Model diseases for studies of autoimmunity.

FRIDA DALIN
The events triggering autoimmune diseases are to large extent unknown and model diseases are an important tool in studies aiming to elucidate molecular mechanisms in autoimmunity. Autoimmune Addison’s disease (AAD) is a rare disease characterized by autoimmune destruction of adrenal glands and most patients with AAD have autoantibodies against the enzyme 21\#hydroxylase in the adrenal cortex. The autoimmune destruction in AAD is however suspected to be initiated by T cells. One of the most important investigations in this thesis was to characterize the T cell response in AAD. It could be concluded the T cells in AAD patients respond to three immunodominant epitopes on the 21-hydroxylase.

In addition, this thesis aims to gain updated data on comorbidities, replacement therapy, autoantibody profiles, and metabolic factors in AAD. A cohort of 660 AAD patients was studied and it was found that AAD patients are prone to develop other autoimmune conditions. AAD is one of three main disease components Autoimmune Polyendocrine Syndrome type 1 (APS-1), a rare disorder caused by mutations in the AutoImmune REgulator gene (\textit{AIRE}) that can be potentially fatal without timely diagnosis. Screening for autoantibodies against interferon-\(\omega\), interferon-\(\alpha 4\), and interleukin-22 revealed four new APS-1 patients among the AAD cohort, confirmed by the presence of disease causing mutations in the \textit{AIRE} gene.

Cancer Associated Retinopathy (CAR) is a paraneoplastic phenomenon arising as a consequence to an autoimmune response triggered by a malignant neoplasm present in the body. This disease is devastating and it is valuable to identify new biomarkers associated with CAR, not least from a tumor diagnostic perspective. In this thesis, a patient with osteosarcoma and CAR was studied and by screening of a proteom array, and the novel CAR autoantigen Aryl hydrocarbon receptor interacting protein-like 1 (AIPL1) was identified.

In conclusion, this thesis covers studies on T cell and B cell responses in AAD. Moreover, it includes an update on clinical and immunological characterisation of AAD patients. Finally, a novel autoantigen in CAR was identified and proposed as a diagnostic marker for the paraneoplastic syndrome.

\textit{Frida Dalin, Department of Medical Sciences, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.}

© Frida Dalin 2015

ISSN 1651-6206
urn:nbn:se:uu:diva-265276 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-265276)
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


Reprints were made with permission from the respective publishers.
Contents

Introduction ................................................................................................................. 9
  The Immune System and Immunological Tolerance ................................................. 9
  Autoimmunity and Autoimmune Disease ................................................................. 10
  Autoimmune Polyendocrine Syndrome type 1 ......................................................... 10
  Autoimmune Addison’s disease ............................................................................... 11
  Paraneoplastic Syndromes and Cancer-Associated Retinopathy ......................... 13

Current investigations .............................................................................................. 15
  Aims ......................................................................................................................... 15
  Materials and Methods ........................................................................................... 15
    In vitro Transcription and Translation (Manuscripts I, II, III) ......................... 15
    Protein Array Screening (Manuscript I) ............................................................... 16
    Immunofluorescence Staining (Manuscript I) .................................................... 16
    Recall Assay (Paper I) ......................................................................................... 16
    Ex vivo ELISPOT ................................................................................................. 17
    Granzyme B Assay (Paper I) .............................................................................. 18
    Statistical Analysis (Manuscript II) ................................................................... 18

Results and Discussion ............................................................................................ 18
  Cytolytic 21-hydroxylase-specific CD8+ T cells mediate adrenal cortex destruction in autoimmune Addison's disease patients (Paper I) .......................................................................................... 18
  Identification of Aryl Hydrocarbon Receptor-Interacting Protein-Like 1 (AIPL1) as a novel retina-specific autoantigen in Cancer-Associated Retinopathy (Manuscript I) .............................................. 22
  Clinical, immunological and epidemiological characteristics of Autoimmune Addison’s disease (Manuscript II) ................................................................. 24
  Identification of subclinical APS-1 patients within the Swedish Addison registry (Manuscript III) ........................................................................................................... 29

Conclusions ............................................................................................................... 32
Future Perspectives .................................................................33
Summary of Thesis in Swedish.................................................34
Acknowledgement.....................................................................37
References..............................................................................39
Abbreviations

17α-OH   17α-hydroxylase
21-OH   21-hydroxylase
AAD   Autoimmune Addison’s disease
AADC   Aromatic L-amino acid decarboxylase
ACTH   Adrenocorticotropic hormone
AIRE   Autoimmune regulator
APC   Allophycocyanin (fluorescent dye)
APS-1   Autoimmune polyendocrine syndrome type 1
AR   Autoimmune retinopathy
BCR   B cell receptor
BSA   Bovine serum albumin
CAR   Cancer-associated retinopathy
CRH   Corticotropin releasing hormone
CY3   Indocarbocyanine (fluorescent dye)
ELISPOT   Enzyme-linked immunosorbent spot
ERG   Electroretinography
FITC   Fluorescein isothiocyanate (fluorescent dye)
GAD65   Glutamate decarboxylase 65
HPA   Hypothalamic-pituitary-adrenal
IA-2   Islet cell antigen 2
ICS   Intracellular staining
IFN   Interferon
IL   Interleukin
ITT   In vitro transcription and translation
KCNRG   Potassium channel regulator
MHC   Major histocompatibility complex
mTECs   Medullary thymic epithelial cells
NALP5   NACHT, leucine rich repeat and PYD containing 5
PBMC   Peripheral blood mononuclear cell
PerCP   Peridinin chlorophyll (fluorescent dye)
RLBA   Radio-ligand binding assay
SOX10   SRY (sex determining region Y)-box 10
T1DM   Type 1 diabetes mellitus
T2DM   Type 2 diabetes mellitus
TCR   T cell receptor
TMB   (3,3′,5,5′-tetramethylbenzidine)
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Enzyme Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPH</td>
<td>Tryptophan hydroxylase</td>
</tr>
<tr>
<td>TPO</td>
<td>Thyroid peroxidase</td>
</tr>
<tr>
<td>TGM2</td>
<td>Tissue transglutaminase 2</td>
</tr>
</tbody>
</table>
Introduction

Autoimmune diseases include a spectrum of more than 80 different disorders, estimated to affect 5% of the population. Many diseases that were previously considered to be idiopathic are in fact suspected to have autoimmune origin [1]. Autoimmune diseases usually have complex inheritance patterns and occur as a consequence when several factors such as heredity and environment interact [2-4]. These parameters make it difficult to study autoimmune diseases, which hamper the development of new therapies [5]. Autoimmune disease models are therefore very important for understanding the pathogenesis of this broad disease group.

The Immune System and Immunological Tolerance

The human immune system is a complex organization of several types of cells and molecules that protect us from invading microorganisms [6]. It is divided into two fundamentally different types of responses: the innate response and the adaptive response. The innate response is the non-specific, first line defence against an invading microorganism and occurs to the same extent every time the pathogen is encountered, whereas the adaptive response is improved upon repeated exposure to the same infection [6]. The adaptive immune system consists of several cell types, although the two major ones are bone marrow-derived B cells and thymus-derived T cells, both originating from a common pluripotent lymphoid progenitor. These cells possess highly specialized receptors for a given antigen: the soluble or transmembrane B cell receptor (BCR) and the transmembrane T cell receptor (TCR) [7].

Once a functional TCR is produced, immature T cells undergo a number of selection events. First, the TCR is tested for its antigen-recognition ability against molecules present in the thymus. Medullary thymic epithelial cells (mTECs) present self-antigens on their surface, bound to major histocompatibility complex (MHC) molecules. This promiscuous expression of self-antigens is regulated by the autoimmune regulator gene (AIRE)[8]. Only lymphocytes whose receptors bind weakly to self-antigens bound to the body’s MHC molecules are selected to survive and develop further. This process is termed **positive selection**. Lymphocytes with receptors that bind strongly to self-antigens are selected to undergo apoptosis; this is termed
negative selection and is an important in preventing self-reactive lymphocytes from damaging the body’s own tissue. In this way, immunological tolerance to self-antigens is established [6, 9, 10].

