone (PTH), which together with 1,25-dihydroxyvitamin D (calcitriol) regulates the uptake of calcium from the intestines, the resorption of calcium in kidneys and bone (Figure 4). However, PTH and calcitriol differ in their structure and synthesis. PTH is a polypeptide hormone consisting of 84 amino acids whereas calcitriol is a steroid hormone/cholesterol based substance entering the body through food, which is converted to its activated form by two enzymatic reactions involving the enzymes 25-hydroxylase and 1,25 hydroxylase^{53, 54}.

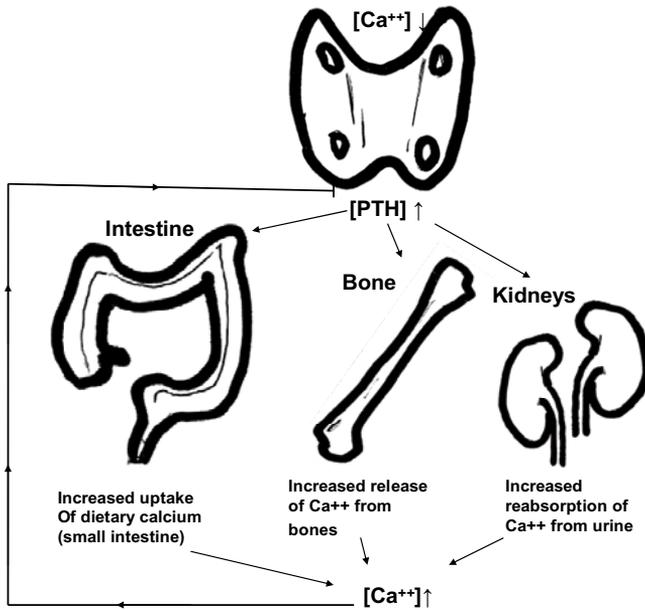


Figure 4. The role of PTH in calcium homeostasis, simplified. In fact, other hormones such as vitamin D, calcitonin and parathyroid related peptide (PTHrP) are also important players in this system.

Chief cells in the parathyroid gland can sense the extracellular calcium concentration through a G-protein coupled receptor localized on their surface membrane, assigned as the calcium sensing receptor (CaSR). CaSR has been previously suggested as the target autoantigen in autoimmune hypoparathyroidism⁵⁵; however, conflicting reports exist^{17, 56}. Moreover, CaSR has been shown to be almost ubiquitously expressed^{57, 58}.

The Lungs

Higher organisms such as mammals have through evolution lost their ability to deposit oxygen. Due to high metabolism and size of the organisms, uptake of oxygen through diffusion is not sufficient. Hence, more sophisticated respiratory organs such as gills and lungs have evolved. Gills were presumably the first respiratory organs and their remnants can still be seen in human embryos and in adults in the form of branchial cleft cysts^{59, 60}. Humans can survive without food for weeks, without water for days, but only less than five minutes without oxygen. The main function of the lungs is to provide continuous gas exchange between inspired air and the blood in the pulmonary circulation, supplying oxygen and removing carbon dioxide, which is then cleared from the lungs by subsequent expiration. Survival depends on this process being reliable, sustained and efficient, even when challenged by disease or an unfavourable environment⁵⁹.

Embryonic Development of the Human Lungs

In humans, the lungs start to develop in approximately 4-week-old embryo, when the respiratory diverticulum appears as an outgrowth from the ventral wall of the foregut, which will later represent the mainstem bronchi and lobes⁶⁰. Hence, major parts of the lungs including the epithelial cells of the respiratory tracts are of endodermal origin. The muscular and vascular components of the lungs originate from the splanchnic mesoderm surrounding the foregut⁶⁰. Taken together, lungs can be considered as organs that are embryonically closely related to the gastrointestinal channel.

Ion Channels of the Lungs

The best way to understand why ion channels are present in the lung is to consider their role through evolution. Due to osmotic pressure, fish in both salt and freshwater need an active mechanism to compensate for the passive electrolyte losses through diffusion over their body surfaces. Therefore, they need ion channels which probably have been maintained and conserved through evolution. Hence, ion channels are important for maintenance of the osmotic balance in fish⁶¹.

About 100 ml of sputum/mucus fluid is produced each day in the respiratory tract by the mucosal membranes' serous producing surface cells^{62, 63}. The purpose of this secretion is to 1) transport particles out of the airways, 2) provide antimicrobial environment, and 3) humidify the inspired air and prevent excessive fluid loss from the airway surface by its hydrophilic nature^{64, 65}. The importance of proper viscosity in the bronchial mucin comes to mind when patients with deficiency in this system are in need of medical care, as best exemplified by cystic fibrosis patients.

The lungs of individuals with cystic fibrosis are subject to bacterial colonization and infections from early age. These bacteria thrive in the altered mucus with low viscosity, which protects them from the immune cells of the host and antibiotics: The lungs respond to repeated damage by thick secretions and chronic infections by gradually remodeling the lower airways, a process called bronchiectasis, that makes infection even more difficult to eradicate⁶⁶.

