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Peripheral immunity in patients with autoimmune endocrine diseases and the influence of physiological adaptions during pregnancy

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Abstract

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Type 1 diabetes (T1D), Hashimoto's thyroiditis (HT), Graves' disease (GD), and autoimmune Addison's disease (AD) appear to share immunogenetic mechanisms. This idea is not novel, as "autoimmune tautology" is an established concept. An issue with previous studies is that no or few simultaneous comparisons between these autoimmune endocrine diseases have been made. Due to methodological limitations, immune deviations associated with these diseases have also been examined for a limited number of immune cell lineages and analytes. High-dimensional single-cell mass cytometry was thus employed to phenotypically characterise all peripheral $CD45^+$ cell lineages, whilst immune-related proteins in plasma and cell supernatants were analysed by proximity extension assay. Patients with new-onset T1D, HT, and AD had altered frequencies of distinct clusters within antigen-presenting and cytotoxic cell lineages. Importantly, previously unreported alterations of rare cell subsets from patients with HT and AD were identified. The systemic immunoprofile of patients with autoimmune endocrine diseases was in general similar. However, an increased abundance of CDCP1 and SLAMF1 in plasma from patients with T1D, HT, and GD might reflect a higher degree of inflammation and lymphocyte activation.

Pregnancy in healthy women entails two important features: 1) an increase in fractional β-cell area and 2) peripheral immunomodulation. The effects of pregnancy on T1D remain nevertheless equivocal, as there are conflicting results on β-cell function and longitudinal analyses on peripheral immunity are lacking. β-cell function and the plasma proteome in pregnant women with long-standing T1D (L-T1D) were therefore examined during three occasions: 1) first trimester, 2) third trimester, and 3) two months postpartum. An oral glucose tolerance test was performed to measure both fasting and stimulated C-peptide concentrations in plasma. Plasma proteins related to cell regulatory and immunological processes were analysed by proximity extension assay. Glucose-induced insulin secretion was regained in pregnant women with L-T1D, which decreased slowly after parturition. The plasma proteome was dynamic during gestation, although few analytes were functionally linked. A recovered β-cell function might be related to elevated plasma levels of prolactin, prokineticin-1, or glucagon. Moreover, reduced plasma levels of proteins related to leukocyte migration, T cell activation, and antigen-presentation might have further protected an improved β-cell function.

Keywords: Type 1 diabetes, Hashimoto´s thyroiditis, Graves´ disease, autoimmune Addison´s disease, antigen-presenting cells, B cells, non-classical monocytes, NK cells, memory CD8+ T cells, CDCP1, SLAMF1, inflammation, lymphocyte activation, pregnancy, β-cell function, C-peptide, plasma proteome, prolactin, prokineticin-1, glucagon, oral glucose tolerance test, mass cytometry, proximity extension assay

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Till min familj som har stöttat mig i åratal att förstå min egen typ 1 diabetes

"Livet är inte en lång semester, utan en ständig lärotid"

Ur Otrohet av Paulo Coelho

List of Papers

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

I **Magnusson L**, Barcenilla H, Pihl M, Bensing S, Espes D, Carlsson P-O, Casas R. (2020) Mass Cytometry Studies of Patients with Autoimmune Endocrine Diseases Reveal Distinct Disease-Specific Alterations in Immune Cell Subsets. *Front Immunol*, 11:288. doi: 10.3389/fimmu.2020.00288

II **Magnusson L**, Espes D, Casas R, Carlsson P-O. (2020) Increased Plasma Levels of the Co-stimulatory Proteins CDCP1 and SLAMF1 in Patients with Autoimmune Endocrine Diseases. *Front Immunol*, 11:1916. doi: 10.3389/fimmu.2020.01916

III **Magnusson L**, Espes D, Caballero-Corbalán J, Schwarcz E, Casas R, Carlsson P-O. Pregnancy Induces Pancreatic Insulin Secretion in Women with Long-Standing Type 1 Diabetes. *Manuscript*