Autoimmunity and Autoimmune Disease

Despite the mechanism of self-tolerance, some self-reactive lymphocytes do mature and cause autoimmunity when the organism begins producing an abnormal immune response to its own tissue. This generally involves self-reactive cytolytic T cells and autoantibody-producing self-reactive B cells [11]. Sometimes pathogens express antigens that resemble host molecules, either by chance or as an immune escape mechanism. This can result in activated lymphocytes cross-reacting with self-antigens and consequently induction of autoimmunity. If autoimmunity leads to pathological changes such as tissue destruction, the condition is recognized as an autoimmune disease [5].

Autoimmune diseases are often classified as either organ-specific, where the autoimmune reaction is localized to an isolated organ, or systemic, where the autoimmunity is widespread in the body [12]. The inflammation or cellular destruction occurring in an autoimmune disease may be caused directly by T cells or indirectly by T cells that sustain autoantibody responses.

The presence of serum autoantibodies have proven to be valuable markers for many autoimmune diseases and is an important tool in diagnosing and predicting diseases development since autoantibodies may be present years before clinical symptoms [13]. Such biomarkers can be measured when the autoantigen is known. It is therefore of great interest to identify autoantigens associated with various autoimmune diseases.

Autoimmune Polyendocrine Syndrome type 1

Autoimmune Polyendocrine Syndrome type 1 (APS-1) is a multi-organ autoimmune disorder caused by mutations in the AutoImmune REgulator gene (AIRE), located on chromosome 21q22 [14]. The AIRE protein is a unique transcriptional regulator, located in the nucleus of mTECs, where it promotes the promiscuous expression of thousands of peripheral organ-specific autoantigens in the thymus during T cell maturation. This is a step critical for the induction of immunological self-tolerance [9, 15, 16].

The hallmarks of APS-1 are hypoparathyroidism, chronic mucocutaneous candidiasis, and adrenal failure. However, there are today more than 20 different autoimmune endocrine and non-endocrine manifestations that have been identified in APS-1 [17, 18]. Patients with APS-1 develop autoantibodies to multiple tissue-specific antigens [17, 19, 20] and the presence of these autoantibodies is used to recognize and diagnose the syndrome (table 1). The
The majority of patients have three to five different disease manifestations [21, 22].

A hallmark of APS-1 is chronic mucocutaneous candidiasis. Circulating neutralizing anti-cytokine autoantibodies, such as several type I interferon isoforms, e.g., interferon-ω (IFNω) and interferon-α4 (IFNα4) [23, 24] and IL-17/IL-22 [25-27] are present in the vast majority of APS-1 patients. It is hypothesized that autoantibodies against the interleukins may be responsible for the chronic mucocutaneous candidiasis infections present in almost all APS-1 patients, since these cytokines play key roles in inducing defensins, small proteins important for the immune response to pathogens on mucosal surfaces [27]. Measuring these serum autoantibodies is an important diagnostic tool, as they are found in almost all APS 1 patients before manifestations of most symptoms [20, 28].

Table 1. Disease component associated with different target autoantigens in APS-1.

<table>
<thead>
<tr>
<th>Disease component</th>
<th>Related autoantigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal failure</td>
<td>21-OH, 17α-OH, SCC</td>
</tr>
<tr>
<td>Gonadal failure</td>
<td>SCC</td>
</tr>
<tr>
<td>Hypoparathyroidism</td>
<td>NALP5</td>
</tr>
<tr>
<td>Diabetes</td>
<td>GAD65, IA-2</td>
</tr>
<tr>
<td>Respiratory symptoms</td>
<td>KCNRG</td>
</tr>
<tr>
<td>Intestinal symptoms</td>
<td>TPH</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>AADC</td>
</tr>
<tr>
<td>Alopecia</td>
<td>TH</td>
</tr>
<tr>
<td>Vitiligo</td>
<td>SOX9, SOX10</td>
</tr>
<tr>
<td>?1)</td>
<td>IFNω, IFNα4</td>
</tr>
<tr>
<td>Chronic Mucocutaneous Candidias?</td>
<td>IL-22, IL-17A</td>
</tr>
</tbody>
</table>

1) No known associated disease component.

Autoimmune Addison’s disease

Autoimmune Addison’s disease (AAD) is a primary adrenal failure caused by autoimmune destruction of the adrenal cortex. The disease was first described by Thomas Addison in 1855 [29]. The principal symptoms of Addison’s disease are fatigue, reduced appetite, weight loss, salt cravings, and hyperpigmentation of the skin due to elevated adrenocorticotropic hormone levels [30, 31]. An important hallmark of AAD is the presence of autoantibodies targeting the steroidogenic 21-hydroxylase (21-OH), an intracellular key enzyme in cortisol and aldosterone production, selectively expressed in the adrenal cortex [32]. Circulating adrenal cortex autoantibodies can be detected several years before clinical onset; they therefore serve as predictive markers for the disease [33]. At diagnosis, >90% of Addison patients have 21-OH autoantibodies, and the prevalence slowly declines with disease...
duration, due to decreased size of the remaining adrenal cortex tissue [34, 35]. Less than 0.5% of the general population have 21-OH autoantibodies. However, 50% of people with autoantibodies to 21-OH never develop Addison’s disease [33], the autoantibodies may thus not be the causative factor of AAD but rather an indication of a T cell mediated destruction of the adrenal cortex.

As a consequence of the adrenal gland destruction, patients become incapable of producing sufficient steroid hormones such as glucocorticoids (e.g. cortisol) and mineralocorticoids (e.g. aldosterone) [36]. The adrenal glands constitute together with hypothalamus and pituitary glands the hypothalamic–pituitary–adrenal (HPA) axis, mainly regulated by the corticotrophin-releasing hormone (CRH) and the adrenocorticotropic hormone (ACTH) (figure 1). The HPA-axis activity is controlled by the negative feedback of cortisol on the hypothalamus and the pituitary gland (figure 1). It helps control reactions to stress and regulates many body processes such as mood, sexuality, and metabolism [37]. In Addison’s patients, the lack of endogenous cortisol production impairs the negative feedback loop and results in elevated CRH and ACTH levels.

![Figure 1. The normal function of the Hypothalamic-Pituitary-Adrenal (HPA) axis and its regulation. The hypothalamus releases CRH which upregulates the secretion of ACTH by the anterior pituitary. ACTH in turn acts on the adrenal cortex to stimulate cortisol production. Cortisol has a negative feedback effect on both the hypothalamus and the anterior pituitary to decrease CRH and ACTH release respectively.](image)

Untreated, AAD can lead to the life-threatening condition Addisonian crisis, characterized by very low blood pressure and ensuing coma [30]. Treatment of Addison patients should include glucocorticoid and mineralocorticoid replacement, whilst the benefit of androgen substitution remains debatable [36, 38]. The ideal glucocorticoid substitution therapy would mimic the endogenous circadian cortisol rhythm, although this is not possible to obtain with the current oral replacement regimens that generate periods of supra- and infra-physiological cortisol levels [39]. Overtreatment with glucocorticoids has been connected to negative metabolic consequences e.g. impaired
glucose tolerance, dyslipidaemia, hypertension, and osteoporosis [40-42]. It is believed that today’s non-optimized replacement therapy may contribute to the increased mortality and morbidity reported in AAD [43].

Autoimmune Addison’s disease is frequently associated with other non-adrenal organ-specific autoimmune disorders, most commonly hypothyroidism, and the patient then suffers from an autoimmune polyendocrine syndrome, APS [31, 38]; non-endocrine conditions such as vitiligo and celiac disease, however, are also typically associated with Addison’s disease [44]. APS-1 is the monogenic disorder mentioned above whereas APS-2 is a much more common and genetically complex entity characterized by two or more concurrent autoimmune endocrinopathies, e.g. AAD, autoimmune hypo- or hyperthyroidism and type 1 diabetes [45-47]. The associated diseases are often connected with the presence of autoantibodies against a specific antigen (table 1). After 21-OH autoantibodies, anti-thyroid peroxidase (TPO), associated with hypothyroidism is most frequently found in AAD patients. The third most common autoantibodies are parietal cell antibodies, associated with pernicious anemia. However, autoantibodies against many other autoantigens have been associated with Addison’s disease and APS-1, e.g. 17α-OH, SCC, and GAD65 [47]. Other autoantibodies are considered to be specific for APS-1, such as IFNα, IFNα4, and IL-22.