Much is known about the activity and regulation of chloride and sodium channels in the lungs^{67, 68}. Examples of such channels are cystic fibrosis transmembrane conductance regulator Cl-channel (CFTR), and epithelial Na⁺ channel (ENaC). However, little is known about potassium channels in the lungs although several different types of potassium channels have been discovered and described in recent years⁶⁹.

This thesis concerns identification of a new protein in the lungs designated KCNRG. The structure KCNRG suggests that it has a regulatory function on potassium channels.

Obstructive Lung Disorders

Airway obstruction can occur due to excess of mucus in the airways (e.g. in cigarette-smoke caused chronic bronchitis) or broncho-constriction due to inflammation (e.g. in asthma). Disorders that impair air flow to the bronchioles of the lungs, leading to insufficient breathing, are categorized as obstructive lung diseases. During the exhalation, the diameter of the patient's airways decreases, in turn increasing the airway resistance that inhibits the patient's ability to empty their lungs (air trapping). This disease group can be distinguished from other lung disorders by a functional test named spirometry (meaning measuring of breath) (Figure 5).

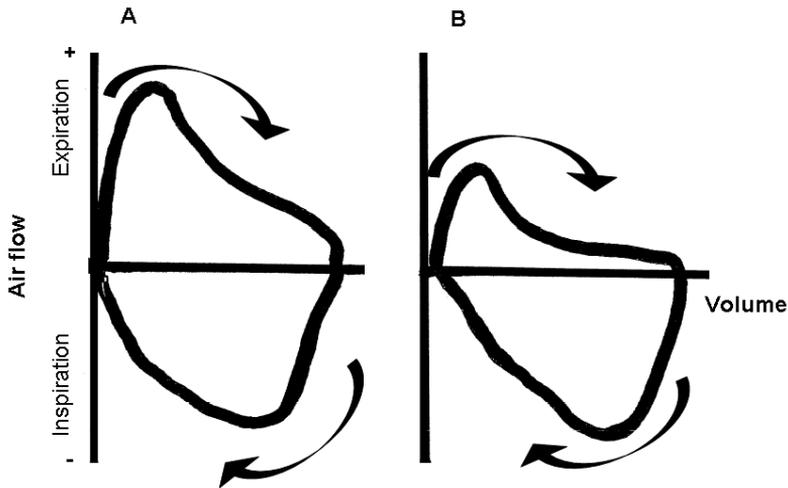


Figure 5. Flow-volume loop in spirometry. The Y-axis represents air flow. Positive values represent expiration and negative values represent inspiration. A) Typical flow-volume loop in a healthy person. B) Typical flow-volume loop in a person with obstructive lung disorder.

Asthma is a common disease in the general population and a typical form of obstructive lung disease. It occurs due to chronic inflammation in the airways, particularly in the parts of the airways which are not supported by cartilage ring. This chronic inflammation leads to hyper-sensitivity, which in turn can lead to bronchospasm and then dyspnoea due to impaired airflow in expiration. The chronic inflammation also causes an increase of the sputum production causing cough⁷⁰. Today asthma can be treated by using corticosteroid inhalations, bronchodilators and mucolytic drugs⁷¹, and sometimes other drugs such as leucotrien-antagonists and newer immune-modulating drugs⁷².

Another lung diseases of obstructive nature is chronic obstructive lung disease (COPD), affecting about 5% of the general population. COPD consists of broncho-obstruction and emphysema, and it is induced and provoked by smoking.

This thesis describes both the discovery of APS-1 patients suffering from respiratory symptoms similar to obstructive lung disease and the identification of an autoantigen that could explain the pathomechanism of this rare disease component.

The AIRE Deficient Mouse - an Animal Model for APS-1

In the last decade, several lines of AIRE-deficient mice, mimicking the genetic mutation in APS-1 patients, have been established^{4, 29, 73}. Except for poor fertility (Hässler et al., unpublished observations), these mice have displayed a very mild clinical phenotype with no destruction of the organs affected in APS-1 patients. Nevertheless, these mice have lymphocytic infiltrates in several organs and presence of autoantibodies has been shown by indirect immunofluorescence²⁹. It has been shown that the genetic background of the mouse strain on which AIRE deficiency is introduced affects the severity of the autoimmunity⁷⁴. In a recent report, the role of environmental danger signals on development of autoimmunity in AIRE-deficient mice has been evaluated; however, the experimental exposure of AIRE-deficient mice to danger signals in form of different ligands for Toll-like receptors did not affect the onset and outcome of autoimmunity⁷⁵. Microarray studies on mTECs from AIRE-deficient mice leads to the proposal of the promiscuous organ-specific autoantigen expression theory²⁹. This theory has had a major impact on understanding the role of AIRE in central tolerance.

