Biomarker Discovery in Tissue-specific Autoimmune Disease

NILS LANDEGREN
Abstract


Autoimmune diseases encompass a diverse group of disorders that collectively affect 5% of the population. Despite large clinical variability, autoimmune disorders share a common etiology in that they all develop from immune responses against self. T-cell receptors and antibodies recognize distinct self-molecules and direct destructive effector mechanisms to the target organs. Characterization of autoimmune targets can help in the understanding autoimmune disease features and is of additional importance for subsequent use in clinical diagnosis.

Rare monogenic disorder can provide an access to the study and understanding of mechanisms underlying common and more complex diseases. Autoimmune polyendocrine syndrome type 1 (APS1) is an autosomal recessive disorder caused by mutations in the AIRE gene, and is a valuable model of tissue-specific autoimmune disease. APS1 patients develop multiple autoimmune disease manifestations and display autoantibodies against the affected tissues.

Recent development in protein array technology has opened a novel avenue for explorative biomarker studies in autoimmune disorders. Present-day protein arrays contain many thousands of full-length human proteins and enable autoantibody screens at the proteome-scale. In the current work I have utilized proteome arrays to perform a comprehensive study of autoimmune targets in APS1. Survey of established autoantigens revealed highly reliable detection of autoantibodies, and by exploring the full panel of 9000 proteins we further identified three novel, major autoantigens. Our findings revealed a marked enrichment for tissue-specific immune targets and further suggest that only a very limited portion of the proteome becomes targeted by the immune system in APS1. This work identifies prostatic transglutaminase 4 as novel male-specific autoantigen. In the mouse model of APS1 we could link TGM4 immunity with a tissue-destructive prostatitis, a compromised prostatic secretion of TGM4 and with defect in the establishment of central immune tolerance for TGM4. Our findings suggest prostate autoimmunity is a major manifestation in male APS1 patients with potential role in development of subfertility. In this doctoral work we also report on collecting duct autoantibodies in APS1 patients with interstitial nephritis and on the identification of aquaporin 2 as a collecting duct autoantigen. Collectively, the present investigations provide an overview-perspective on the autoimmune target repertoire in APS1 and identify novel autoimmune manifestations of the syndrome.

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List of Papers

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Abbreviations

ALPS5  Autoimmune lymphoproliferative syndrome, type 5
BLAST  Basic Local Alignment Search Tool
APS1   Autoimmune polyendocrine syndrome type 1
BSA    Bovine serum albumin
DDC    Aromatic L-amino acid decarboxylase
GAD    Glutamate decarboxylase
GAL    GenePix array list
GST    Glutathione s-transferase
HLA    Human leukocyte antigen
HOXB7  Homeobox B7
IFNA   Interferon alpha
IFNW   Interferon omega
IgG    Immunoglobulin G
IPEX   Immunodysregulation, polyendocrinopathy, and enteropathy, x-linked
IVIG   Intravenous immunoglobulin
MAGEB  Melanoma antigen family B
MIM    Mendelian inheritance in man
NFAT5  Nuclear factor of activated T-cells 5
mTEC   Medullary thymic epithelial cells
PDILT  Protein disulfide isomerase-like testis expressed
RA     Rheumatoid arthritis
SD     Standard deviations
SLE    Systemic lupus erythematosus
TGM    Transglutaminase
Introduction

Definition and classification of autoimmune diseases

- How to recognize an autoimmune disorder when you see one

Autoimmune diseases encompass a clinically diverse group of disorders that collectively affect 5% of the population. Despite large variability in disease expression, autoimmune disorders share a common etiology – they all develop from a self-reactive response elicited by the adaptive immune system. Although easily defined in theory, it is more challenging to recognize an autoimmune disease in practice, and systems therefore have been suggested for the definition of autoimmune disease. In 1957, Earnest Witebsky noted that rabbits immunized with thyroid extracts developed features mimicking those of patients suffering from chronic thyroiditis. He believed his observations in rabbits supported an autoimmune pathogenesis of thyroiditis in man. Witebsky further suggested a set of criteria that could be used in the recognition of autoimmune diseases, similar to what Robert Koch and Friedrich Loeffler earlier had introduced for infectious disease. Witebsky's defining criteria for autoimmune disease have been revisited and adapted more recently (Table 1).

Firstly, autoimmune diseases share some typical features that can serve as circumstantial evidence for an autoimmune etiology. These include coinheritance and comorbidity with established autoimmune diseases and strong association with certain human leukocyte antigen (HLA) haplotypes. Other typical features of autoimmune disease are therapeutic response to immunomodulatory agents, such as cortisone, methotrexate, anti-TNF alpha etc, or to autoantibody/B-cell depleting therapy, including plasmapheresis, intravenous immunoglobulin (IVIG) and anti-CD20 therapy. Lymphocytic infiltration of affected organs is further a typical pathological findings consistent with but not specific for an autoimmune etiology.

Indirect and direct evidence for an autoimmune etiology could, according to Witebsky, only be obtained from studies in experimental animals. If disease features can be successfully induced in experimental animals by active immunization, as was the case in Witebsky’s thyroiditis model, this would represent indirect evidence for an autoimmune etiology. If disease features could be reproduced in a healthy recipient by only passive transfer of auto-
antibodies, or T-cells as later added, the criteria for direct evidence would be met.

Pathogenicity of autoantibodies in diseases such as myasthenia gravis or Graves’ disease can be demonstrated by transfer of patient serum to experimental animals, or as aptly illustrated in the natural situation, in a newborn who has received autoantibodies by active transplacental immunoglobulin G (IgG) transport from a mother affected by the disease. Pathogenicity of autoantibodies has, however, only been demonstrated in a small number of diseases, and for most diseases generally regarded as autoimmune it is believed that tissue-destruction is effectuated by T-cells and not by autoantibodies. For example, newborns of mothers suffering from Addison’s disease do not develop adrenal disease despite receiving 21-hydroxylase autoantibodies during the fetal period. As T-cells recognize their antigen in association with HLA, it is a challenge to demonstrate pathogenic effect of patient derived T-cells in experimental animals. In fact, few diseases have fulfilled the criteria for direct evidence of an autoimmune etiology.

<table>
<thead>
<tr>
<th>Circumstantial evidence</th>
<th>Coheritability with autoimmune diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLA association</td>
</tr>
<tr>
<td></td>
<td>Therapeutic response to immunomodulatory agents</td>
</tr>
<tr>
<td></td>
<td>Lymphocytic infiltration of target organs</td>
</tr>
<tr>
<td>Indirect evidence</td>
<td>Successful induction of disease symptoms in experimental animals by active immunization</td>
</tr>
<tr>
<td>Direct evidence</td>
<td>Successful induction of disease symptoms in experimental animals or newborns by passive immunization (transfer of autoantibodies or T-cells)</td>
</tr>
</tbody>
</table>

Table 1. Autoimmune disease criteria. Adapted from Witebsky.

Autoimmune diseases may be classified as being either systemic or tissue-specific (Table 2). The former group includes diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis and Sjögren’s syndrome. In this group of disorders, disease activity may for long periods be confined to distinct organs – to the joints in RA patients, to the salivary and lacrimal glands in patients with Sjögren’s syndrome or to the skin in patients with systemic sclerosis, but may also at periods flare up with severe systemic manifestations or additional organ involvement such as glomerulonephritis or alveolitis. Immunomodulatory agents such as cortisone, methotrexate and anti-TNF alpha, are effectively used to hamper inflammatory activity. Systemic autoimmune diseases are further associated with autoantibodies against ubiquitous molecules, typically nuclear components such as DNA, histones, topoisomerase, ribonuclear RNA etc.

Tissue-specific autoimmune diseases can be exemplified by type 1 diabetes, Addison’s disease and autoimmune gastritis. In these disorders, the autoimmune response is directed against distinct tissues, and is not associated
with systemic manifestations. The affected tissue is in general completely destroyed, thereby the autoimmune disease activity becomes self-limiting. Substitution therapy is often required to compensate the function of failing tissues, such as insulin in type 1 diabetes, steroids in Addison’s disease and vitamin B12 in autoimmune gastritis. Typical autoantigens in tissue-specific autoimmune diseases are molecules with restricted expression pattern and key functions in the affected tissues.

<table>
<thead>
<tr>
<th></th>
<th>Systemic autoimmune disease</th>
<th>Tissue-specific autoimmune disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Typical diseases</strong></td>
<td>RA, SLE, systemic sclerosis, Sjögren’s</td>
<td>T1 diabetes, Addison’s disease, autoimmune thyroiditis</td>
</tr>
<tr>
<td><strong>Typical therapy</strong></td>
<td>Immunomodulatory (cortisone, methotrexate, anti-TNF alpha)</td>
<td>Substitution (insulin, steroids, vitamin B12)</td>
</tr>
<tr>
<td><strong>Typical autoantigens</strong></td>
<td>Ubiquitous molecules (DNA, histones, topoisomerase)</td>
<td>Tissue-specific molecules (GAD2, CYP21A2, TPO)</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of systemic and tissue-specific autoimmune diseases.