The Swedish Addison Registry is the world’s largest collection of clinical data and blood from patients with AAD. To estimate the total number of Addison patients in Sweden, individuals with both a diagnosis of AAD and on combination treatment with hydrocortisone/cortisone acetate and fludrocortisone were identified in 2013 through the Swedish National Patient Registry and the Swedish Prescribed Drug Registry [48]. With this approach, the number of patients with AAD in Sweden was estimated to be 1305. Assuming this number is still valid, the Swedish Addison Registry covers approximately 57% of the AAD patients in Sweden.

Paraneoplastic Syndromes and Cancer-Associated Retinopathy

Paraneoplastic syndromes are rare, tumour-associated disorders that are not a direct effect of the tumour itself. These conditions are either mediated by humoral factors, such as hormones or cytokines secreted by the tumour, or mediated by an immunological response against the tumour [49, 50]. Immunologically mediated paraneoplastic syndromes arise when the immune response against the tumour cross-reacts with healthy tissue where the same or cognate antigens are normally expressed. The location where the autoantigen is expressed will therefore determine the type of organ or tissue involved [51]. Some autoimmune diseases arise almost exclusively as paraneoplastic
manifestations whereas many autoimmune diseases can occur either in presence or in absence of an underlying malignancy [52]. Paraneoplastic retinopathies can be divided into cancer-associated retinopathy (CAR) and melanoma-associated retinopathy (MAR). These are autoimmune retinal degenerative disorders, occurring in a small subset of patients with systemic cancer. CAR is characterized by sudden, bilateral, and painless vision loss and photosensitivity, and is associated with anti-retinal autoantibodies [53-55].

Paraneoplastic syndromes occur in 7-10% of patients with malignant neoplasms. The number of patients with visual symptoms of CAR and undiagnosed malignancy are continuously increasing with growing physician awareness and improved diagnostic tools and criteria. Nevertheless, it still remains a significant diagnostic and therapeutic challenge [56]. The latency time from the presentation of vision loss to detection of tumour may be several years; hence the presence of autoantibodies may serve as a predictive marker of an underlying neoplasm [53, 57].

CAR is most commonly associated with breast cancer, followed by lung and gynaecological cancers. Different malignancies are often associated with specific CAR antigens, e.g. breast cancer is associated with autoantibodies against α-enolase and transductin-α, while cervical cancers are associated with anti-recoverin antibodies. There are also a number of unknown antigens with known molecular weights identified by Western blotting, that are associated with different malignancies. Several different methods are used to evaluate the presence of anti-retinal antibodies: immunofluorescence testing, western blot testing, and ELISA testing. However, testing for antiretinal antibodies are not currently standardized among laboratories, hence the results are not always concordant between different testing sites [58]. There is a need for standardized testing methods and identification of new CAR associated biomarkers.
Current investigations

Aims

I. Investigate the mechanisms behind the T cell mediated destruction of the adrenal glands in autoimmune Addison's disease.

II. Identify new retinal structure/s and autoantigen/s in patients suffering from cancer-associated retinopathy.

III. Study patients with autoimmune Addison’s disease to gain deeper insights into clinical and immunological features to provide upgraded data regarding autoimmune comorbidities, replacement therapy, autoantibody profiles, and metabolic factors.

IV. Investigate the frequency of undiagnosed cases of APS-1 in the Swedish Addison Registry, and evaluate the prediction value of currently available diagnostic serological and genetic markers for the diagnosis of APS-1.

Materials and Methods

In vitro Transcription and Translation (Manuscripts I, II, III)

Full-length cDNA clones were used to produce recombinant $^{35}$S-radiolabeled protein using the TnT® system (Promega). Trichloroacetic acid precipitation and scintillation counting measured the $^{35}$S-methionine incorporation. 20 000 counts per minute (cpm) of the $^{35}$S-radiolabeled recombinant protein were incubated with patient or blood donor serum over night at 225 rpm at 4°C. The immune reactions were transferred to 96-well microtiter filtration plates (Millipore) and were incubated with protein-A for 45 min at 225 rpm at 4°C. On each plate, a positive standard, an APS-1 patient with previously known high titre autoantibodies against the antigen of interest, and a negative standard, 4% bovine serum albumin (BSA), were included. As positive standard in manuscript I, a commercial anti-AIPL1 antibody was used. The radioactivity of the immunoprecipitated material was measured with a liquid scintillation counter (Wallac 1450 MicroBeta, Perkin-Elmer). The autoantibody detection results were expressed as an index: $[cpm \text{ in the unknown sample} -$
cpm in negative standard) ÷ (cpm in the positive standard – cpm in the negative standard) * 100]. The upper limit of the normal range was defined as the mean index value for blood donors plus 3 standard deviations (SD) for manuscripts II and III, and 4 SD in manuscript I. For 21-OH autoantibody analysis in manuscript II and III, the limit for positive index values was based on maximum accuracy cut-off estimations from a recent interlaboratory study [59].

Protein Array Screening (Manuscript I)
A human proteome microarray (HuProt™) (CDI Laboratories, Baltimore, MD), containing 19,394 proteins covering approximately 80% of the protein coding genes, was screened with serum from a patient with CAR and osteosarcoma. The proteome array was incubated with serum in dilution 1:2000 for 90 min, at 50 rpm at room temperature followed by washing and addition of secondary detection anti-human Alexa 647 conjugated antibody (Life Technologies) in the dark for 50 min at 50 rpm at room temperature. The array was then washed and scanned using a CapitalBio LuxScan HT24 microarray scanner.

Immunofluorescence Staining (Manuscript I)
Human retinal cryosections were fixed with 4% paraformaldehyde for 10 min followed by permeabilization, and blocking at room temperature. The sections were incubated with human serum overnight at 4°C, followed by incubation with secondary anti-human antibody conjugated to Alexa 488. After washing and blocking, a rabbit anti-AIPL1 antibody in dilution 1:100 was added and the sections were incubated for 1 h, followed by washing and incubation with a secondary anti-rabbit antibody conjugated to Alexa 594. The sections were washed again and mounting reagent with DAPI was added. The immunofluorescent labelling was evaluated using an Olympus Fluoview1000 confocal microscope.

Porcine eye cryosections were fixed in ice-cold acetone for 2 min followed by blocking at room temperature. The sections were incubated with human serum in dilution 1:400 overnight at 4°C. Antibody binding was detected using a FITC-conjugated goat anti-human antibody in dilution 1:100. The sections were washed, and mounting reagent with DAPI was added. The immunofluorescent labelling was evaluated under a Zeiss LSM 510 META confocal microscope.

Recall Assay (Paper I)
Overlapping 18-aa peptides spanning the entire 21-OH protein dissolved in DMSO (40 µg/ml) were used to stimulate patient PBMCs either individually
or as a combined pool. Six million cells per well were stimulated with 1 µg/ml 21-OH peptide pool in RH-10 medium with IL-7. On day 3, RH-10 medium containing IL-2 was added and the cells were cultured with this medium until day 13, when they were washed to remove IL-2 and incubated in RH-10 overnight. On day 14, 200 000 cells were pulsed with individual peptides or the pool of peptides for 5 h in presence of brefeldin A, followed by washing and staining for the surface markers CD8 conjugated to APC-H7, CD4 conjugated to PerCP, CD3 conjugated to V500, CD107 conjugated to PE together with LIVE/DEAD viability marker for 30 min on ice. The cells were then washed and fixed in fixation solution overnight at 4°C. The following day, the cells were permeabilized and stained for intracellular for cytokine secretion with FITC conjugated antibody against IFNγ for 30 min on ice. Cells were washed and re-suspended in FACS buffer for acquisition by flow cytometry (FACSCantoII) and data were analysed using FlowJo (TreeStar).