Current Investigation

Aims

- I. Identification of serological similarities between APS-1 patients and AIRE-deficient mice.
- II. Identification of the parathyroid autoantigen in APS-1, as an attempt to elucidate the pathomechanism of this disease component.
- III. Identification of novel autoantigens in AIRE-deficient mice.

Results and Discussion

Comparative Serological Studies on APS-1 and AIRE-Deficient Mice (Paper I)

In order to be able to study the factors that trigger autoimmunity in APS-1 patients and to better understand the disease pathways, an animal disease model has been necessary. Until today different strains of AIRE-deficient mice have been generated^{4, 29, 74}. This mouse model displays some autoimmune features such as infertility and lymphocytic infiltrates in several organs. However, studies on AIRE-deficient mice have consistently shown that the phenotype in these animals is considerably milder than the APS-1 patients.

In the studies contributing to Paper I, we examined the presence of autoantibodies in sera from AIRE-deficient mice using immunofluorescence. We found that, in contrast to wild type mice, sera from AIRE-deficient mice stained liver and renal parenchyma (Figure 6).

Next, we sought to test whether these mice had autoantibodies to the same autoantigens as human APS-1 patients. Despite several attempts using the radioimmunoprecipitation-based autoantibody assay described in Paper II and IV, we could not detect any autoantibodies in sera from AIRE-deficient mice that could recognize any of the known human autoantigens. Together with the immunofluorescence results, these negative findings suggested that AIRE-deficient mice in fact had autoantibodies; nonetheless, these

autoantibodies were probably directed against other, yet undiscovered, autoantigens.

A disadvantage with our approach was that we searched for the presence of mouse autoantibodies directed against human autoantigens. A first spontaneous reflection on this approach could be that mouse autoantibodies should not be able to bind human autoantigens since their TCR and BCRs have never met these autoantigens. APS-1 associated autoantigens are, however, evolutionary conserved proteins with high homology between species. Moreover, it has also been shown that autoantibodies in general are usually directed against evolutionary conserved epitopes and hence inter-species recognition of autoantigens could be expected^{76,77}.

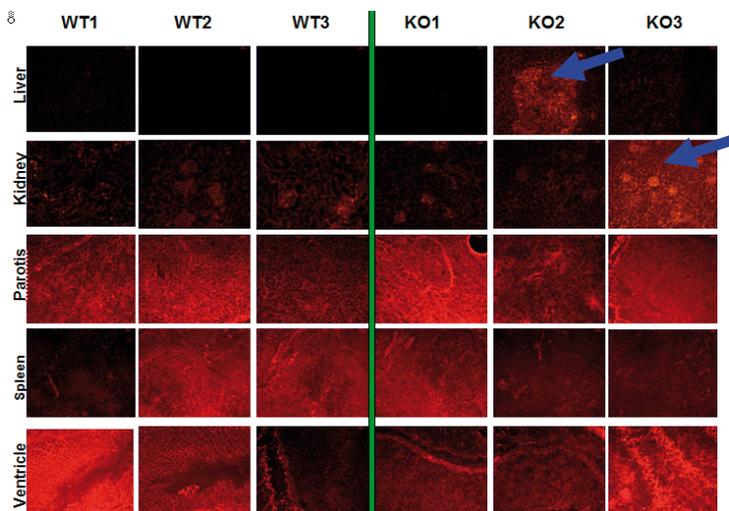


Figure 6. Indirect immunofluorescence on 6 μ m cryosections from different tissues from wild type mice, using sera from wild type mice (WT) (left panels) or AIRE-deficient mice (KO) (right panels). Red color is from the secondary antibodies conjugated to the fluorochrome CY3. The staining pattern in parotid glands, spleen and ventricle demonstrates the problem with background staining when trying to assess presence of autoantibodies in sera from AIRE-deficient mice. However, some of the AIRE-deficient mice displayed presence of specific autoantibodies directed against tissues such as liver and kidneys (blue arrows).

In order to completely confirm the absence of mouse autoantibodies directed against the known autoantigens in human APS-1, our collaborators in Finland cloned the mouse orthologs of the 14 known human autoantigens and used the protein products of these clones to evaluate the serum reactivity in AIRE-deficient mice. Except for the autoreactivity against insulin (Paper I, Figure 2), none of the other autoantigens were recognized by AIRE-deficient mouse sera. Although autoantibodies directed against insulin are indeed very good prediction markers for development of

type-1 diabetes^{78, 79}, insulin is not considered an autoantigen in human APS-1⁴⁶. Moreover, methods to assess anti-insulin autoantibodies have shown varying results in different laboratories compared to methods to detect other autoantibodies such as GAD-65⁷⁹.