**Autoantibodies**

- A hallmark of autoimmune diseases

Autoimmune responses can fundamentally be defined at the molecular level by the specific interaction between T-cell or B-cell receptors and a distinct self-molecule. Autoimmune diseases typically involve a combined cellular and humoral response with the cognate target specificities\(^\text{10-12}\), and while the T-cell response is often directly responsible for tissue damage the B-cell response is in general better exploited for disease monitoring. Characterization of autoimmune targets is critical for understanding autoimmune disease mechanisms and can often help to explain disease features such as the target specificity of the autoimmune insult. This is well exemplified by autoimmune adrenalitis, characterized by autoantibodies against 21-hydroxylase\(^\text{13}\). 21-hydroxylase is an enzyme that is involved in the production of glucocorticoid and mineralocorticoid steroid hormones and that is exclusively expressed in the adrenal cortex. The expression pattern of 21-hydroxylase therefore explains the targeted destruction of the adrenal cortex seen in Addison’s disease. A subset of patients with Addison’s disease harbor autoantibodies against side chain cleavage enzyme\(^\text{14}\), which is expressed not only in the adrenal cortex but also in the gonadal steroidogenic cells. Importantly, the presence of autoantibodies against side chain cleavage enzyme in female patients with Addison’s disease is associated with an increased risk of developing autoimmune ovarian failure\(^\text{15}\). This observation pertinently illustrates how autoim-
mune responses are governed by the expression pattern of the target antigen and how the definition of autoantigens can help in the understanding of disease features.

Parts from being markers of cell-mediated autoimmunity, autoantibodies may also have a direct causative role in disease development. Autoantibodies may cause disease by exerting functional effect on receptors – either inhibiting signal transduction or causing a constitutive hyper-activation. In myasthenia gravis for examples, autoantibodies block the function of the nicotinic acetylcholine receptor at the motor endplate, thereby disrupting neuromuscular signal transmission and causing fluctuating weakness in the patient. In Graves’ disease, on the other hand, autoantibody binding causes a constitutive activation of the thyroid stimulating hormone receptor (TSHR) and subsequent overproduction of thyroid hormone. Autoantibody binding may further inhibit enzymatic activity of humoral factors or clear them from circulations, as exemplified by autoantibodies against ADAMTS13 in patients with immune mediated thrombocytopenic purpura or FVIII autoantibodies in acquired hemophilia A. Autoantibodies against extracellular matrix proteins may cause matrix destabilization or to provoke complement activation. This is exemplified by Goodpasture syndrome, where autoantibodies against collagen 4 alpha 3, present in the glomerular and alveolar basement membranes, cause glomerulonephritis and pulmonary hemorrhage. Likewise, in acquired epidermolysis bullosa autoantibodies against collagen 7 alpha 1, which is an anchoring fibril in the dermal–epidermal basement zone, cause severe subepithelial blistering.

Defining autoantibodies is not only important for understanding autoimmune disease mechanisms but also for clinical diagnosis. While being easily accessible in peripheral blood, autoantibodies have the potential to reveal autoimmune disease processes in internal organs with high degree of certainty. Autoantibody markers have acquired a central role both in research and in clinical diagnosis of autoimmune disorders, providing means for secure diagnosis, stratification of patients with respect to the underlying etiology and for disease prediction. For comparison it can be noted that despite enormous efforts the last decades to identify tumor markers, serological markers still have a very limited role in the clinical diagnosis of patients with cancers. Autoantibody markers, on the other hand, are absolutely central in the clinical diagnosis of autoimmune diseases such as type 1 diabetes, Addison’s disease, autoimmune thyroiditis, celiac disease and many more. Autoantibody markers may not only help support diagnosis of an ongoing disease but can also predict future development of disease. Predictive markers are most useful in evaluating individuals with increased risks, such as patients with autoimmune diseases who face an increased risk of developing a second one, or individuals with strong family history of autoimmune diseases. Autoantibody markers are further important in providing etiological information – indicating an autoimmune genesis rather than other disease etiologies. This
quality is highly valuable in genetic studies for example, where autoantibody assessment can help securing a homogenous disease cohort and to avoid blunting the analyses with the inclusion of misdiagnosed patients. I have included examples of autoimmune diseases with well-defined autoantigens below (Table 3).

<table>
<thead>
<tr>
<th>Autoimmune diseases</th>
<th>Autoantigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoimmune thyroiditis</td>
<td>TPO&lt;sup&gt;26&lt;/sup&gt;</td>
</tr>
<tr>
<td>Graves’ disease</td>
<td>TSHR&lt;sup&gt;17&lt;/sup&gt;</td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>GAD2&lt;sup&gt;27&lt;/sup&gt;</td>
</tr>
<tr>
<td>Addison’s disease</td>
<td>CYP21A2&lt;sup&gt;13&lt;/sup&gt;</td>
</tr>
<tr>
<td>Autoimmune ovarian failure</td>
<td>CYP11A1&lt;sup&gt;14&lt;/sup&gt;</td>
</tr>
<tr>
<td>Autoimmune gastritis</td>
<td>ATP4A&lt;sup&gt;28&lt;/sup&gt;</td>
</tr>
<tr>
<td>Celiac disease</td>
<td>TGM2&lt;sup&gt;29&lt;/sup&gt;</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>CHRNA1&lt;sup&gt;16&lt;/sup&gt;</td>
</tr>
<tr>
<td>Neuromyelitis optica</td>
<td>AQP4&lt;sup&gt;20&lt;/sup&gt;</td>
</tr>
<tr>
<td>Goodpasture syndrome</td>
<td>COL4A3&lt;sup&gt;21&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acquired epidermolysis bullosa</td>
<td>COL7A1&lt;sup&gt;22&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thrombotic thrombocytopenic purpura</td>
<td>ADAMTS13&lt;sup&gt;18,19&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acquired haemophilia A</td>
<td>F8&lt;sup&gt;20&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 3. Examples of autoimmune diseases with well-defined autoantigens. Thyroid peroxidase (TPO), thyroid stimulating hormone receptor (TSHR), glutamate decarboxylase 2 (GAD2) – also known as GAD65, cytochrome P450 family 21 subfamily A polypeptide 2 (CYP21A2) – also known as 21-hydroxylase, cytochrome P450 family 11 subfamily A polypeptide 1 (CYP11A1) - also known as side chain cleavage enzyme, ATPase H+/K+ exchanging alpha polypeptide (ATP4A) - subunit of the gastric proton pump, T transglutaminase 2 (TGM2) – also known as tissue transglutaminase, cholinergic receptor nicotinic alpha 1 (CHRNA1) – subunit of the pentameric nicotinic acetylcholine receptor, aquaporin 4 (AQP4), collagen type IV alpha 3 (COL4A3), collagen type VII alpha 1 (COL7A1), ADAM metallopeptidase with thrombospondin type 1 motif 13 (ADAMTS13) and coagulation factor VIII, procoagulant component (F8).

Biomarker discovery

- **How to find a novel autoantigen**

Identifying novel autoimmune targets is important for understanding and diagnosing autoimmune diseases. But how can it be achieved? As shown by review of past discoveries, many important autoantigens have been identified in dedicated, hypothesis driven efforts. For example, the identification
of the major autoantigen type 1 diabetes was a result of observations of the rare neurological disorder stiff person syndrome\textsuperscript{31} and the subsequent focused investigation of glutamic acid decarboxylase as a candidate autoantigen\textsuperscript{27}. Similarly, the identification of AQP4 as the autoantigen in neuromyelitis optica came about following the recognition of a characteristic autoantibody staining pattern and then a focused candidate approach\textsuperscript{30}.

However, in many cases hypotheses and inspired guesses cannot take you all the way and systematic approaches are instead required. Mass spectrometry presents one such opportunity, typically used in combination with 2D gel separation. An alternative approach is to clone the autoantigen from a cDNA library by immunoscreening, which has proved successful in many studies in APS1\textsuperscript{32-36}. Proteome arrays have emerged as a novel avenue for explorative biomarkers studies.