![Figure 2. Overview of the recall assay.](image)

**Ex vivo ELISPOT (Paper I)**

Plates were coated at 4°C overnight with capture antibody in coating buffer and the next day washed followed by 1 h blocking at 37°C. The plates were washed again and 500 000 cells per well were incubated with 10 µg/ml individual peptide or peptide pool were incubated overnight at 37°C. The plates were washed before adding biotin detection antibody, then incubated for 2 h at 37°C followed by washing and adding of Streptavidin-ALP and the plates were then incubated for 1 h at room temperature. Finally, the plates were washed before developing by adding substrate solution for 10 min in dark at room temperature. Rinsing the plates thoroughly with cold water stopped development and the plates were left to dry completely before spots were counted using an AID EliSpot Reader.
Granzyme B Assay (Paper I)

20 000 CD8⁺ T cells were co-cultured with 40 000 target cells from the HLA-A2–expressing tumour line NCI H295 overnight followed by measurement of Granzyme B secretion in the supernatant using ELISA. The ELISA plates were coated overnight with anti-Granzyme B antibody before addition of the supernatant and then incubated overnight at 4°C. The next day, the plates were washed and incubated with biotin antibody, followed by addition of ExtrAvidin Peroxidase. The plates were developed using TMB solution.

Statistical Analysis (Manuscript II)

T-test was used when investigating correlations between a non-categorical variable with a categorical variable. Chi²-tests and Fisher’s exact test were used when investigating correlation between proportions. Linear logistic regression was used for analysis of 21-OH autoantibody index and disease duration. For comparisons of the SAR and MONICA cohorts linear logistic regression was used for BMI and binary logistic regression for hypertension, hyperlipidaemia, and type 2 diabetes. Glucocorticoid replacement dose (mg/day) was recalculated to hydrocortisone equivalent dose (mg/m²/day) accordingly:

\[
\text{Hydrocortisone dose x 1 + Cortisone acetate dose x 0.8 + Plenadren dose x 0.806 + Prednisolone dose x 4 + Dexametasone dose x 26.667} \sqrt{\frac{\text{length} \times \text{weight}}{3600}}
\]

All the above statistical analyses were performed using the SAS v9.4, Prism v6 and SPSS 23.0 software. Significance was accepted if p<0.05 for all analyses in this study.

Results and Discussion

Cytolytic 21-hydroxylase-specific CD8⁺ T cells mediate adrenal cortex destruction in autoimmune Addison's disease patients (Paper I)

The target antigen for autoimmune Addison’s disease (AAD), 21-OH, is exclusively expressed in the adrenal cortex [60]. The mechanisms behind
destruction of the adrenal glands in AAD remain unclear and it is speculated that T cells mediate the disease.

In this study, we wanted to identify the immunodominant T cell epitope/s on the 21-OH protein. To do this, peripheral blood monocytes (PBMCs) were isolated from 20 patients with AAD with high 21-OH antibody titres and 7 healthy controls. The PBMCs were incubated with overlapping 18-aa peptides spanning the entire 21-OH protein for 13 days in a recall assay. At day 15, the IFNγ secretion from CD4+ and CD8+ T cells respectively was quantified via FACS. Expanding the patient’s PBMCs with a pool of peptides revealed that the majority of the 20 Addison’s disease patients had high frequency of 21-OH–specific CD8+ and CD4+ T cells, compared with healthy controls (figure 3, upper panels). Expanding PBMCs with each individual peptide revealed that the majority of patients had CD8+ T cells capable of recognizing 21-OH337–354 and/or 21-OH428–445, and CD4+ T cells recognizing the peptide 21-OH 207–224 (figure 3, lower panels). Frequencies of 21-OH specific T cell responses were also compared between patients diagnosed with Addison’s disease for up to 20 years previously, revealing that although the magnitude of the 21-OH–specific T cell response decreased over time, it is still present even at the point when the adrenal cortex is hypothesized to be completely destroyed. This indicates a continuous stimulation of 21-OH–specific T cells, perhaps mediated by ectopic expression of 21-OH in other tissues or by pathogens expressing a cross-reactive epitope.

Figure 3. Upper panels: The production of IFNγ from (A) CD8+ and (B) CD4+ T cells in response to 21-OH in AAD patients or healthy controls under re-stimulation
with the 21-OH peptide pool or unstimulated (DMSO). Lower panels: 21-OH peptide pool bulk cultured PBMCs were re-stimulated with individual peptides, DMSO or the 21-OH pool and stained intracellularly for IFNγ secretion.

By ex vivo ELISPOT assay using patients' PBMCs we could show that the same immunodominant peptides as in the recall assay evoked T cell responses. The ELISPOT assay provides a better understanding of the magnitude of 21-OH–specific T cells as the recall assay indicates a memory T cell response rather than representing the actual frequency of 21-OH–specific T cells. By ex vivo ELISPOT assay, we identified which epitope on the immunodominant peptide 21-OH337–354 evoked the strongest T cell response by incubating PBMCs with overlapping 9-mers spanning the 18 amino acid long peptide. The 9-mer 21-OH342–350 (LLNATIAEV) elicited the strongest response and was subsequently used for sorting CD8⁺ T cell clones to perform functional studies. The T cell clones were stained with HLA-A2 and HLA-B8 tetramers loaded with the peptides 21-OH342–350 and 21-OH428–435 to confirm the specificity and HLA restriction of the T cell response.

The CD8⁺ T cells were tested in VITAL and Granzyme B assay and for their ability to recognize 21-OH–expressing cells. In the VITAL assay, the CD8⁺ T cells recognized target cells pulsed with 21-OH342–350 peptide, but not with irrelevant peptides. These data provide direct evidence that the T cell populations derived from PBMCs of Addison’s disease patients contain HLA-A2–restricted CD8⁺ T cells that recognize the peptide 21-OH342–350. In the Granzyme B assay, the 21-OH342–350–specific HLA-A2–restricted CD8⁺ T cell clone was co-cultured with the HLA-A2–expressing tumour cell line NCI H295, and an increase of Granzyme B secretion in the supernatant, as measured by ELISA was observed. Addition of HLA-A2–blocking antibody BB7.2 reduced NCI H295 recognition (figure 4).

**Figure 4.** Granzyme B secretion detectable in the supernatant by ELISA when 21-OH specific T cells are cocultured with NCI H295 cells. The response is increased when exogenous 21-OH342–350 peptide is pulsed to target cells before coculture and reduced when the A2 blocking antibody, BB7.2 is added.
The ability of 21-OH–specific CD8$^+$ T cell clones to recognize endogenous 21-OH protein may be directly linked to the progression of Addison’s disease. Since only a minority of individuals with 21-OH autoantibodies develop Addison’s disease within 5 years [33], future investigations of T cell responses in these individuals and the ability to predict disease progression are of major importance, and may possibly result in diagnostic and therapeutic opportunities. A long-term vision would also be to use the understanding of T cell response initiation in Addison’s disease to induce responses against adrenal cortex cancer; a 21-OH–positive tumour with very poor prognosis that often afflicts younger people.
Aryl Hydrocarbon Receptor-Interacting Protein-Like 1 (AIPL1) is a novel retina-specific autoantigen in Cancer-Associated Retinopathy (Manuscript I)

Paraneoplastic syndromes constitute symptoms of tissue damage remote from a malignant neoplasm present in the body and in many cases the paraneoplastic symptoms arise due to an immunological reaction. In this study, we identify a novel autoantigen in a patient with cancer-associated retinopathy (CAR) concomitant with malignant osteosarcoma. The patient was a previously healthy 15-year-old female patient with two years’ history of pain in her right calcaneus and simultaneous development of increased photosensitivity and bilateral rapid visual loss. Optical coherence tomography (OCT) showed a macular atrophy with photoreceptor thinning and electroretinogram (ERG) demonstrated a reduction of rod-cone function by more than 90% as compared to a normal response some months before the patient became blind. When the patient’s symptoms in her calcaneus were investigated with magnetic resonance imaging (MRI) and subsequent biopsy, it revealed a small-cell tumour that was later characterized as osteosarcoma.