Taken together, the efforts to find common serological markers for both APS-1 patients and the mouse model were fruitless. The reasons for the differences in the serological pattern of APS-1 patients and AIRE-deficient mice could be due to i) different environmental factors that trigger autoimmunity i.e. molecular mimicry caused by a microorganism that only infects humans⁸⁰⁻⁸², ii) different physiology in mice and human i.e. the APS-1 autoantigens may have a differential expression pattern in mice⁸³ or only have a minor physiologic role in mice and hence not being enough prominent to be targeted by the immune system, or iii) due to the fact that AIRE may control different genes in human and mice. In fact, it has recently been shown that the genetic background of the mouse strain used for generation of the AIRE-deficient mice influences the autoimmune feature of the animal⁷⁴.

As a final point, these results raise two important questions: i) is the AIRE-deficient mouse a good and sufficient disease model to study APS-1? ii) Is there a possibility that some human individuals with AIRE-deficiency, similar to AIRE-deficient mice, present only mild autoimmune symptoms and hence never get the diagnosis of APS-1?

Identification of Parathyroid Autoantigen in APS-1 (Paper II)

Hypoparathyroidism is one of the cardinal disease components of APS-1 and also one of the most difficult manifestations in the clinical management of APS-1. Hitherto, the pathomechanism for hypoparathyroidism has been unknown. The calcium sensing receptor was previously suggested as the autoantigen for autoimmune hypoparathyroidism⁵⁵; however, conflicting results to this statement have been reported^{17, 56}. Our aim was therefore to identify the parathyroid autoantigen in APS-1. The study started by immunoscreening of a bovine parathyroid cDNA library using sera from APS-1 patients with hypoparathyroidism. Unfortunately, we could not retrieve any relevant autoantigen for hypoparathyroidism from the bovine cDNA library. Some interesting findings could, however, still be extracted from this library as described in Paper IV.

Consequently, a new cDNA library from human parathyroid glands was constructed. Immunoscreening of the human library resulted in identification

of a new autoantigen named NLR family pyrin domain containing 5 (NALP5).

NALP5 (Figure 7) is a member of NACHT, leucine-rich repeat and PYD-containing protein (NALP) family. NALP5 is also known as maternal antigen that embryos require, (MATER). NALPs constitute a large subfamily of the CATERPILLER protein family⁸⁴ and consist of 14 members designated NALP1-14. NALPs are characterized by an N-terminal PYD domain proposed to be involved in protein-protein interactions, a central NACHT domain with a potential NTPase activity⁸⁵, an NAD domain with unknown function and C-terminal leucine-rich repeats (LRRs) believed to be involved in molecular pattern sensing and protein-protein interactions^{84, 86}. The NALP molecules are proposed to be important components of the “inflammasome”, a group of intracellular molecules similar to the Toll-like receptors in terms of pathogen molecular pattern recognition. An important role of NALP5 molecules might therefore involve pattern recognition. NALPs activate the immune system and are reported to be involved in multiple autoinflammatory disorders such as gout, vitiligo and activation of the immune system upon vaccination⁸⁷⁻⁹¹. The tissue expression of NALP5 was suggested to be specifically restricted to oocytes⁹² and an essential role for NALP5 had been addressed to early development since embryos to NALP5-deficient mice did not survive longer than the two-cell stage⁹².



Figure 7. Schematic illustration of the domains of NALP5

Strikingly, our observation was not the first finding of NALP5 as an autoantigen. NALP5 was, in fact, previously described as an autoantigen in mice thymectomized at day 3 of life (D3TX) which is a frequently used animal model for several organ-specific autoimmune disorders⁹³. D3TX mice develop several organ-specific autoimmune diseases. Among those, autoimmune ovarian disease (AOD) is one of the most studied manifestations. AOD is an equivalent to hypogonadism or autoimmune ovarian insufficiency seen in female APS-1 patients^{94, 95}.

Evaluation of NALP5 as an autoantigen in APS-1 using a standard autoantibody assay^{96, 97} revealed that 41% of the APS-1 patients had autoantibodies directed against NALP5. NALP5 was not structurally related to any of the other well established APS-1 autoantigens¹⁷, which reduced the probability for cross-reactivity of the autoantibodies directed against the other autoantigens in APS-1 such as members of the cytochrome P450 family including 21OH and SCC.

Correlation studies (Paper II, Table 1), demonstrated that the presence of NALP5 autoantibodies was significantly correlated to hypoparathyroidism. For hypoparathyroidism, the sensitivity and specificity of the NALP5 autoantibody test was 49% and 100%, respectively. For hypogonadism, a significant correlation was also found. NALP5 autoantibodies could be found in both male and female patients with APS-1. Notably, all of the patients with both hypogonadism and NALP5 autoantibodies were females. This could be because AOD affects female patients much more than ATF affects male patients with APS-1³³.

In the D3TX mice, NALP5 was suggested as the oocyte-specific autoantigen correlated to AOD. Interestingly, AOD is also diagnosed in half of all APS-1 patients causing either premature ovarian failure or delayed puberty³³. As NALP5 is expressed in ovaries, it may also have a function there as a disease-relevant autoantigen. In support of this suggestion are our results showing that NALP5-specific autoantibodies are significantly correlated with autoimmune ovarian insufficiency (Paper II, Table 1).