**Proteome arrays**

- Three-fourth of a human on a single slide

Protein array technology enables parallel studies of thousands of proteins and opens for novel ways to explore autoimmune diseases. The principle of spotting antigen onto a surface for use in autoantibody detection was examined already in the early 1960’s. George Feinberg demonstrated how minute amounts of antigen could be deposited onto a coated microscope glass slide using a fine capillary tube\textsuperscript{37} and how the system could then be applied for detecting autoantibodies against thyroglobulin in sera from patients with autoimmune thyroiditis\textsuperscript{38}. It would, however, take another couple of decades before the protein array technology took off on a greater scale. Proteome arrays followed in the wake of the DNA microarray revolution during the 1990’s, benefitting from the development of instruments for high-density array printing and fluorescence scanning. In a pioneering work, Heng Zhu and Michael Snyder constructed a protein array that included a majority of the open reading frames in yeast\textsuperscript{39}. The technology was commercialized through a start-up company, Protometrix, which was later acquired by Invitrogen (now Life Technologies). A human proteome array was developed, ProtoArray®, containing over 9000 full-length human proteins. In this system, proteins are expressed as glutathione s-transferase (GST)-fusions in insect cells using baculovirus vectors, purified and then spotted in duplicate onto nitrocellulose-coated glass slides. The ProtoArray® recently got a strong competitor. CDI laboratories launched the HuProt® array that contains almost 20,000 human full-length proteins, covering around three-fourths of the human protein coding genes. Proteome arrays have been used for various applications in life science research, including studies of protein-protein interaction\textsuperscript{39}, DNA-binding\textsuperscript{40}, RNA-binding\textsuperscript{41} and post-translational
modification. They have also gained popularity in the industry for validating binding specificity of affinity reagents. And importantly, proteome arrays provide a very attractive system for autoantibody biomarker studies. For this application arrays are first incubated with diluted patient and control sera followed by incubation with a fluorescence labeled anti-human IgG. An anti-GST detection reagent can also be applied to evaluate protein content. Fluorescence signals are recorded using a microarray scanner that excites the fluorophores with lasers and detects the emitted light, thereby producing a false-color image of the IgG and the GST channels (Figure 1). The scanned image must then be aligned with a GenePix array list (GAL) file that contains the array target identities in each position before the data can be accessed.

**Figure 1.** Cropped image of a proteome array. The image shows the IgG channel of a microarray slide that has been probed with a serum sample.

### Autoimmune disease models

- **Of mice and men**

Rare monogenic disorders have proved invaluable for the understanding of common and more complex disorders such as Alzheimer’s disease and dyslipidemia. Most autoimmune diseases develop from a complex setting that involve a combination of environmental factors and a polygenic genetic background. There are, however, a few autoimmune diseases that develop from mutations in a single gene (Table 4). These rare disorders, and their corresponding mouse models, have been instrumental for the mechanistic understanding of autoimmune disease. APS1 (Mendelian inheritance in man (MIM) number 240300) is an autosomal recessive disorder that features multiple tissue-specific autoimmune manifestations. APS1 has been a main focus in this thesis work and will be discussed in further detail in the coming section. A mouse model of APS1 has been generated that recapitulates fea-
tures of APS1. Immunodysregulation, polyendocrinopathy, and enteropathy, x-linked (IPEX) (MIM# 304790) is a severe x-linked disorder that debuts in infancy. Individuals with IPEX typically develop enteropathy, type 1 diabetes and dermatitis, but may also suffer from additional autoimmune manifestations, such as hypothyroidism, thrombocytopenia, autoimmune hemolytic anemia, lymphadenopathy, nephritis and hepatitis. Scurfy is an x-linked mouse mutant that features multiple autoimmune manifestations and has been extensively studied as a spontaneous autoimmune disease model. The Scurfy mouse and IPEX were both linked to mutations in the forkhead box P3 (FOXP3) gene. FOXP3 encodes a transcription factor that is necessary for the development and function of T-reg cells. IPEX and the Scurfy mouse have illustrated an important role of peripheral tolerance mechanisms in the development of autoimmune disease. The cytotoxic T-lymphocyte-associated protein 4 (CTLA4) is costimulatory molecule expressed by activated T-cells. CTLA4 knock out mice develop lymphocytic infiltration in multiple organs, and has been studied as an autoimmune disease model for two decades. A human disease counterpart was recently described - autoimmune lymphoproliferative syndrome, type 5 (ALPS5) (MIM#616100) – a dominant autosomal disorder due to mutations in CTLA4. Individuals with ALPS5 develop lymphocytic infiltration in multiple organs, including the gastrointestinal tract, lungs and brain. These rare monogenic disorder and their mouse models exemplify different pathogenic routes that all emanate in tissue-destructive autoimmunity, illustrating the variety of mechanism that can be at play in the development of autoimmune disease.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Disease (MIM#)</th>
<th>Animal model</th>
<th>Main mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIRE</td>
<td>APS147,48 (240300)</td>
<td>Aire-deficient mouse47,48</td>
<td>Defect of central immune tolerance</td>
</tr>
<tr>
<td>FOXP3</td>
<td>IPEX50 (304790)</td>
<td>&quot;Scurfy” Foxp3-deficient mouse49</td>
<td>Defect of T-regs</td>
</tr>
<tr>
<td>CTLA4</td>
<td>ALPS552,53 (616100)</td>
<td>Ctla4-deficient mouse51</td>
<td>Defect of T- and B-cell homeostasis</td>
</tr>
</tbody>
</table>

Table 4. Monogenic autoimmunity syndromes and their mouse models. Mendelian inheritance in man number, MIM#.
Autoimmune polyendocrine syndrome type 1

- A rare disease that features many common components

APS1 is a rare monogenic disorder that has been central for the understanding of central immune tolerance and its role in tissue-specific autoimmune disease. After few case reports in the mid 20th century, Neufeld provided the first description on a larger series of patient with APS1 in 1980. Ahonen and Perheentupa ten years later presented a detailed description and longitudinal follow-up of a series of Finnish patients with APS1. APS1 has also been reviewed more recently.

APS1 is clinically defined by three hallmark components; chronic mucocutaneous candidiasis, hypoparathyroidism and adrenal failure, of which two are required for diagnosis. The first manifestation typically presents in infancy or early childhood and is in most patients represented by candidiasis. With increasing age APS1 patients typically accumulate a number of disease manifestations in endocrine and non-endocrine organs, such as gonadal failure, type 1 diabetes, autoimmune gastritis, autoimmune hepatitis, vitiligo, alopecia and tubulointerstitial nephritis (Table 5). Infertility is common in both male and female APS1 patients. Female infertility in APS1 can be explained by autoimmune ovarian failure. Gonadal failure is, however, rare in male APS1 patients, and the causes for male infertility in APS1 remains poorly understood.

A number of autoantigens have been identified in APS1. Most autoantigens in APS1 are intracellular molecules with tissue-specific expression, many being enzymes. Several autoantigens in APS1 are also involved in autoimmune disorders in the general population, supporting APS1’s relevance as a disease model and valuable system for biomarker discovery. Examples of APS1 autoantigens with extended relevance include GAD2, which is the major autoantigen in type 1 diabetes, CYP21A, CYP11A1 and CYP17A1, which are steroid cell autoantigens in patients with Addison's disease and autoimmune ovarian failure, gastric intrinsic factor (GIF), which is a major autoantigen in autoimmune gastritis, and the recently identified pulmonary autoantigen BPIFB1 that is associated with interstitial lung disease. Although many autoantigens have been identified in APS1 over the years, the repertoire of immune targets has never been studied in a comprehensive way. The understanding has remained limited of how molecules become autoimmune targets in APS1.
Clinical components of APS1

<table>
<thead>
<tr>
<th>Clinical component</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic mucocutaneous candidiasis</td>
<td>75-100%</td>
</tr>
<tr>
<td>Hypoparathyroidism</td>
<td>82-96%</td>
</tr>
<tr>
<td>Adrenal failure</td>
<td>60-73%</td>
</tr>
<tr>
<td>Gonadal failure</td>
<td>12-43%</td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>12%</td>
</tr>
<tr>
<td>Autoimmune gastritis</td>
<td>9-16%</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>10-15%</td>
</tr>
<tr>
<td>Intestinal dysfunction</td>
<td>20%</td>
</tr>
<tr>
<td>Exocrine pancreatic failure</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Chronic kerato-conjunctivitis</td>
<td>15%</td>
</tr>
<tr>
<td>Vitiligo</td>
<td>4-15%</td>
</tr>
<tr>
<td>Alopecia</td>
<td>13-37%</td>
</tr>
<tr>
<td>Enamel hypoplasia</td>
<td>70-80%</td>
</tr>
<tr>
<td>Spleen hypo/aplasia</td>
<td>9%</td>
</tr>
<tr>
<td>Tubulointerstitial nephritis</td>
<td>9%</td>
</tr>
<tr>
<td>Pulmonary symptoms</td>
<td>&lt;10%</td>
</tr>
</tbody>
</table>

Table 5. Disease manifestations of APS1 and their prevalences

**AIRE**

- **Molecular mechanisms behind immune tolerance**

The immune system must meet two challenging tasks – it must be capable of eliciting strong responses against foreign antigens of all possible sorts to provide efficient protection against invading organisms, and at the same time, it must remain inert against endogenous antigens to avoid attacking healthy self-tissues. To achieve this, the adaptive immune system must prepare a very broad repertoire of unique T- and B-cell receptors with different binding capacities and then be rendered tolerant specifically against self. The identification of the AIRE gene and following studies in Aire-deficient
mice brought light on the processes by which the immune system learns to tolerate self.