By screening a proteome array with patient serum, the retina and pineal gland specific protein Aryl hydrocarbon receptor interacting protein-like 1 (AIPL1) was identified as the immune target. Evaluation of AIPL1 as an autoantigen in CAR using a radioligand-binding assay with 73 healthy blood donors and 100 other patient sera from patients with CAR and different malignancies, or autoimmune retinopathy (AR), revealed that AIPL1 is an uncommon autoantigen among CAR/AR patients. To localize the target antigen in the retina, human retinal tissue was immunostained with serum from the patient with osteosarcoma and CAR, a healthy control, and a commercial monoclonal AIPL1 antibody. The patient’s autoantibodies were directed towards the interphotoreceptor matrix in the retina. Confocal images showed that the AIPL1 is targeted in rod photoreceptor cells (figure 5). These results are consistent with previous studies showing that AIPL1 is expressed in both developing cone and rod photoreceptors but restricted to rod photoreceptors in the adult retina, suggesting that AIPL1 is essential for development of normal rod and cone photoreceptor function but is only required for rod survival in the adult [61]. The symptoms of CAR depend on whether the rod or cone function is primarily disrupted. In this case, with the major autoantigen being AIPL1, expressed in rods, the signs are often constriction of the visual field with impaired dark adaptation [62].
Figure 5. Indirect immunofluorescence staining of human retinal tissue with serum sample from the CAR patient (dilution 1:50) and AIPL1 antibody (dilution 1:100). Green represents secondary antibody conjugated to Alexa 488, staining structures recognized by the patient serum. Red represents secondary antibody conjugated to Alexa 594 staining structures recognized by the monoclonal anti-AIPL1. Blue represents DAPI stained nuclei. CAR serum stains the rod photoreceptors and also cones (green). The anti-AIPL1 antibody stains mainly rods (red). The merged image shows an overlap between anti-AIPL1 antibody and CAR patient serum (yellow). The synaptic region of photoreceptor cells is strongly labelled. Scale bars represent 20 \( \mu \)m in all sub-panels.

Homozygous or compound heterozygous mutations in the gene encoding AIPL1 on chromosome 17p13 cause the disease Leber congenital amaurosis 4 (LCA4) (OMIM# 604393). Patients affected by LCA4 are diagnosed at birth or in the first months of life with nystagmus, severely impaired vision or blindness, and absence of rod and cone function, as demonstrated by ERG [63]. Sohocki et al. identified the disease causing nonsense mutation W278X in AIPL1 in 2000 and it is estimated that mutations in the AIPL1 gene might account for approximately 20% of LCA in isolated populations [64] and 7% worldwide [65]. The human aryl hydrocarbon receptor interacting protein-like 1 is named for its similarity to the human aryl hydrocarbon receptor-interacting protein (AIP) and belongs to the FK506-binding protein (FKBP) family. In the retina, it acts as a specific chaperone for phosphodiesterase-6 (PDE6), a visual effector enzyme, which is important in phototransduction. Mutations in AIPL1 result in destabilization of PDE6 and thereby disturbed phototransduction, resulting in LCA4 [66].
Intriguingly, AIPL1 is only expressed in photoreceptor cells and the pineal gland. This co-expression suggests that the pineal gland is a potential target for autoimmune disorders. The pineal gland has a diurnal release of melatonin, resetting circadian rhythm. However, the exact function of AIPL1 in this tissue remains elusive although it is not unlikely that AIPL1 mutations may also give rise to pineal-associated abnormalities in LCA4 patients [64].

When the same protein is deficient due to either genetic mutations or autoimmune destruction, the autoimmune disorder is considered to be a phenocopy of the genetic disorder, meaning that CAR could be an acquired autoimmune phenocopy of the inherited disease LCA4. In phenocopic disorders, the shared molecular targets are often important proteins with key functions in various tissues. In this context, this suggests that AIPL1 is a plausible key protein in the retina.

In retrospect, it is apparent that the patient presented with CAR long before the tumour was diagnosed. This is nothing unique, paraneoplastic symptoms precede the diagnosis of the neoplasm more often than is usually recognised [53, 67]. This empathises the challenge to recognize when a symptom are associated with an underlying malignancy and the importance of the identification of paraneoplastic autoantibodies also from a tumour diagnostic perspective. Facilitating earlier diagnosis and treatment could in this particular case perhaps at least partly have saved the patient’s vision. The retinal damage is to the largest extent considered to be irreversible; nevertheless, tumour removal may reduce the disease’s progression, underlining the importance of early diagnosis.

Clinical and immunological characteristics of Autoimmune Addison’s disease (Manuscript II)

In order to make an updated characterization of Addison patients, a comprehensive clinical and immunological study was conducted on 660 patients with autoimmune Addison’s disease in the multi-centre Swedish Addison Registry (SAR). This is the largest patient cohort available internationally. Because autoimmune Addison’s disease (AAD) is a rare disease, deeper insights into clinical and immunological features are needed to optimize monitoring of these patients. Clinical data from registration forms were evaluated and presence of autoantibodies against 13 different autoantigens were determined in all patients.

All patients were screened for autoantibodies against associated antigens 21-OH, 17α-OH, SCC, SOX10, AADC, TPO, GAD65, TGM2, deamidated gliadin, parietal cells, and the APS-1 indicative autoantigens IFNγ, IFNa4, and IL-22. Patients with any of the APS-1 suggestive autoantibodies were also screened for conventional APS-1 associated autoantibodies; TH, TPH, KCNRG, CPY1A2, and NALP5.
This study validates some previous findings and provides upgraded data regarding gender differences, autoimmune comorbidities, replacement therapy, autoantibody profiles and, for the first time in such a large cohort, data on metabolic factors.

The proportion of 21-OH autoantibody positive patients was 83% and these patients had significantly shorter disease duration (mean 15.6 years) than the 21-OH negative patients (mean 23.9 years). Our study also revealed a significant negative correlation ($r=-0.26$) between 21-OH autoantibody levels and disease duration (figure 6). A similar correlation has previously been seen in a smaller cohort (n=94) of Norwegian AAD patients [34]. Nevertheless, 21-OH autoantibodies were often detectable in the circulation a long time after AAD diagnosis, which is intriguing since it is believed that the autoimmune attack results in complete adrenal gland destruction and with this, the presence of the autoantigen.

![Figure 6](image)

**Figure 6.** Prevalence of 21-OH autoantibodies among 660 patients in the SAR cohort. Panel A shows 21-OH autoantibody index value plotted against disease duration ($R^2=0.068$; p<0.0001). Panel B shows the proportion of 21-OH autoantibody positive patients in different disease duration intervals.

We could confirm that AAD more commonly affects women than men [34] and the mean age at diagnosis was significantly higher for women, 36.7 years, than for men with 31.1 years (95% CI: 3.45-7.76; p<0.0001), which is concurrent with previous studies [34, 68, 69]. This might mirror the fact that the disease actually develops later in life among women, or that the time from that the disease develops until diagnosis is longer in women, which in turn might be a consequence of women being more neglected in the healthcare system than men, as similar tendencies have been observed for patients with acute coronary syndrome [70, 71]. Another hypothesis might be that women are protected from developing autoimmunity by pregnancy [72, 73]. The majority of the patients in the SAR (62%) had one or more associated autoimmune disease with a woman to man ratio of 1.03:0.64 (p<0.0001). The finding that women with AAD have a higher degree of autoimmune comorbidity as compared to men with AAD is in line with the trend seen in the general population [74, 75]. Hypothyrodism (p<0.0001),
hyperthyrodism (p=0.0028), hypogonadism (p=0.0015), and alopecia (p=0.0454) had significantly higher frequencies among women than men in our study. The female preponderance of hypogonadism may partly have two explanations; women get more obvious symptoms than men, i.e. amenorrhea, in contrast to the more diffuse symptom erectile dysfunction [46] or that the gonadal autoantigen is more protected in men because of the blood-testis barrier.