In keeping with the correlation studies, the tissue expression profiling of NALP5 demonstrated a restricted expression to the parathyroid glands and ovaries. Together, these findings provide a body of evidence supporting the original finding of NALP5 as a major parathyroid autoantigen in APS-1. However, one remaining concern is the lack of evidence to support the notion that NALP5 autoantibodies contribute to destruction of the parathyroid glands⁹⁸. A simple way to elucidate this issue would be start experiments to detect NALP5 autoantibodies autoreactive T cells that target NALP5 in the animal model for APS-1. Additionally, prospective studies in which presence of NALP5 autoantibodies are assessed before the onset of the clinical disease could contribute to the further understanding of the role of NALP5 in immunopathogenesis of APS-1 associated hypoparathyroidism⁹⁸.

Evaluation of NALP5 as a Common Autoantigen in both APS-1 Patients and the AIRE-Deficient Mouse Model (Paper III)

Considering that no common autoantigen exists in both APS-1 patients and AIRE-deficient mice, which we described in Paper I, we found identification of NALP5 very striking. Especially, since NALP5 had previously been suggested as an autoantigen in D3TX mice. In Paper III, we evaluated NALP5 as an autoantigen in AIRE-deficient mice. We found that AIRE-deficient mice did not have autoantibodies directed against the human ortholog of NALP5. Nevertheless, elevated levels of autoantibodies directed against the murine ortholog of NALP5 could surprisingly be detected in 20% of the animals studied (Paper III, Figure 1). This finding suggested NALP5 to be the first clinically relevant autoantigen common to both APS-1 and AIRE-deficient mice. We found no statistically significant difference in presence of NALP5 autoantibodies when comparing AIRE-deficient mice on B6 background with Balb/c background (Paper III, Figure 2). Interestingly, murine NALP5 autoantibodies were only present in female animals. This latter would be in line with the known oocyte expression of NALP5. However eosin/hematoxylin staining of parathyroid glands from AIRE-deficient mice revealed lymphocytic infiltrates suggesting that the parathyroid glands were also target for autoimmunity (Paper III, Figure 3). The parathyroid expression of NALP5 could be confirmed by our PCR analysis of murine parathyroid cDNA in which NALP5 transcripts could be detected. However, using commercially available antiserum against murine NALP5, we failed to detect protein expression of NALP5.

Further studies are currently being performed to complete our understanding of the NALP5 autoantibodies in AIRE-deficient mice.

Identification of KCNRG as a Novel Pulmonary Autoantigen (Paper IV)

As mentioned above, the bovine parathyroid library never resulted in identification of a parathyroid autoantigen. However, from that library, we retrieved a clone encoding a protein named KCNRG with predominant expression in the lungs (Paper IV, Figure 2). From the start, we could not find the clinical relevance of this clone. Two years after the discovery of this clone, we observed that patients with autoantibodies directed against the protein also suffered from mild to moderate pulmonary symptoms.

We were not able to detect KCNRG transcripts by PCR on human parathyroid cDNA, suggesting that KCNRG expression is absent or very low in human parathyroid glands. By immunohistochemistry we found that KCNRG protein was specifically expressed in the epithelial cells of the

terminal bronchiols of the lungs, previously unrecognized autoimmune target in APS-1 (Paper IV, Figure 2).

Through an informal international network of endocrinologist taking care of APS-1 patients, we recruited additional cases of APS-1 with respiratory symptoms (Paper IV, Table 1). In total we found eight patients and detected KCNRG autoantibodies in seven of these eight APS-1 cases with pulmonary symptoms. In two cases, the outcome of the respiratory symptoms was fatal. This suggests that KCNRG autoantibodies are useful markers for pulmonary disease in APS-1 and useful tools for clinicians caring for these patients. The KCNRG autoantibody test provides the possibility to assess whether pulmonary symptoms in an APS-1 patient are due pulmonary autoimmunity which can be life threatening, or whether these symptoms are due to concurrent obstructive lung disease or lower respiratory tract infections.

Although pulmonary autoimmunity hitherto has not been considered as a component of APS-1, the AIRE-deficient mice display pulmonary pathology of variable severity depending on the background strain. Interestingly, the expression of KCNRG is also decreased in mTECS of AIRE-deficient mice in the same manner as NALP5 (Hamish Scott, unpublished data). AIRE-deficient mice on C57BL6 and Balb/c background display modest pulmonary disease whereas AIRE-deficient mice on NOD and SJL background strain have severe and fatal lung pathology similar to the histological findings in our APS-1 patients⁷⁴. Phenotypic variability within the patients that we present may therefore depend on genetic background in humans.