The gene determinant of APS1 was identified through linkage analyses and positional cloning in families with APS1 and was named the *Autoimmune Regulator* (*AIRE*). The novel gene contained domains typically found in transcription factors, suggesting a role in gene regulation. *AIRE* demonstrated a restricted expression pattern, limited to distinct cells in the thymic medulla and also in a rare set cells in the lymph nodes and spleen, consistent with a function in immune regulation. *Aire*-deficient mice were developed on different backgrounds as a model of APS1 and to study *Aire* gene mechanisms. The *Aire*-deficient mouse reproduced APS1 features including multi-organ autoimmunity and infertility, but also differed from APS1 with a less aggressive autoimmunity phenotype on most mouse backgrounds and with a distinct set of target tissues. It was found that *Aire* expression predominantly occurred in the medullary thymic epithelial cells (mTECs) – a cell type known to be involved in negative T-cells selection. Naïve T-cells undergo a two-step selection process that ensures that (i) they are capable of binding to HLA and are therefore potentially useful (positive selection) and yet (ii.) do not bind with excessive affinity to self-molecules and therefore become potentially harmful (negative selection). It had remained enigmatic how T-cells could be exposed to tissue-specific molecules that would not be expected to be present in the thymus. In studies of mTECs isolated from *Aire*-deficient and wild type mice it was shown that *Aire* promoted an ectopic display of a broad range of tissue-specific molecules in the thymus. In this way, *Aire* was necessary for inducing tolerance against tissue-specific molecules. Although subsequent studies have indicated a broader role of *Aire* in function such as mTEC development and in peripheral tolerance mechanisms, *Aire*’s role in the establishment of central immune tolerance is believed to be its main *modus operandi* in the prevention of autoimmune disease.

The prostate

*A male gland*

The mature human prostate is a tubuloalveolar gland composed of secretory ducts lined by a pseudostratified columnar epithelium and surrounding fibromuscular stroma. The prostate is responsible for delivering proteins and ions to the semen, providing a milieu for sperm maturation. Key functions of prostatic secretion include regulating semen gelation and liquidification. Prostate secretory proteins are also important for coating the sperm and supporting sperm capacitation.
The development of the prostate begins around the 10th embryonic week with the growth of prostatic buds from the urogenital sinus. Androgen secretion from the newly formed male gonads initiates the developmental process and is thereafter needed for continued growth and ductal branching. During puberty the prostate enters second phase of development, again driven by testosterone. The prostate then matures into an active secretory gland and starts producing a battery of secretory proteins. Pubertal prostate maturation can be monitored in boys by measuring serum levels of prostate-specific antigen.

Prostate disorders such as prostatitis can affect fertility in men, although the involved mechanisms are poorly defined.

Figure 2. Human prostate tissue, showing secretory ducts lined with pseudostratified columnar epithelium and surrounding fibromuscular stroma.

Transglutaminases

- A family of protein cross-linkers

Transglutaminases are a family of enzymes that play important roles in many bodily functions and also have been implicated in the pathogenesis of a broad range of familial and acquired disorders (Table 6). This group of enzymes has further gained popularity in the food industry as “meat glues”
and is widely used in the production of imitations food such as crabsticks (invented and patented in Japan 1973)

The human transglutaminase family consists of nine genes that encode eight enzymes and one non-catalytic protein. All enzymes in the family are composed of four domains, and acquire confirmations that alter between a compact form, in the absence of Ca$^{2+}$, and an extended and active form, in presence of Ca$^{2+}$. The catalytic repertoire of transglutaminases include a variety of post-translational protein modifications that can be divided into three main reactions; transamidation, esterification and hydrolysis. Importantly, transglutaminases catalyze the formation of covalent bonds between glutamine and lysine residues - thereby cross-linking proteins.

Transglutaminases are expressed in most human tissues. While tissue transglutaminase (TGM2) is found throughout the body, other members of the transglutaminase family carry out key functions in specialized tissues and display restricted expression patterns. TGM2 have important roles both outside and inside of cells. Extracellular TGM2 activity is important for matrix stabilization and functions such as wound healing, angiogenesis and bone remodeling. In the cells TGM2 is involved in the regulation of fundamental cellular processes including apoptotic signaling. Another important member of the transglutaminase family, factor XIII (F13A1), is responsible for the final step in the coagulation cascade when fibrin is cross-linked to form a stable clot. Familial deficiency of F13A1 is associated with hemophilia. TGM1, TGM3, TGM5 are transglutaminases that all show predominant expression in squamous epithelia and especially the in epidermis and hair follicles. TGM1 and TGM5 are expressed in the spinous and granular layers while TGM3 is confined to the upper granular layer of epidermis. These transglutaminases cross-link components of the cornified cell envelope and are important in the process of keratinization and for skin barrier integrity. Mutations in TGM1 and TGM5 are linked with monogenic skin disorders - ichthyosis, congenital, autosomal recessive 1 (MIM# 242300) and Peeling skin syndrome, acral type (MIM# 609796), respectively. TGM6 is a transglutaminase predominantly expressed in central nervous tissue and is associated with monogenic spinocerebellar ataxia (MIM#613908). Erythrocyte band protein 4.2 (EPB4.2) is the only family member without enzymatic activity, and is an integral part of the red blood cell cytoskeleton. Mutations in EPB4.2 cause spherocytosis (MIM#612690).

Interestingly, several members of the transglutaminase family have been identified as autoantigens in different autoimmune disorders. The molecular target of endomyseal autoantibodies in patients with celiac disease was identified as TGM2. TGM2 autoantibodies are today an important biomarker in the clinical diagnosis of celiac disease. A subset of patients with celiac disease develops a skin disorder characterized by severely itching eruptions called dermatitis herpetiformis. Patient with dermatitis herpetiformis harbor autoantibodies against epidermal transglutaminase.
toms, typically cerebellar ataxia, develop in small portion of the patients with celiac disease. These rare complications have been linked with autoantibodies against neuronal TGM6\(^8^2\), phenocopying monogenic spinocerebellar ataxia caused by TGM6 mutations. Acquired FXIII deficient hemophilia, caused by autoantibodies that inhibit the function of F13A1\(^8^3\), is another example of an autoimmune phenocopy related to transglutaminase autoantibodies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tissue distribution</th>
<th>Monogenic disorders (MIM#)</th>
<th>Autoimmune disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGM1</td>
<td>Squamous epithelia</td>
<td>Ichthyosis, congenital, autosomal recessive 1 (242300)</td>
<td>-</td>
</tr>
<tr>
<td>TGM2</td>
<td>Ubiquitous</td>
<td>-</td>
<td>Celiac disease(^2^9)</td>
</tr>
<tr>
<td>TGM3</td>
<td>Squamous epithelia</td>
<td>-</td>
<td>Dermatitis herpetiformis(^6^5)</td>
</tr>
<tr>
<td>TGM4</td>
<td>Prostate epithelium</td>
<td>-</td>
<td>APS1(^8^4)</td>
</tr>
<tr>
<td>TGM5</td>
<td>Squamous epithelia</td>
<td>Peeling skin syndrome, acral type (609796)</td>
<td>-</td>
</tr>
<tr>
<td>TGM6</td>
<td>Neuronal tissue</td>
<td>Spinocerebellar ataxia 35 (613908)</td>
<td>Gluten ataxia(^8^2)</td>
</tr>
<tr>
<td>TGM7</td>
<td>Testis and lung</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F13A1</td>
<td>Blood circulation, thrombocytes</td>
<td>Factor XIII A deficiency (613225)</td>
<td>Acquired FXIII deficient hemophilia(^8^3)</td>
</tr>
<tr>
<td>EPB42</td>
<td>Erythrocytes</td>
<td>Spherocytosis, hereditary, type 5 (612690)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6. The human transglutaminase family and associated monogenic and autoimmune disorders. Mendelian inheritance in man number, MIM#.

Transglutaminase 4

- A prostatic transglutaminase

TGM4 is a member of the transglutaminase family that is exclusively produced by the prostate gland and that has been implicated with different functions in male reproductive physiology. TGM4 was cloned from prostate cDNA library by Ho and colleagues in 1992\(^8^5\), and was subsequently shown to have tissue-specific and androgen-driven activity\(^8^6,8^7\). However, many years before cloning and initial description of the TGM4 gene, studies had identified important roles of a transglutaminase present in semen. It was found that transglutaminase-mediated modifications of the sperm surface were important for masking the sperm from immune attack in the female reproductive tract\(^8^8,8^9\). Transglutaminase activity was also found to be critical for sperm capacitation – the process whereby the sperm differentiates to become fully mature fertile cells\(^9^0,9^1\). TGM4 has further been shown to be a key regulator of semen viscosity. TGM4 cross-links gel forming proteins in
the semen, such as semenogelins, promoting semen coagulation. TGM4 knock out mice fail to form a copulatory plug and display severely reduced male fertility. Although studies point towards multiple functions of TGM4 in reproductive physiology, little is currently known about the role of TGM4 in human disease.
Current investigations

Aims
The aim of the present investigations was to characterize mechanisms in tissue-specific autoimmune disease utilizing APS1 as a model disorder. We also sought to evaluate proteome arrays as tools for autoimmune disease biomarker discovery.