The most common concomitant diseases were hypothyroidism, followed by type 1 diabetes, pernicious anaemia, and hyperthyroidism. In the SAR cohort, 264/660 (40%) had hypothyroidism, which is a considerably higher proportion than in previous studies, with frequencies between 6% and 22% [35, 46]. In the general population, hypothyroidism affects less than 1% of people [35, 75]. TPO autoantibodies were seen in 57% of the SAR population with a significant association to hypothyroidism. These results suggest that TPO antibodies and development of hypothyroidism should be monitored regularly in Addison’s patients. The frequency of type 1 diabetes (T1DM) was 11% among the Addison patients, concurrent with previous studies (7-12%) [35, 46], which could be compared to a prevalence of T1DM in the general population of 0.5% [76]. However, we did not see any significantly higher frequency of T1DM among men, which was to be expected as it is more common among men in the general population [77]. GAD65 autoantibodies were detected in 23% of the cohort, showing a clear association to T1DM (p<0.0001). The prevalence of pernicious anaemia was 11% among the AAD patients in the SAR, similar to previous results [46]. However, 33% of the AAD patients were positive for parietal cell autoantibodies, strongly associated with pernicious anaemia. This suggests that the actual number of patients suffering from pernicious anaemia is under reported in our patient cohort and that B12 deficiency should be monitored regularly in Addison’s patients. Surprisingly, only 17 (2.6%) had transglutaminase (TGM2) autoantibodies in this cohort, and 15 (2.3%) had diagnosed celiac disease. These numbers are not significantly higher than the estimated 1% of the general Western world population having celiac disease [78-80]. However, only five of the patients with celiac disease diagnosis had TGM2 autoantibodies suggesting that 12 patients with TGM2 autoantibodies do not yet have a diagnosis; hence the actual number of celiac disease patients is rather 27 (4.1%). This number is still lower than 5.6-7.9% in previous studies [35, 81]. A higher prevalence of celiac disease was previously shown among AAD patients with APS-1 [82], which may partly explain the low incidence of celiac disease in this study, where APS-1 patients are excluded.

There was an overall discrepancy between serum autoantibody findings and clinical diagnoses of corresponding disease. For example, 374 patients had TPO autoantibodies but only 264 had hypothyroidism and 153 patients had GAD65 autoantibodies while only 71 had T1DM. GAD65 autoantibodies in non-diabetic patients might indicate subclinical insulitis, which in time
can progress to overt T1DM [83, 84], suggesting these patients should be regularly followed up.

We also analysed if the autoimmune components were diagnosed before, at the same time as, or after AAD diagnosis. This revealed that the median time from AAD diagnosis to hypothyroidism diagnosis was 0 years (Q1-Q3; -1;5 years) (p <0.0001), possibly reflecting that the patient is screened for numerous diseases when getting the AAD diagnosis. Another explanation would be that an increase in TSH due to low cortisol is misinterpreted as hypothyroidism. From AAD diagnosis to pernicious anaemia diagnosis, the median time was +10 years (Q1-Q3; 0;22 years) (p<0.0001). No other associated diseases showed any significant differences in time of diagnosis. However, hypertension, hyperlipidaemia, and type 2 diabetes take 15-20 years to develop; mirroring these may be unfavourable side effects from long-term glucocorticoid substitution therapy.

Glucocorticoid replacement was predominantly covered by regular hydrocortisone (89%) with a mean dose of 28.0 mg/day (std dev 8.5), divided into one (12.9%), two (49.5%), three (33.0%), or four (3.3%) daily doses. The mean daily hydrocortisone dose was slightly higher than 15-25 mg/day, which is generally the recommendation today [85]. When glucocorticoid doses were normalized to body surface and recalculated to hydrocortisone equivalent dose, the mean dose was 14.8 mg/m²/day (std dev 4.4). Very little data exist on the actual doses used in AAD cohorts, however, in a Norwegian study, the hydrocortisone equivalent daily dose, recalculated from cortisol, was 32.4 mg/day [35] and a south African study reports a median hydrocortisone dose of 23.8 mg/day [41].

The hydrocortisone equivalent dose was compared for the different glucocorticoid replacement regimens, where patients receiving hydrocortisone modified release tablet Plenadren® (n=52) had a 2.8 mg/m²/day (95% CI: 1.56-4.04; p<0.0001) lower mean total hydrocortisone equivalent dose, and patients with prednisolone (n=3) had a 5.1 mg/m²/day (95% CI 0.07-10.08; p=0.047) lower hydrocortisone equivalent dose than patients on regular hydrocortisone substitution (figure 7).
The ideal glucocorticoid replacement therapy aims to mimic the endogenous cortisol rhythm [86, 87], and minimize unfavourable metabolic side effects and decrease in quality of life. This means low cortisol levels in the evening, gradually rising during the night and peaking in the morning before waking, followed by a decrease during the day [88]. However, today’s conventional glucocorticoid replacement therapy inadequately imitates this pattern. A too high hydrocortisone dose and non-physiological peaks in cortisol levels are associated with unfavourable metabolic side effects and decrease in quality of life [86, 87, 89], and previous studies show that hydrocortisone doses exceeding 30 mg/day more severely impair the quality of life compared to lower doses [86, 90, 91].

Mineralocorticoid substitution was utilized by 89% of the patients with a mean dose of 0.093 mg/day (std dev: 0.054 mg/day). The recommended daily dose is 0.05-0.2 mg [31] but data suggests that 0.2 mg of fludrocortisone is needed to maintain adequate sodium and water balance [92]. This indicates that the patients on mineralocorticoid substitution in the SAR are receiving too low doses for appropriate effects. The patients not receiving fludrocortisone substitution (11%) had a 1.2 mg/m²/day higher mean hydrocortisone equivalent dose as compared to those receiving fludrocortisone replacement (95% CI: 0.16-2.30; p=0.0240). The long-term effects of being without or on too low a mineralocorticoid dose and compensate this with higher glucocorticoid dose are not known. No correlation between daily fludrocortisone dose and incidence of hypertension, hyperlipidemia, or type 2 diabetes could be seen.

When analysing the occurrence of metabolic factors, 553 patients in the AAD cohort (25-75 years of age) were compared to the population-based control cohort MONICA (MONItoring of Trends and Determinants of CArdiovascular Disease) (n=3627) from northern Sweden. The SAR cohort con-
tained a significantly higher proportion of women (59.7%) than the control cohort (50.9%) (p<0.0001). The comparison revealed that despite higher than recommended average doses of glucocorticoid replacement therapy among the Addison’s patients, they did not have a higher risk of type 2 diabetes or hyperlipidaemia, usually more frequent in AAD patients with glucocorticoid replacement than in the general population. Furthermore, the AAD patients had a lower risk of hypertension than the control population, and BMI was significantly lower in patients with AAD compared with the control subjects, mean difference 1.27 kg/m²* (95% CI 0.86-1.68 kg/m²) (*adjusted for age and sex), p<0.0001).

Long-term glucocorticoid treatment is generally associated with increased BMI [93, 94]. The finding that the Addison patients in this study have lower BMI may concur with the lower risk of having hypertension among the Addison cohort. The results are contradictory to some previous studies, showing that patients with primary adrenal insufficiency have an increased mortality risk with cardiovascular diseases as the leading cause of death [68, 95]. However, in these studies the cause of death is not considered and the cardiovascular mortality among AAD patients may be partly explained by that the cause of death may be acute cortisol deficiency during the intensive care rather than from cardiovascular causes. In summary; all these results on metabolic effects indicate that the patients in the SAR are receiving an adequate glucocorticoid replacement therapy.

Identification of subclinical APS-1 patients within the Swedish Addison registry (Manuscript III)

Autoimmune Addison’s disease is a major disease component in autoimmune polyendocrine syndrome type 1 (APS-1). Therefore, following the comprehensive autoantibody testing in the previous study, patients in the Swedish Addison registry positive for any of the autoantibodies against IFNω, IFNα4, or IL-22 were classified as possible undiagnosed APS-1 patients and were further screened for conventional APS-1 associated autoantibodies against TH, TPH, KCNRG, CYP1A2, and NALP5. In this study, also the APS-1 patients in the SAR were included, resulting in 677 Addison patients. Of these patients, 34 had IFNω, IFNα4, and/or IL-22 autoantibodies. Fourteen of these (41%) had autoantibodies against more than one of these three autoantigens (figure 8). Thirteen of the 34 AAD patients with APS-1 autoantibodies fulfilled the clinical criteria for APS-1 and already had an established APS-1 diagnosis. Four of the remaining 25 suspected new APS-1 patients had disease-causing mutations in AIRE. This leaves 21 patients with IFNω, IFNα4, and/or IL-22 autoantibodies but without APS-1.
Figure 8. Autoantibody profiles for 677 patients in the Swedish Addison Registry. Overview of the different patient subgroups from the antibody screening and sequencing is shown to the left. Diagram over the autoantibody profiles of the suspected (n=25) and known (n=13) APS-1 patients is shown to the right. Each circle represents one of the three autoantibodies IFNω, IFNa4, and IL-22. The number outside the circle represents patients without any of the autoantibodies. Known APS-1 patients are indicated by *.