As for the function of KCNRG, the information is still limited. What we know so far is that: i) Two human splice variants of KCNRG encoding 31- and 26-kDa isoforms have been characterized by ourselves and others⁹⁹. ii) KCNRG has a homology to the cytoplasmic tetramerization domain of voltage gated potassium channels and it inhibits potassium fluxes in vitro, suggesting that KCNRG may function as a potassium channel regulating protein⁹⁹. iii) We have experimentally confirmed the tendency of KCNRG to form tetramers in vitro (Paper IV, Supplementary Figure 2).

Although the exact role of KCNRG in the lungs remains to be determined, a role of potassium channels in histamine-induced bronchoconstriction and plasma exudation has been postulated, and drugs interfering with potassium channels have been proposed to treat bronchoconstriction^{100, 101}. It is also well recognized that autoantibodies to calcium and potassium channels cause autoimmune disease such as the Lambert-Eaton Myasthenic Syndrome¹⁰².

Conclusions

- I.** The autoantibody pattern is, in almost all cases, very different between APS-1 patients and the AIRE-deficient mice. This may be because AIRE controls different genes in men and mice.
- II.** Autoantibodies directed against NALP5 are present in 49% of the APS-1 patients with hypoparathyroidism. The presence of these autoantibodies is significantly correlated to hypoparathyroidism, suggesting NALP5 as a major parathyroid autoantigen in APS-1. The NALP5 autoantibody assay will be important for the timely diagnosis of APS-1 and APS-1 associated hypoparathyroidism.
- III.** NALP5 constitutes an autoantigen in AIRE-deficient mice. The autoantibody test for murine NALP5 may be of considerable use for assessment of autoimmunity in AIRE-deficient mice. Further studies are needed to determine factors that provoke development of NALP5 autoantibodies.
- IV.** A subset of APS-1 patients present with respiratory symptoms. The majority of these patients harbor autoantibodies directed against KCNRG, a protein with predominant expression in the epithelial cells of terminal bronchiole.

Future Investigations

- To further characterize NALP5 and its role in parathyroid physiology and pathology.
- To investigate the environmental factors that influence development of NALP5 autoantibodies in AIRE-deficient mice.
- To evaluate KCNRG as tumor antigen in different epithelial cell derived lung carcinomas.

Identification of NALP5 in parathyroid glands is indeed a valuable finding. Due to the predominant expression of NALP5 in parathyroid glands, this molecule could serve as a good marker for diseases in the parathyroid glands. Moreover, the tissue-specific expression of NALP5 makes the molecule attractive as a target to treat diseases in parathyroid glands such as parathyroid tumors. Although rare, parathyroid cancer is a neoplasm with poor prognosis, specially when the cancer has metastasized¹⁰³. In this disease immunotherapeutic methods in which the immune system is primed against a tumor-specific antigen could be an option and NALP5 could be such a candidate antigen.

In the nearest future, we plan to study expression of NALP5 in different parathyroid tumor samples as compared to normal parathyroid glands. In the event that NALP5 is overexpressed in parathyroid tumors, we intend to start evaluating NALP5 as a tumor marker.

Additionally the molecular function of NALP5 is still unknown and intriguing. The NALP protein family has attracted considerable interest during the recent years in the scientific literature^{87, 89, 91, 104-108}. The function of some of the NALPs has started to be elucidated. For NALP5, only the oocyte expression and its importance for oogenesis have been assessed. Hence, it will be interesting to investigate the molecular function of NALP5. Our preliminary data point at NALP5 responding to changes in extra-cellular calcium concentration (Figure 8).

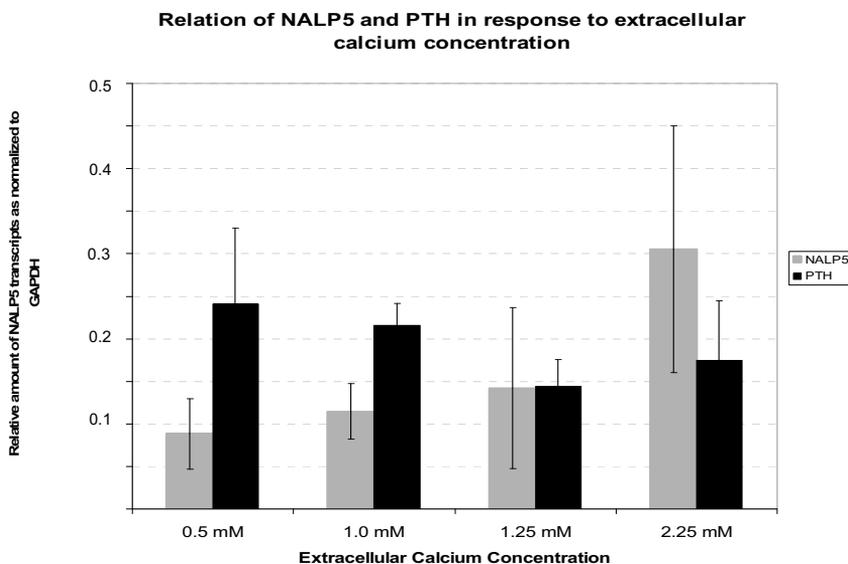


Figure 8. Bovine parathyroid chief cells response to changes in the extra-cellular calcium concentration. Expression levels of messenger RNA for PTH and NALP5 has been determined using quantitative PCR.