Our specific aims were to:
• Perform a comprehensive study of autoimmune targets in APS1 (Paper I)
• Investigate mechanisms underlying male infertility (Paper II) and kidney disease (Paper III) in APS1

Materials and methods
The most important methodology in this thesis work is described in brief below. Further details on materials and methods can be found in the individual papers.

Patient cohorts
APS1 is a very rare disease but is seen at higher frequency in certain populations including the Finns. Our study cohort comprised patients with APS1 from Finland, Sweden and Norway. All patients met the clinical diagnostic criteria for APS1, requiring two of the hallmark components; chronic mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency, or at least one of the hallmark components in siblings or children of individuals with APS1. Most of the patients had also been genotyped and found with mutations in the AIRE gene.

Aire-deficient mice
The mouse model of APS1, the Aire knock out mouse, spontaneously develops multiple tissue-specific autoimmune disease manifestations and display
autoantibodies against the affected tissues. In this work we used the \textit{Aire}-deficient mouse to gain better understanding our findings of prostate autoimmunity in APS1 patients. Mice were kept in a pathogen-free, barrier facility at the University of California San Francisco (UCSF). All procedures were approved by the UCSF Institutional Animal Care Committee and Veterinary Services, and adhered to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

**Protein array screening**

We used protein arrays containing over 9000 human proteins (ProtoArray®, Life Technologies) to perform a broad characterization of immune targets in APS1. Arrays were probed at a serum dilution of 1:2000 and otherwise according to the supplier’s protocol. Arrays were scanned using a GenePix 4000B microarray scanner. The GenePix® Pro microarray (v6.1) software was used for alignment and data acquisition.

**Radio-ligand binding autoantibody assays**

Radio-ligand binding assays were used to screen sera for autoantibodies. cDNA clones encoding genes of interest were transcribed and translated \textit{in vitro} in the presence of $^{35}$S-labeled methionine. Radio-labeled protein was immunoprecipitated with serum samples (2.5 µl) in 96-well filtration plates. Autoantibody-antigen complexes were immobilized to protein A Sepharose, and radioactivity was measured in a liquid scintillation counter (Wallac 1450 MicroBeta, PerkinElmer).

**Immunofluorescence labeling of kidneys and laser-scanning confocal-microscopy**

We used indirect immunofluorescence technique to screen patient sera for kidney autoantibodies. Sections of fresh-frozen rat kidney tissue were incubated with patient and control sera, and autoantibodies were detected using a fluorescence labeled anti-human IgG. The target cell specificity of patient autoantibodies was defined in co-stainings with markers of different tubular segments. AQP2 was used to label the collecting ducts (Figure 3) and AQP1 to label the proximal tubuli. Images were taken using a Zeiss Laser Scan Microscope 700.
Construction and screening of a collecting duct cDNA Library

To define molecular targets of collecting duct autoantibodies we constructed and screened a collecting duct cell cDNA library. Inner medullary collecting duct cells were isolated from rat kidneys. mRNA was extracted, converted to cDNA and cloned into a Zap Express vector. The cDNA library was screened with patient sera. Isolated clones were amplified with PCR, Sanger sequenced and identified using the Basic Local Alignment Search Tool (BLAST) http://blast.ncbi.nlm.nih.gov/Blast.cgi (Figure 4).

Figure 4. The collecting duct library was screened with serum from patients with kidney disease and collecting duct autoantibodies. Here shown, a filter with one positive clone present in multiple copies.
Results and discussion

Paper I

APS1 features multiple tissue-destructive manifestations and autoantibody responses against the affected tissues. Although several autoantigens have been defined in APS1 in separate studies\textsuperscript{14,32-34,36,67,94-96} the repertoire of immune targets has not been assessed in a comprehensive way. The aim of Paper I was to use proteome arrays to perform a broad characterization of autoimmune targets in APS1. With this effort we sought to gain better understanding of recognized autoimmune manifestations of APS1 and also to identify novel target organs. Further, as multiple known APS1 autoantigens were present in the protein panel, our screen would allow for a stringent evaluation of the proteome array performance.

We used proteome arrays containing over 9000 full-length human proteins to study autoimmune targets in APS1. Arrays were probed with serum samples from 51 patients with APS1 and 21 healthy control subjects and autoantibodies were detected using a fluorescence labeled anti-human IgG. Multiple known autoantigens were present in the panel, providing excellent means to evaluate the performance of autoantibody detection. All known autoantigens were replicated in the screen, showing elevated signals specifically among the patients (Figure 5). All proteins were spotted twice on the array in neighboring positions, and the results for duplicates of the established autoantibodies were well aligned (Figure 6). The proteome array results were further very consistent with our in-house radio-ligand binding autoantibody assays in classifying patients as autoantibody positive or negative. Collectively, the known targets revealed a highly reliable detection of autoantibodies in our proteome array screen.

![Figure 5. Previously identified autoantigens were replicated in the proteome array screen, here exemplified by GAD2 autoantibodies.](image-url)
Patients with APS1 develop complex combinations of autoimmune manifestations. We assessed correlations between the known autoantibodies to better understand how individual autoimmune responses were related in APS1. The homologous antigens IFNA1 – IFNW1 and GAD1 – GAD2 showed pairwise strong correlations. Interestingly, we also observed a correlation between TPH1 and DDC, which are structurally unrelated proteins but both involved in the monoamine synthesis and co-expressed in serotonergic neuronal and enterochromaffinic cells. The correlation between TPH1 and DDC was thereby probably not explained by shared epitopes and autoantibody cross-reactivity but rather by a shared target cell specificity.

Around 100 different mutations have been described in APS1\(^\text{97}\), and parts from two \textit{AIRE} mutations that are associated with distinct non-APS1 autoimmunity syndrome\(^\text{97,98}\), no reliable genotype to phenotype relation has been established for \textit{AIRE} mutations\(^\text{99}\). We used our data for the known autoantibodies to study potential effects of \textit{AIRE} genotype on autoimmune expression. We divided the patients with APS1 into three major groups; i. patients with the Finnish mutation R257X, ii. patients with the 13 base pair deletion in exon 8 and iii. patients who were heterozygous for type of \textit{AIRE} mutation or who displayed rare \textit{AIRE} gene mutations, and we clustered the patients with respect to data for the known autoantibodies. While the healthy control subjects formed a distinct cluster separated from the patients, the APS1 patients did not show tendency for clustering according to type of \textit{AIRE} mutation. \textit{AIRE} genotype thereby did not appear to be an import determinant of the autoantibody responses in APS1.
Cytokine autoantibodies have been implicated with the pathogeneses of candidiasis and are further valuable markers in the diagnosis of APS1\textsuperscript{95,96,100}. Multiple cytokine species were present in our panel, which allowed us to perform a broad characterization of cytokine autoantibodies in the APS1 patients. In review of 49 cytokines present in the array we specifically observed reactivity against the expected targets, namely the type 1 interferon specifics, IL22 and IL17A. Responses against the different interferon species were strongly correlated, consistent with a scenario of shared autoepitopes and autoantibody cross-reactivity. IFNA4 produced the strongest autoantibody signal of the investigated interferons, suggesting IFNA4 was a dominating autoantigen and possibly better suited than other interferons for diagnostic autoantibody assays.

Our survey of known targets indicated a highly robust detection of autoantibodies and we next explored the full protein panel in search for novel immune targets. We first excluded all targets that failed to generate high signals in any of the investigated sera. To identify patient-specific signals we next introduced cutoff values for all targets at three standard deviations (SD) above the mean for the healthy control subjects and compared the frequency of positive individuals between the patients and the controls. The top 35 targets, with unadjusted p-values ranging from $8 \times 10^{-19}$ to 0.05 (Fisher’s exact test), were selected for further investigations. In our top selection there were several targets that were located in direct vicinity to known autoantigens on the array, suggesting these signals had appeared as result of printing contaminations. The signals for suspected artifacts were strongly correlated with the expected sources of contamination (Figure 7 ad 8). We could therefore use the signal correlations between array targets as to identify and exclude printing contaminations in our data set (Figure 9). In our top selection we found 14 targets that showed strong and unexpected correlations with nearby located known autoantigens. After exclusion of the identified artifacts our annotated set contained eleven type one interferon species (IFNW1, IFNA13, IFNA21, IFNA4, IFNA17, IFNA1, IFNA5, IFNA2, IFNA6, IFNA8 and IFNA14), another six previously defined autoantigens (TPH1, IL22, DDC, GAD1, GAD2 and GIF), and four novel candidate autoantigens; transglutaminase 4 (TGM4), melanoma antigen family B 2 and 4 (MAGEB2 and MAGEB4) and protein disulfide isomerase-like testis expressed (PDILT). The signals for MAGEB2 and MAGEB4 were correlated, suggesting the two related molecules were both targets of a shared cross-reactive response. MAGEB2 autoantibodies were detected in 21 out of 51 APS1 patients and PDILT autoantibodies were present in 19 of 51 APS1 patients, while absent in controls.
Figure 7. Printing contaminations were visible on the scanned arrays. The IgG channel for an array probed with patient serum reveals gradual signal washout in spots adjacent to strong signaling antigens. All proteins were represented in duplicate in adjacent positions on the array.