It can be speculated that the patients with IFN/IL-22 autoantibodies without disease causing AIRE mutations or clinical manifestations indicative for APS-1 may be more closely related to each other than the rest of the Addison cohort or other APS-1 patients. However, by clustering all genetic variants of AIRE, this does not seem to hold. Since previous studies show that these autoantibodies are present in almost all APS-1 patients before manifestations of most symptoms [20, 28], it is important to keep these patients under observation for signs of late onset APS-1.

Patients with autoantibodies against IFNω, IFNa4, or IL-22 and no clinical symptoms typical of APS-1 and no identified disease causing AIRE mutations may indicate an intermediate form of APS-1, and these patients should be kept under observation. Since APS-1 patients can present with a wide range of clinical phenotypes, we suggest that all Addison patients should be screened for IFN/IL-22 antibodies. IFNω antibodies are almost always present before APS-1 is diagnosed and persistent, making them excellent disease markers, particularly for early or atypical patients who have not developed the full APS 1 picture [24]. Furthermore, APS-1 diagnosis should especially be considered in patients developing AD in childhood, and when a new patient is diagnosed, all siblings should be investigated for APS-1 manifestations and AIRE mutations [96].

Recent findings by Oftedal et al suggests that mono-allelic mutations in the PHD1 domain of AIRE can partially or completely disrupt the AIRE protein in a dominant negative manner giving milder phenotypes with later onset as compared to classical autosomal-recessive inherited APS-1. This can be explained by that some wild-type AIRE tetramers are still formed inducing some degree of self-tolerance [97]. However, the sequencing of
these patients in our study did not reveal such mono-allelic variants in the PHD1 domain (exon 8 and 9 of *AIRE*).
Conclusions

I. Both CD4\(^+\) and CD8\(^+\) 21-OH specific T cells are abundant in a large proportion of Addison’s patients both in vivo and ex vivo assays. CD8\(^+\) T cells are able to lyse 21-OH\(^+\) target cells, consistent with a potential mechanism for disease pathogenesis.

II. AIPL1 is a novel paraneoplastic retinal autoantigen, a finding demonstrating autoimmune-mediated CAR to be a phenocopy of the inherited disorder Leber congenital amaurosis type 4 (LCA4). We suggest that assessment of anti-retinal antibodies against AIPL1 might be useful not only to diagnose the retinal disease, but also to guide the clinician in searching for an underlying undiagnosed tumour or metastatic spread of cancer.

III. Patients with AAD are prone to develop autoimmune comorbidities; therefore these patients should be carefully monitored. The replacement therapy needs to be adjusted to follow recommended doses, however, the AAD patients do not have more unfavourable metabolic side effects than the control population.

IV. Four hitherto novel APS-1 patients in the SAR were identified by autoantibody screening. The patients had typical APS-1 autoantibody profiles, AIRE mutations, and fulfilled the clinical criteria for APS-1. The importance of screening for APS-1 autoantibodies should not be underestimated when it comes to diagnosing possible APS-1 that is not clinically overt. Since the clinical presentation of APS-1 is variable and often takes years to develop, patients having autoantibodies indicative for APS-1 should be further examined for mutations in the AIRE gene to help earlier diagnosis and allowing treatments to prevent serious complications.
Future Perspectives

This thesis describes the use of autoimmune model diseases to gain a better understanding of how and why autoimmunity arises. In the first paper, we identified three immunodominant epitopes in patients with autoimmune adrenal insufficiency. A better understanding of this initiation may open up for development of a T cell mediated immunotherapy against the aggressive cancer adrenal cortical carcinoma, a rare endocrine malignancy with limited treatment options and poor prognosis [98, 99]. Today’s treatment is based on complete surgical removal of the adrenals, but in half of the patients this is impossible due to metastasized disease [100]. Research has been conducted on immunotherapies in adrenal cortical carcinoma; however, adoptive T cell therapy is a new approach that not yet has been approved for this type of cancer [101, 102]. Using an adrenal cortex carcinoma cell-line (NCI-H295), the cytolytic ability of the T cells selected for the immunodominant epitope of 21-OH could be investigated ex vivo. This experiment can be considered as an initial study to assess the possibility of using adoptive T cell therapy in adrenal cortex carcinoma.

Deeper studies on paraneoplastic syndromes in general and the AIPL1 antigen in particular is needed for increased understanding of the T cell response to AIPL1 protein and other known paraneoplastic antigens, e.g. to explore if any of the autoantigens associated with CAR have shared epitopes. It is also of great interest to evaluate more CAR patient serum samples on proteome arrays to identify possible new autoantigens. It would also be of interest to study the role of AIPL1 in the pineal gland. Moreover, development of a multiplex testing with known paraneoplastic autotigens on a custom-made protein array would improve diagnostics for paraneoplastic syndromes.

Longitudinal studies on a group of patients with Addison’s disease would be needed to monitor how several factors such as 21-OH antibody titres, T cell responses, and development of associated endocrinopathies, alter with disease progression.
Vårt immunförsvar består av ett komplext nätverk som skyddar oss mot angrepp från bakterier, virus och parasiter. Två centrala celltyper som ingår i immunförsvarset är antikroppsproducerande B-celler, och cytotoxiska T-celler.

En av immunförsvarets grundläggande uppgifter är att urskilja vilka strukturer som tillhör den egna kroppen och inte angripa dessa. Trots allt sker dessa självdödsaktiga angrepp emellanåt och fenomenet kallas autoimmunitet och en sådan aktivering av immunförsvarset kan ge upphov till autoimmuna sjukdomar. De brukar delas in i organspecifika, där specifika organ angrips av immunförsvaret, eller systemiska, där autoantigener är spridda i hela kroppen. Trots att autoimmuna sjukdomar är vanligt förekommande är mycket lite känt om de mekanismer som har betydelse för dess uppkomst.

Ett kännetecken för många autoimmuna sjukdomar är att B-celler producerar antikroppar mot särskilda antigen. Genom att upptäcka dessa antikroppar kan man diagnostisera många sjukdomar och det är därför viktigt att identifiera dessa sjukdomsmarkörer som är specifikt associerade med olika autoimmuna sjukdomar. För att underlätta sökandet av nya sjukdomsmarkörer använder man sig av olika sjukdomsmodeller.

Den här avhandlingen beskriver studier av sjukdomsmechanismer i tre olika autoimmuna sjukdomar; Autoimmun kronisk binjurebarksvikt (Addisons sjukdom), Autoimmunt Polyendokrint Syndrom typ 1 (APS-1) och det paraneoplastiska syndromet Cancerassocierad Retinit (CAR).


Jag har studerat T-cellers roll i Addisons sjukdom, då det är mest troligt att det är T-celler som initierar sjukdomen och i sin tur aktiverar antikroppsproducerande B-celler. Genom att ingående förstå mekanismerna bakom hur Addisons sjukdom initieras skulle man i förlängningen kunna använda autoreaktiva 21-OH specifika T-celler som vaccinering vid binjurebarkscancer där de då skulle attackera tumörcellerna. Jag kunde se att T-cellerna känner
igen tre olika immundominanta epitoper på 21-OH proteinet och att aktive-
rade T-celler från patienter med Addisons sjukdom känner igen och attack-
rar binjurebarkcancerceller som uttrycker 21-OH.