In future experiments, we intend to knock down the expression of NALP5 in bovine parathyroid chief cells using RNAi and to study the changes in expression of other genes as well as the physiological function of these cells. With these approaches, we expect to get a better understanding of the molecular functions of NALP5 in the parathyroid glands.

As for the mechanisms leading to targeting of NALP5 as an autoantigen, it would be interesting to study the AIRE-deficient mice. One could for example test the influence of different diets or infections on the development of NALP5 autoantibodies. A possible experiment would be to use AIRE-deficient mice as sentinel animals in cages with well defined content of pathogens.

The discussion on NALP5 as a possible tumor antigen in parathyroid cancers is of course also valid for KCNRG. Soon, we will try to recruit tumor biopsies from different subtypes of lung carcinoma and by immunohistochemistry evaluate the expression of KCNRG.

Finally, we are continuing our attempts to identify other tissue-specific autoantigens in APS-1.

Summary of the Thesis in Swedish

Populärvetenskaplig sammanfattning på svenska

Under evolutionen har alla däggdjur utvecklat ett sofistikerat immunsystem med främsta funktionen att skydda oss mot inkräktande mikroorganismer. För att åstadkomma detta, är det nödvändigt att immunsystemet skall kunna skilja mellan själv och icke-själv.

Under vissa omständigheter klarar immunsystemet inte att skilja mellan själv och icke-själv vilket leder till att immunsystemet av misstag attackerar kroppsegna strukturer och förstör dessa. Detta fenomen kallas för autoimmunitet. Det finns en rad olika autoimmuna sjukdomar som kan exemplifieras med folksjukdomarna insulinberoende diabetes, reumatoid artrit och multipel skleros. Dessa sjukdomar kan innebära stort lidande för den enskilda patienten och är dessutom mycket kostsamma för samhället.

Idag finns det många sjukdomar som anses vara autoimmunt betingade och ett flertal sjukdomar med tidigare okänt mekanism som misstänks bero på autoimmunitet. Autoimmuna sjukdomar uppvisar i regel ett komplext nedärvningsmönster, vilket innebär att de uppkommer när flera olika faktorer som t.ex. arv, miljö och slump samverkar. Komplexiteten av sjukdomsalstrande parametrar gör dessa sjukdomar svåra att studera och försvårar därmed utvecklingen av nya behandlingsmetoder. För att kunna förstå autoimmuna sjukdomar, och på sikt använda dessa kunskaper till behandlingsmetoder, är autoimmuna sjukdomsmodeller av stor betydelse.

Autoimmunt polyendokrint syndrom typ 1 (APS-1) är en sällsynt autoimmun sjukdom som framför allt förekommer i genetiskt isolerade befolkningar, exempelvis på Sardinien och persiska judar. Patienter med APS-1 utvecklar autoimmunitet mot flertal olika organ. Sjukdomen karakteriseras av flera delkomponenter som t.ex. autoimmunt betingat underfunktion i bisköldkörtlarna (autoimmunt hypoparatyreoidism), autoimmunitet mot binjurebarken (Addisons sjukdom) och diabetes mellitus. Trots att APS-1 betraktas som en sällsynt sjukdom är de ingående sjukdomskomponenterna, i sina isolerade former, vanligt förekommande i befolkningen t.ex. insulinberoende diabetes, som är att betrakta som en folksjukdom.

Man har på senare år identifierat den gen som skyddar mot av APS-1 och visat att mutationer i denna gen leder till utveckling av APS-1. Till skillnad från de komplexa autoimmuna sjukdomarna orsakas APS-1 av defekter i en enda gen, vilket gör den lämplig för studier som syftar till att öka förståelsen om uppkomsten av autoimmunitet.

Denna avhandling inkluderar studier som syftar till att kartlägga uppkomstmekanismen av två sjukdomskomponenter vid APS-1. I dessa studier fann vi nya markörer för diagnostik av autoimmun bisköldkörtelunderfunktion samt autoimmuna lungsymptom. Dessutom har studier genomförts för att jämföra immunreaktionen hos APS-1 patienter med immunreaktionen hos musmodellen för sjukdomen.

Förhoppningen är att dessa resultat på sikt skall kunna användas till att utveckla nya terapimetoder för APS-1 och även andra autoimmuna sjukdomar.