Figure 8. Effects of printing contamination could be seen in array targets adjacent to the known APS1 autoantigens, here exemplified by DDC and neighboring targets. Results for 21 healthy subjects are shown to the left and 51 patients with APS1 to the right.
Figure 9. Printing contamination artifacts were identified by assessing the correlation in autoantibody signal. Unexpected correlated signals from targets located close to known autoantigens were identified as printing contaminations and excluded (marked in Italic).

In Paper I we validated our candidate autoantigens, MAGEB2 and PDILT, using a radio-ligand binding autoantibody assay in an extended cohort of APS1 patients. We could confirm our findings in the discovery cohort and further replicate MAGEB2 and PDILT as valid autoantigens in a validation cohort of 43 APS1 patients. In total, MAGEB2 autoantibodies were detected in 31 out of 92 (34%) patients with APS1 while PDILT autoantibodies were present in 28 out of 93 (30%) of the APS1 patients. Investigations in a number of control cohorts further suggested MAGEB2 and PDILT autoantibodies were highly specific for APS1.

Previous studies suggest MAGEB2 and PDILT are specifically expressed in male germ cells\textsuperscript{101-104}. Studies in mice further suggest MAGEB genes are also expressed in oocytes and are important in female gametogenesis\textsuperscript{105}. We validated the expression of MAGEB2 and PDILT across a panel of different human tissues using digital droplet PCR. MAGEB2 and PDILT were specifically detected in testicular tissue (ovarian tissue was not available for analyses). Tissue stainings further showed that MAGEB2 was expressed in germ cells across all differentiation stages while PDILT was confined to germ cells of late differentiation stages. Collectively, our findings indicated MAGEB2 and PDILT were major gonadal autoantigens in APS1.

In this work we present the first comprehensive study of autoimmune targets in APS1. While the \textit{Aire}-dependent genome has been well characterized in mice, and suggest around 4000 genes are positively controlled by \textit{Aire} in mTEC\textsuperscript{106,107}, it has remained unknown how many of these molecules that become immune targets in APS1 patients. Here we investigated the protein products of around one third of the human genes, and although our review of known targets indicated a highly reliable detection of known autoantibodies,
we found only around 20 proteins that were frequent immune targets in APS1. Our findings suggest a very limited portion of the proteome become targeted by the immune system in APS1. This is in contrast with the very broad gene repertoire controlled by Aire in the thymus and brings important questions on what other factors are required for break of tolerance. One important aspect that must be considered here is that antigen presentation is HLA dependent, and that only a subset of molecules are expected to possess necessary requirements for being presented on HLA efficiently and to render strong immune responses. B-cell tolerance and peripheral tolerance mechanisms may also be expected to further limit the number of molecules that escape the filter of tolerance.

Our proteome-wide assessment also enabled us to explore patterns of shared features between the immune targets. Firstly, we could note that most of the immune targets showed tissue-specific expression. The tissue expression has been determined for a great majority of human genes by mRNA sequencing, as part of the Human Protein Atlas project\textsuperscript{108} \url{http://www.proteinatlas.org}. While only 12\% of the human genes are annotated as being tissue enriched, more than half of the immune targets in our screen belonged to this category. Further, while almost half of the genes in the genome are expressed in all tissues we found no immune targets in our proteome array screen of that sort. This marked enrichment for tissue-specific immune targets goes in hand with the biology of AIRE-deficiency and AIRE’s function in specifically compensating the lack of tissue-specific genes in the thymus.

In this study we also report on MAGEB2 and PDILT as two novel, major gonadal autoantigens. MAGEB2 is a member of a gene family that is clustered on the dosage-sensitive sex reversal region of the X-chromosome\textsuperscript{101}. PDILT is a testis-specific protein with disulfide isomerases homology that functions in a germ cell-specific chaperone complex necessary for male fertility\textsuperscript{102-104}. Autoantibodies against MAGEB2 and PDILT did not associate with any of the disease manifestations that had been recorded in the APS1 patients. Future studies are needed to understand the role of these novel autoantigens in APS1.

To this day most proteome array studies have been carried out in disorders without an established autoimmune etiology, where internal controls in the form of known autoantigens have not been available and where it has further proved challenging to identify novel bona fide autoantigens. Our screens with APS1 sera permitted a more stringent assessment of the proteome array performance than has been possible in earlier studies. The known autoantigens in the panel revealed a highly robust detection of autoantibodies but also exposed artifacts. We established a strategy to identify printing contamination artifacts in a relatively unbiased fashion by assessing the signal correlations between the targets. Data from a proteome array contaminated with printing artifacts can be rescued once the artifacts have been identi-
fied. This type of artifacts would, however, render the data from focused array useless, where antigens of interest are typically spotted in adjacent positions. To our knowledge we are the first to report on printing contamination artifacts on proteome arrays despite many earlier studies using the ProtoArray®. This illustrates the value of starting out with well-characterized and informative autoimmune disease sera that can be evaluated before embarking into the unknown of idiopathic and non-autoimmune disorders. Overall our screen in APS1 showed results that are very encouraging for future proteome arrays studies in other autoimmune diseases.

**Paper II**

Infertility is common in both male and female APS1 patients\(^6^0,1^0^9\). While female infertility in APS1 can be explained by autoimmune ovarian failure, the mechanisms behind male infertility have remained poorly understood. In this study we followed up on our finding of TGM4 as a prostate autoantigen and we utilized the mouse model of APS1 to better understand the role of prostate autoimmunity in APS1.

We conducted a focused search for male-specific signals in our proteome-wide data that could reflect autoimmune responses against the male reproductive organs. One single target in the panel, the prostate-specific enzyme TGM4, differed significantly between the male and female patients. We utilized a radio-ligand binding autoantibody assay to confirm TGM4 as a valid male-specific autoantigen in an extended APS1 cohort and in a broad clinical control material. TGM4 autoantibodies were present in 26 out of 46 (56%) male patients and in one out of 47 female patients, while absent in controls with different autoimmune disorders, prostate disorders and males with idiopathic infertility (Figure 9).

![Figure 10. TGM4 autoantibodies were investigated in male (n=46) and female (n=47) patients with APS1 and in control subjects (n>400) using a radio-ligand binding assay.](image-url)
Several members of the transglutaminase family have been identified as autoantigens in different autoimmune disorders\textsuperscript{29,65,82,83} - TGM2 being the major autoantigen in celiac disease\textsuperscript{29}. To investigate the target specificity of TGM4 autoantibodies in APS1, we immunoprecipitated TGM4 and TGM2 protein with sera from patients with APS1 and celiac disease for comparison. APS1 patient sera specifically reacted with TGM4 and not with TGM2 while sera from patients with celiac disease only recognized TGM2.

Previous studies suggest TGM4 is specifically expressed in the prostate epithelium\textsuperscript{85-87}. We validated TGM4 expression in multiple human tissues on both mRNA and protein levels (Figure 11). TGM4 expression was specifically detected in the prostate and was further restricted to distinct regions in the gland, consistent with previous reports\textsuperscript{110}.

![Figure 11. TGM4 staining in human prostate tissue, labeling a subset of epithelial cells in prostatic ducts.](image)

The prostate is a male organ, and we specifically observed TGM4 autoantibodies in the male patients. However, although the prostate is formed during embryonic development it fully matures into an active secretory organ first during puberty. Studies on other prostate secretory molecules in human\textsuperscript{75} and on TGM4 in mouse\textsuperscript{111} suggest TGM4 expression begins during early puberty, around the age of ten to twelve years in boys. We therefore looked at the prevalence by age of TGM4 autoantibodies in the male APS1 patients. Notably, all males below the age of 13 years were negative for TGM4 autoantibodies (Figure 12). To pinpoint the relation between TGM4 autoantibodies and puberty we selected six male APS1 patients of post-pubertal age who were all positive for TGM4 autoantibodies, and we analyzed serum sample
that had been collected from the patients over the years to determine when TGM4 autoantibodies first appeared. In all the investigated males TGM4 autoantibodies were detected first between the age of 12 to 16 years (Figure 12). Importantly, none of the males had developed TGM4 autoantibodies before the age of an expected pubertal debut.