Vidare har jag studerat en stor kohort Addisonpatienter som är registre-
rade i Svenska Addison Registret (SAR) för att karaktärisera denna grupp av
patienter och undersöka en mängd olika faktorer såsom associerade auto-
immuna sjukdomar, förekomst av autoantikroppar, metabola biverkningar av
medicinering mm. Jag valde ut 660 patienter ur SAR som hade autoimmun
binjurebarksvikt, vilket gör den här studien till världens största studie av Ad-
disonpatienter, och kunde bl.a. se att 83 % hade antikroppar mot 21-OH som
sakta avtog med ökad sjukdomstid. De vanligaste associerade sjukdomarna
är hypothyroidism följt av typ 1-diabetes och perniciös anemi. Vid jämförelse
med en kontrollkohort verkade Addisonpatienterna inte ha högre risk för att
utveckla typiska metabola biverkningar såsom högt BMI, hypertension, hy-
perlipidemi och typ 2-diabetes av sin medicinering, vilket var oväntat eftersom dessa är förväntade effekter vid långvarig glukokortikoids substi-
tue-ring.

En extrem form av autoimmunitet är den ovanliga sjukdomen APS-1 där
patienten utvecklar många olika organspecífiska autoimmuna sjukdomskom-
ponenter. APS-1 orsakas av att en viktig gen, Autoimmun Regulator (AIRE),
som kontrollerar immunförsvarets förmåga att inte angripa kroppsega
strukturer är muterad och resulterar i ett icke fungerande protein. Patienter
med APS-1 har höga nivåer autoantikroppar mot ett antal kända vävnadsspe-
cifika autoantigen. Samma autoantikroppar som identifierats hos APS-1
patienter kan också förekomma vid isolerade former av de autoimmuna
sjukdomskomponenterna. Det har tidigare visat sig att olika signaleringsmo-
lekylor som interferon-ω, interferon-α4 och interleukin-22 är bra biomark-
rer för sjukdomen då majoriteten av alla APS-1-patienter har antikroppar
mot dessa proteiner. Därför kan de användas för att hitta misstänkta odi-
gnostiserade APS-1-patienter bland riskgrupper; såsom patienter med auto-
immun kronisk binjurebarksvikt (Addisons sjukdom). Genom att undersöka
677 patienter ur Svenska Addisonregistret för autoantikroppar mot dessa
karaktäristiska APS-1-markörer, studera deras kliniska sytomm och sekven-
sera AIRE genen för att hitta sjukdomsframkallande mutationer identifierade
jag fyra nya APS-1-patienter i kohorten. Det är av stor vikt att APS-1-
patienter får rätt behandling, då obehandlad sjukdomen är livshotande.

Vidare har jag studerat en patient med det paraneoplastiska syndromet
cancerassosierad retinit. Paraneoplastiska syndrom uppstår när det finns en
tumör i kroppen som uttrycker en mängd olika antigen vilket resulterar i en
massiv aktivering av immunförsvaret i syfte att bekämpa tumören. Av en
slump kan tumören uttrycka proteiner som normalt även uttrycks någonstans
i övriga kroppen, och immunförsvaret kan då missriktas och attackera
droppsegen vävnad med höga nivåer autoantikroppar som följd. Dessa auto-
antikroppar finns ofta i cirkulationen innan tumören upptäcks och kan såle-
des vara användbara biomarkörer ur ett tumördiagnostiskt perspektiv. Patienten som jag har studerat var en ung flicka med hastigt försämrad syn och smärtor i en fot. Först när patienten var helt blind upptäcktes att orsaken till smärtan var skelettcancer i hälbenet, vilket resulterade i diagnosen osteosar­kom och cancerassocierad retinit. Jag identifierade det retinaspecifika anti­genet AIPL1 som målet för patientens autoimmuna angrepp på retina. Man kan spekulera i att med en mer utvecklad diagnostik av CAR som inkluderar den nya biomarkören AIPL1, hade en tidigare diagnos getts och patientens syn delvis kunnat räddas.

Sammanfattningsvis bidrar denna avhandling till ökad förståelse för bakomliggande mekanismer vid autoimmuna sjukdomar och kan ligga till grund för vidare studier som syftar till att utveckla diagnostik och terapier.
Acknowledgements

The work of this thesis was mainly carried out at the Department of Medical Sciences at Uppsala University. However, many experiments were performed at Center for Molecular Medicine at the Karolinska Institute and at the Weatherall Institute of Molecular Medicine at University of Oxford, where I have met incredibly talented and helpful people. I would like to express my gratitude to all of them, with a special thanks to the following people:

The heads of the Department of Medical Sciences at Uppsala University Lars Rönnblom and Eva Tiensuu Jansson.

My main supervisor Mohammad Alimohammadi for always having a solution to every problem and for great discussions. Thank you for encourage me to do things in good time and for still being patient with me when I did not.

My co-supervisor professor Olle Kämpe for his great knowledge, generosity, cooking expertise, and for taking such good care of the research group.

My other co-supervisor Sophie Bensing for her strong support, realistic advice and for all the inspiring discussions and good suggestions.

Äsa Hallgren, for knowing everything and for all the help and support over the years. Thank you for always having time and patience, and for being a great friend also outside of the lab.

My fellow commuters (and PhD colleagues) at Karolinska: Nils Landegren and Daniel Eriksson. Thank you for making every day in the lab easier and for all the great scientific and not-so-scientific discussions during fikas. I would also like to thank the new lab colleagues at CMM for being so friendly and for welcoming us to the new lab.

Friends and co-workers in Uppsala: Kerstin Ahlgren, Anna Lobell, Anna-Stina Sahlqvist, Brita Ardesjö-Lundgren, Magnus Isaksson, Fredrik Rorsman, Håkan Hedstrand, and Gerli Rosengren Pielberg. I would also like to give a special thanks to all other members of CKMF 2 and 3 for always being friendly and helpful.
I would like to thank all my collaborators, with a special thanks to Vincenzo Cerundolo and Amina Dawoodji and the other co-workers at the Weatherall Institute of Molecular Medicine in Oxford for welcoming me to the lab and making the stay there so worthwhile. Per Dahlquist and Marie Eriksson in Umeå for great statistical inputs. Per Morten Knappskog and Eystein Husebye in Bergen, and pediatric oncologists Josefine Palle and Britt-Marie Frost in Uppsala.

All of my co-authors and of course the patients.

My friends outside the lab Matilda, for being the best BFF ever, and for our new routine, dubious thai buffet lunches. Ebba and Sofie for great BSF discussions. Hanna, Annica, Mic, and Freddis for making life easier.

My parents Kerstin and Rolf for their love, encouragement, and invaluable life advice. My sisters and brothers Linda, Elin, Linus, and Andreas for always being there when needed. A special thank to Linda (again), not only for being my slightly older and considerably wiser sister but also for being my personal statistician with extremely flexible accessibility. I would also like to thank the Winquist family for their incredible hospitality.

My amazing son Frans, thank you for filling my life with happiness. To my joy, my friend, my companion, my love Thomas, thank you for everything! And last but not least I would like to thank Darwin, for being the best dog in the world.
References


59. Falorni, A., et al., Determination of 21-hydroxylase autoantibodies: inter-
laboratory concordance in the Euradrenal International Serum Exchange
60. Winqvist, O., F.A. Karlsson, and O. Kampe, 21-Hydroxylase, a major
62.
61. van der Spuy, J., et al., Predominant rod photoreceptor degeneration in Leber
62. Keltner, J.L., et al., Management and monitoring of cancer-
63. Cremers, F.P., J.A. van den Hurk, and A.I. den Hollander, Molecular genetics
64. Sohocki, M.M., et al., Mutations in a new photoreceptor-pineal gene on 17p
8.
67. Heckenlively, J.R. and H.A. Ferreyra, Autoimmune retinopathy: a review and
68. Berghorsdottir, R., et al., Premature mortality in patients with Addison's
4849-53.
70. Herlitz, J., et al., Characteristics and outcome among women and men
transported by ambulance due to symptoms arousing suspicion of acute
71. Maynard, C., et al., Influence of sex on the use of cardiac procedures in
patients presenting to the emergency department. A prospective multicenter
73. Kung, A.W. and B.M. Jones, A change from stimulatory to blocking antibody
75. Jacobson, D.L., et al., Epidemiology and estimated population burden of
selected autoimmune diseases in the United States. Clin Immunol
rate of diabetes mellitus in a Swedish community during 30 years of follow-up.


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”.)