Detaljer om ingående delarbeten:

Delarbete 1:

I och med att man lyckades identifiera den gen som orsakade APS-1, kunde man under 2001 utveckla en musmodell för APS-1. Detta innebär att man har tagit fram en musstam med samma genetiska mutation som hos APS-1 patienter. Det som har varit förbryllande är att dessa möss är förvånansvärt friska och uppvisar inte samma sjukdomsbild som människor med APS-1. Detta har varit en besvikelse för läkare och forskare inom fältet eftersom man då inte har haft möjlighet att utnyttja musmodellen till att ta reda på vilka olika faktorer som kan inducera eller förebygga sjukdomen. I detta delarbete, har vi systematisk jämfört immunsvaret hos musmodellen för APS-1 med immunsvaret för APS-1 patienter och kommit fram till att musmodellen inte har immunreaktion mot de hittills kända målstrukturerna (s.k. autoantigener) hos APS-1 patienter. Arbetet belyser vikten av att man behöver identifiera gemensamma markörer mellan musmodellen och APS-1 patienter innan man kan komma vidare med jämförande (s.k. translationella) studier.

Delarbete 2:

Bisköldkörtlarna är de senast upptäckta anatomiska organen hos människan och beskrevs av Ivar Sandström 1879 i Uppsala. Dessa körtlar, som också kallas paratyreoideakörtlar, producerar parathormon som är nödvändig för reglering av kalkbalansen i kroppen. Paratyreoideakörtlarna är således livsnödvändiga. Ett klassiskt kännetecken för APS-1 är att patienternas immunsystem slår ut bisköldkörtelfunktionen. Detta leder till allvarliga och svårbehandlade kalkrubbingar hos patienterna som utan korrekt behandling

utvecklar kramper som kan ha dödligt utgång. Man har hittills inte känt till exakt vilka strukturer i bisköldkörtlarna som retar immunsystemet och resulterar i en autoimmun destruktion av dessa körtlar. Denna kunskapslucka har begränsat möjligheten till diagnostik av en begynnande destruktion av bisköldkörtlar. I delarbete 2 identifieras den målstruktur i bisköldkörtlarna som igenkänns och angrips av APS-1 patienters immunsystem. Denna målstruktur, benämnd NALP5, är ett protein med hittills oklar funktion. Identifieringen av NALP5 som mål för immunsystemet har varit en viktig upptäckt som nu underlättar diagnostiken av ett begynnande immundestruktion av bisköldkörtlarna.

Delarbete 3:

Här visar vi att NALP5 är den första påvisade strukturen som angrips av immunsystemet hos både APS-1 patienter och musmodellen för APS-1. Som beskrivet i förklaringen till delarbete 1, är identifieringen av en gemensam nämnare hos APS-1 patienterna och musmodellen för APS-1, mycket viktig. Upptäckten av NALP5 som mål för immunangrepp hos både möss och människor, öppnar många möjligheter till translationella studier, vilka sannolikt kommer att öka förståelsen kring vilka faktorer som kan påverka uppkomsten av autoimmuna reaktioner.

Delarbete 4:

Ungefär fem procent av APS-1 patienterna uppvisar andningssymtom som påminner om astma. I enstaka fall har dessa lungsymptom haft dödlig utgång. Eftersom APS-1 är en ganska allvarlig sjukdom med flera olika sjukdomskomponenter som innebär stort lidande för patienterna, har man hittills inte trott att de lungsymtomen kan ha en direkt koppling till sjukdomen. I delarbete 4 uppmärksammar vi lungsjukdom hos APS-1 patienterna och presenterar resultat som visar att denna sjukdomskomponent sannolikt är autoimmunt betingat. Vi identifierar dessutom exakt vilken struktur som immunsystemet hos APS-1 patienterna känner igen och angriper i lungorna. Denna målstruktur är ett protein benämnt KCNRG och finns i de fina luftrören i lungorna. KCNRG uppvisar likheter med kalium jonkanaler och är förmodligen viktig för att upprätthålla de osmotiska trycket i de fina luftrören. Upptäckterna i delarbete 4 erbjuder därmed möjlighet att man på ett tidigt stadium och med ett enkelt blodprov kan identifiera pågående autoimmunt angrepp mot lungvävnad hos APS-1 patienter så att rätt behandling kan insättas.

Sammanfattning:

Autoimmuna sjukdomar är vanligt förekommande och orsakar stort lidande för drabbade patienter och är även kostsamma för samhället. APS-1 är en ovanlig ärftlig autoimmun sjukdom som har visat sig vara en lämplig modellsjukdom för förståelse av autoimmunitet. I avhandlingen ingår studier som leder fram till upptäckt av diagnostiska markörer för två av sjukdomskomponenterna vid APS-1, nämligen autoimmunt betingat underfunktion i bisköldkörtlar samt autoimmun lungsjukdom. Resultaten bidrar inte enbart till ökad förståelse av autoimmuna sjukdomar, utan är även särskilt intressanta eftersom de ger nya kunskaper om den molekylära funktionen av bisköldkörtlar och lungor.

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