Figure 12. TGM4 autoantibodies were present in a majority of the males of post-pubertal ages but were absent in all males below the age of 13 years. To determine at what age TGM4 autoantibodies first appeared we investigated a time series of serum samples collected from six male who were positive for TGM4 autoantibodies at post-pubertal age. All of the investigated males developed TGM4 autoantibodies first during pubertal age and after the age of prostate maturation.

Our findings suggested prostate autoimmunity was a major manifestation of APS1. To better understand the role of prostate autoimmunity in APS1 we followed up on our findings in the mouse model of APS1. *Aire*-deficient mice spontaneously develop autoimmune manifestations in multiple organs and harbor autoantibodies against the affected tissue. *Aire*-deficient male mice display severely reduced fertility. It has further been demonstrated that male infertility develops from autoimmune disease mechanisms, as it can be induced in healthy recipients by transfer of immune cells from *Aire*-deficient male mice. The autoimmune component responsible for male infertility in *Aire*-deficient mice has, however, not been determined. While
the testes appear to be unaffected in *Aire*-deficient mice, the prostate is a major target of autoimmune destruction.\textsuperscript{48,113,114}

We first investigated the prostate tissue histology in aged *Aire*-deficient mice as compared to wild type controls. The *Aire*-deficient mouse prostates displayed typical signs of an active inflammatory prostatitis, with massive mononuclear cells infiltrates in the interstitial tissue (Figure 13). qPCR assessments of immune cell markers indicated the prostate infiltrating lymphocytes were dominated Th1 cells – a subset that has previously been shown to be critical for autoimmune pathology in APS1.\textsuperscript{115}

We next sought to determine whether TGM4 was involved in the development of prostatitis in the *Aire*-deficient mouse. We developed a radioligand binding assay for murine TGM4 to screen a large number of mice for TGM4 autoantibodies. Strikingly, TGM4 autoantibodies were present in all *Aire*-deficient male mice while absent in all female *Aire*-deficient mice and in all male and female wild type mice (Figure 14).

Studies in TGM4 knock out mice have shown that TGM4 is necessary for male fertility.\textsuperscript{93} We therefore investigated whether prostatic TGM4 secretion was affected in the *Aire*-deficient mice and could explain their compromised fertility. We assayed TGM4 mRNA levels by qPCR in prostate tissue collected from *Aire*-deficient and wild type mice. TGM4 mRNA was absent in prostates from the *Aire*-deficient mice, suggesting TGM4 secreting prostatic ducts had been completely destroyed in the autoimmune insult.

To better understand the mechanisms underlying TGM4 immunity we also investigated whether TGM4 was presented in the thymus under control of *Aire*. mRNA levels of TGM4 and reference markers were determined by qPCR in mTECs from *Aire*-deficient and wild type mice. TGM4 was detected in wild type mTEC’s but was completely absent in the *Aire*-deficient mTEC’s, suggesting TGM4 was expressed ectopically in the thymus in an *Aire*-dependent manner.

Collectively, in the mouse model we could link TGM4 immunity with a tissue-destructive autoimmune prostatitis, a compromised secretion of prostatic TGM4 that could explain male subfertility, and with a defect in the establishment of central immune tolerance for TGM4.
Figure 13. Prostate tissue samples from Aire-deficient (left) and wild type (right) mice. Aire-deficient mice displayed typical signs of an active inflammatory prostatitis, with massive mononuclear cell infiltrates in the interstitial tissue and epithelium.

Figure 14. TGM4 autoantibodies in Aire-deficient male mice (n=23), female Aire-deficient mice (n=24), male wild type mice (n=11) and female wild type mice (n=8).

In this study we identify prostate autoimmunity as an occult manifestation of APS1 and TGM4 as a unique male-specific autoantigen. In the mouse model we further link TGM4 immunity with an autoimmune prostatitis and a compromised secretion of prostatic TGM4. Given of the essential role of the prostate gland\textsuperscript{76} and TGM4 specifically\textsuperscript{88,90,91,93} in male reproduction, prostate autoimmunity may be a contributing factor behind infertility in males with APS1. Future studies investigating prostate tissue samples, semen parameters and recording fertility problems in males with APS1 will be important to better understand the role of prostate autoimmunity in male infertility in APS1.

The limited expression of TGM4, restricted to the post-pubertal male prostate, allowed for a unique observation to be made. In comparisons be-
tween males and females and also between males before and after pubertal onset, it became clear that TGM4 autoantibodies only developed in presence of peripheral antigen. These observations illustrate that peripheral antigen presentation is required in the development of an autoimmune response. This could potentially be exploited for future therapeutic strategies, where peripheral antigen expression is suppressed to hamper autoimmune disease activity.

One single female in our cohort was found positive for TGM4 autoantibodies. The outlier was a 42-year-old female from Finland, and she was consistently positive for TGM4 autoantibodies in repeated assessments. Review of her clinical records revealed that she had medicated with fluoxymesterone, a potent androgenic steroid, for a period of several years preceding serum sampling. The finding was striking, but still, could it explain why she had developed prostate autoantibodies. Still, females don’t have a prostate. In the 17th century Reiner de Graaf described a set of glands surrounding the female urethra that he recognized to be the female counterpart of the male prostate. Under the microscope the periurethral glands looks indistinguishable from the pre-pubertal male prostate. In the normal female hormonal milieu the periurethral glands remain small and quiescent throughout life. However, if a female is exposed to superphysiological levels of androgens, as studied in experimental animals and also in females undergoing sex change, the periurethral glands will grow and transform into and active secretory gland that produces prostate-specific proteins. Androgen exposure in the female patient may therefore have induced the expression of TGM4 in her periurethral glands, providing a source of peripheral antigen needed for igniting TGM4 autoimmunity. As it proved, the female outlier did not disrupt picture but rather provided yet another support for the link between peripheral antigen expression and the development of autoimmunity.

Paper III

Tubulointerstitial nephritis is a severe complication of APS1 that affects around 10% of the patients. The cause is not known. Autoantibodies against tubular cells have been demonstrated in patients with APS1 and kidney disease but the target molecules have remained unidentified. In this work we studied APS1 patients with tubulointerstitial nephritis and end-stage kidney disease to better understand mechanisms underlying kidney disease in APS1.

We studied three patients with APS1 who developed hypertension, hypokalemia and end-stage kidney disease at early age. Kidney biopsies from the patients showed tubular atrophy and interstitial fibrosis, consistent with a diagnosis of tubulointerstitial nephritis. To investigate the hypothesis of an autoimmune pathogenesis of tubulointerstitial nephritis we screened the patients’ sera for autoantibodies against kidney tissue, using fresh frozen
rat kidney sections and indirect immunofluorescence technique. In sera from all three patients we observed a distinct staining pattern, labeling tubular cells in a subset of the tubuli (Figure 15). In co-stainings with markers of different tubular segments we found that patient autoantibodies specifically reacted with the collecting duct cells.

We next sought to define the involved molecular immune targets, and first assessed AQP2 as a candidate autoantigen. A radio-ligand binding assay was used to screen patient and control sera for AQP2 autoantibodies. One of the patients with kidney disease harbored high titer AQP2 autoantibodies, while the two other patients with kidney disease and all controls were negative. The presence of AQP2 autoantibodies could be confirmed in the patient’s serum by western blot using native antigen present in human kidney tissue lysate. AQP2 is a transmembrane pore-shaped protein with polypeptide regions facing the cell exterior and interior. In stainings of permeabilized and non-permeabilized collecting duct cells we found that AQP2 autoantibodies reacted with an external epitope on AQP2. We also investigated whether AQP2 autoantibody binding affected AQP2 mediated water transport, and assayed water permeability in AQP2 transfected xenopus laevis oocytes in presence of patient or control sera. Patient serum labeled the oocyte membrane but did not show any effect on water permeability, suggesting AQP2 autoantibody binding did not interfere with water transport.

As AQP2 proved to be relevant autoantigen for only one and not the two other patients with kidney disease and collecting autoantibodies we then adopted a systematic approach to identify additional collecting duct autoantigens. We isolated mRNA from rat inner medullary collecting duct cells that was converted to cDNA and cloned to a lambda phage library. By screening the library with patient serum we identified Homeobox B7 (HOXB7) and Nuclear factor of activated T-cells 5 (NFAT5) as collecting duct autoantigens in the two other patients with kidney disease. Interestingly, HOXB7 and NFAT5 are both transcription factors with binding elements in the AQP2 promoter\(^1\)\(^2\)\(^-\)\(^4\) (Figure 16).

In tissue-specific autoimmune disorders the immune system typically targets molecules with key functions in the affected tissue and further often involve multiple autoantigens. We were fascinated to find that the three patients with kidney disease and collecting duct autoantibodies recognized distinct molecular targets that all appeared to be linked with AQP2. AQP2 has a key role in collecting duct physiology as the main regulator of water permeability\(^1\)\(^5\), and interestingly, NFAT5 is a major controller of AQP2 gene expression\(^1\)\(^4\). NFAT5 knockout mice display a severe atrophy of the kidney medulla and show markedly inhibited AQP2 expression\(^1\)\(^6\). HOXB7 is a member of an evolutionary conserved family of transcription factors - the homeotic or Hox family. Hox genes are expressed during the embryonic period, and are critical for the organization of the body plan. HOXB7 is ex-
pressed in the kidney anlage - the ureteric bud - and thereafter continuously in its derivatives in the collecting ducts of the adult kidney\textsuperscript{127,128}.

Tubulointerstitial nephritis may develop from a variety of disease processes that can affect the kidneys. Despite associations with autoimmune diseases\textsuperscript{129,130}, the role of autoimmune mechanisms in development of tubulointerstitial nephritis is not clear. Importantly, it has remained undetermined whether tubulointerstitial nephritis can develop from tissue-specific autoimmune mechanisms. Tubular basement membrane autoantibodies\textsuperscript{131,132} and tubular cell autoantibodies\textsuperscript{118,120,121,133} have been found in patients with tubulointerstitial nephritis, but the immune targets have remained unidentified. To our knowledge this study is the first identifying tubular-specific autoantigens in tubulointerstitial nephritis. Hopefully, our findings in these patients can aid the understanding tissue-specific autoimmune disease mechanisms in tubulointerstitial nephritis also beyond APS1.

Figure 15. Autoantibodies against collecting duct cells were found in the patients with APS1 and kidney disease. Autoantibodies were detected using rat kidney tissue sections and indirect immunofluorescence technique.

Figure 16. Conserved transcription factor binding elements for NFAT5 and HOXB7 were identified in the AQP2 promoter.
Concluding remarks and future perspectives

In this thesis work I used proteome arrays to conduct a broad characterization of autoimmune targets in APS1. With this effort we accessed an overview-perspective on autoimmune responses in APS1 that had not been available before. We could study known autoantibodies with high detail and we further identified three novel major autoantigens. In parallel studies in APS1 and its mouse model we identified and characterized the prostate as a novel autoimmune target in APS1 with potential role in male subfertility. The current work illustrates how proteome arrays can be a powerful tool for comprehensive characterization of autoimmune responses, for identifying novel biomarkers and for uncovering disease mechanisms in autoimmune disorders.

The present investigations open for many future study directions. Firstly, the proteome array results for APS1 are encouraging for similar efforts in other autoimmune and suspected autoimmune diseases. Rare autoimmunity syndromes such as IPEX or patients with mutations in CTLA4 would for example but interesting study subjects in this context. Another group of disorders where proteome array studies could be of great value is acquired immune deficiencies, which may develop from autoantibody responses against distinct circulating factors.

Our proteome array results also open for novel, more efficient ways to screen for autoantibodies in APS1. Focused screening panels can be developed that include all known autoantigens in APS1, which would allow for highly informative autoantibody screens in patients with APS1.

The identification of TGM4 autoantibodies in APS1 together with earlier discoveries of autoantigens in the transglutaminase family raises the question whether there are yet more transglutaminases to be implicated with autoimmune diseases. For example TGM1 and TGM5, predominantly expressed in the epidermis, would make attractive candidate autoantigens in autoimmune or idiopathic skin disorders. Likewise TGM7 could be an interesting candidate autoantigen in lung disease or EPB4.2 in hemolytic or aplastic anemia based on the expression patterns.

Ibland kan ovanliga sjukdomar erbjuda en ingång till att förstå vanliga och mer komplexa sjukdomar. Autoimmunt polyendokrint syndrom typ 1 (APS1) är en monogent betingad sjukdom som kännetecknas av autoimmuna angrepp mot flera organ. Sjukdomen orsakas av mutationer i genen AIRE som har till funktion att presentera kroppsegna molekyler för immunsystemet så att immunologisk tolerans av den egna kroppen kan etableras. APS1’s många gemensamma egenskaper med vanligt förekommande organspecifika autoimmuna sjukdomar gör den till en värdefull modell.


I mitt första delarbete använder jag proteome arrays för att genomföra en bred karaktärisering av autoantigen hos individer med APS1, dels för att förstå erkända sjukdomskomponenter av APS1 och dels för att upptäcka nya målorgan för autoimmun attack. I en första analys av kända autoantigen kunde vi konstatera att autoantikroppar pålitligt påvisades. Vi tittade också på kopplingen mellan olika autoantikroppar och om typen av AIRE-mutation var styrande för det autoimmuna svaret. Genom att utforska den fulla uppsättningen proteiner upptäckte vi tre nya autoantigen – MAGEB2, PDILT

Infertilitet är vanligt hos både män och kvinnor med APS1. Medan infertilitet hos kvinnor med APS1 förklaras av autoimmunitet mot äggstockar har orsakerna bakom manlig infertilitet vid APS1 förblivit till största del okända. I mitt andra delarbete följer jag upp fyndet av TGM4 för att förstå betydelsen av prostatas-autoimmunitet vid APS1 och dess möjliga roll i manlig infertilitet. TGM4 är ett enzym som bildas uteslutande i prostatakörteln och som utsöndras till sädesvätskan. Tidigare studier har visat att TGM4 har en viktig funktion i att reglera sädesvätskans viskositet och att stödja spermieutmognad. Möss med utslagen TGM4-funktion visar kraftigt nedsatt manlig fertilitet.

TGM4-autoantikroppar kunde påvisas hos hälften av männen med APS1 medan alla kvinnor utom en med APS1 och alla kontroller var negativa. Eftersom prostatatautmognar först i samband med pubertet undersökte vi förekomsten av TGM4-antikroppar i olika åldrar av männen med APS1. Det visade sig att alla pojkar i åldrar innan pubertet var negativa för prostatata-antikropparna. Vi kunde dessutom följa en grupp män i konsekutivt insamlade serumprov, och fann att prostatata-antikropparna uppträdde först i samband med pubertet.


Njursjukdom är en allvarlig komplikation av APS1 med ofullständigt känd bakgrund. I mitt tredje delarbete specialstuderar jag tre patienter med APS1 som utvecklade terminal njursvikt på basen av tubulointerstitiell nefrit. Vi fann att patienterna uppvisade autoantikroppar som specifikt reagerade med njurens samlingsrör, vilket utgör den sista delen av njurens urinledande gångsystem. Genom att konstruera och undersöka ett cDNA-bibliotek som återspeglade genuttrycket i samlingsrör kunde vi identifiera mål för patienternas autoantikroppar. Intressant nog så reagerade de tre patienterna mot olika molekyler i samlingsrör men som alla var kopplade i en gemensam funktion. En patient reagerade mot aquaporin 2 (AQP2), som är en vattenkanal med specifikt uttryck i samlingsrör och som har viktig roll i att reglera vätskebalansen, medan de två andra patienterna reagerade mot två olika transskriptionsfaktorer i AQP2’s promotorregion; HOXB7 och NFAT5.

Fynden är viktiga i första hand för förståelsen av njursjukdom vid APS1. Nästa fråga att ställa är om liknande mekanismer kan ligga till grund för njursjukdom i befolkningen i stort. Tubulointerstitiell nefrit är en vanlig orsak till njursvikt i befolkningen. Diagnosen baseras på fynd vid patologisk undersökning, där man ser förtvivning av njurens gångsystem och utbredd ärbildning, men de underliggande mekanismerna är till stora delar okända. Trots kopplingar till autoimmuna sjukdomar är betydelsen av autoimmuna sjukdomsmechaniker oklar. Förhoppningsvis kan våra fynd i APS1 vara till hjälp i att utforska autoimmuna sjukdomsmechaniser vid njursjukdom i befolkningen i stort.

Sammantaget visar mina doktorandstudier hur proteome arrays kan vara ett kraftfullt verktyg för att brett kartlägga autoimmuna svar och för att identifiera nya sjukdomsmarkörer. I studierna beskrivs tre nya autoantigen och vi identifierar prostata som ett nytt målorgan för autoimmun attack. Deb proteom-vida screeningen gav ett översiktsperspektiv som inte varit tillgängligt tidigare och tillåt ett nytt sätt att följa hur autoimmuna svar utvecklas hos
patienter med APS1. Resultaten gav insikter och genererade också nya frågor. Varför är antalet autoantigen så begränsat? Varför blir vissa molekyler återkommande autoantigen medan andra inte?

Doktorandarbetet öppnar för flera framtida spår. Försöken med proteome arrays i APS1 inspirerar till fortsatta studier i andra autoimmuna sjukdomar. Resultaten visar också att det skulle vara möjligt att sätta upp mindre, riktade paneler för autoantikroppsscreening i APS1 – något som skulle kunna ha stort värde i den kliniska diagnostiken av patienter med APS1. Fyndet av TGM4 som autoantigen, sammantaget med tidigare studier som identifierat andra transglutaminaser som autoantigen, inspirerar till att undersöka resterande medlemmar enzymfamiljen som kandidatautoantigen i andra autoimmuna sjukdomar.
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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine".)

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