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Abbreviations

21OH	21-α hydroxylase
AD	Autoimmune Addison´s disease
APC	Antigen-presenting cell(s)
CDCP	CUB domain-containing protein
Cq	Quantitation cycle
CyTOF®	Cytometry by Time-Of-Flight
DC	Dendritic cell(s)
E2	17-β-oestradiol
GAD	Glutamic acid decarboxylase
GCG	Glucagon
GD	Graves´ disease
GLP	Glucagon-like peptide
GMS	Gaussian mean-shift
HbA1c	Glycated haemoglobin A1c
HC	Healthy controls
HLA	Human leukocyte antigen
hPL	Human placental lactogen
HSNE	Hierarchical stochastic neighbour embedding
HT	Hashimoto´s thyroiditis
IA2	Tyrosine phosphatase-like protein islet antigen-2
ICP	Inductively coupled plasma
IFN	Interferon
IL	Interleukin
LAMP	Lysosomal associated membrane protein
L-T1D	Long-standing type 1 diabetes
LY	Lymphocyte antigen
Mo	Monocyte(s)
MS	Mass spectrometry
NPX	Normalised Protein eXpression
N-T1D	New-onset type 1 diabetes
OGTT	Oral glucose tolerance test
PBMC	Peripheral blood mononuclear cell(s)
PCA	Principal component analysis
PEA	Proximity extension assay
PG	Progesterone
PRL	Prolactin

PROK	Prokineticin
SLAMF	Signalling lymphocytic activation molecule
T1D	Type 1 diabetes
T4	Thyroxine
Th	T helper cell
TPO	Thyroid peroxidase
TSH	Thyroid-stimulating hormone

Introduction

Autoimmune endocrine diseases

Type 1 diabetes

Diabetes mellitus is a medical term for diseases that are characterised by hyperglycaemia, which can eventually progress to ketoacidosis. This condition can be caused by impaired insulin secretion or insulin receptor signalling, which prevents cellular uptake of glucose. Type 1 diabetes (T1D) is characterised by the loss of functional insulin-producing β-cells due to an autoimmune process. In Sweden, T1D is diagnosed according to the criteria of American Diabetes Association (1) as fasting plasma glucose of ≥ 7.0 mmol/L at two separate occasions or a 2-hour plasma glucose of ≥ 11.1 mmol/L after an oral glucose tolerance test (OGTT). Glycated haemoglobin A1c (HbA1c) is applied for glucose monitoring after diagnosis. Certain allele combinations within the human leukocyte antigen (HLA) class I and II genes mainly contribute to the predisposition for T1D, of which haplotypes DR3/4 and DQ2/8 confer the highest risk, but polymorphisms in non-HLA genes are also associated with T1D (2). There is nevertheless no causal relationship between HLA gene associations and the presence of autoantibodies (3), and genetic risk factors are not a necessity for the development of T1D (4). Several environmental factors, such as enteroviral infections, have been suggested to influence β-cell autoimmunity and disease progression (5). Autoantibodies targeted against β-cell antigens are detected in patients with new-onset T1D (N-T1D), including glutamic acid decarboxylase (GAD)65, tyrosine phosphatase-like protein islet antigen-2 (IA2), and zinc transporter 8 (6). Seroconversion to one or more of these autoantibodies can predict progression towards T1D in both genetic high-risk individuals and the general population (7, 8).

The pathogenesis of T1D is a heterogeneous process, which involves phenotypical and functional alterations of β-cells and immune cells. The idea that all β-cells are passive victims of cell-mediated apoptosis and necrosis is obsolete, as insulin-positive β-cells can persist several years after diagnosis (9-11). Seiron and colleagues reported that insulin-negative islets from patients with N-T1D and long-standing T1D (L-T1D) contain α-cells that express PDX1 (a β-cell specific transcription factor) (9), inferring that dedifferentiation of β-cells contributes to the pathogenesis. Despite that insulin-positive islets are

affected by diffuse infiltration or aggregates of immune cells (10), the prevalence of insulitis amongst seroconverted and diagnosed individuals is low (9, 10). Immune infiltrates mainly consist of cytotoxic $CD8^+$ T cells, but other $CD3^+$ T cells, $CD20^+$ B cells, $CD68^+$ macrophages, and $CD11c^+$ dendritic cells (DC) are also present (10, 12). Thus, Seiron and colleagues postulate that an immune-mediated decrease in islet density leads to β-cell exhaustion, dedifferentiation, and eventually T1D (9).

T1D has been historically described as a T helper cell (Th)1-biased disease, but multifaceted alterations within several peripheral $CD45^+$ cell lineages have been revealed. A study recently reported reduced frequencies of DC subsets, $CD56^{dim}$ NK cell subsets, and effector memory $CD8^+$ T cells in patients with N-T1D, whilst frequencies of naïve, central memory Th1 and Th2, and activated $CD4^+$ T cells in addition to activated $CD8^+$ T cells were increased (13). However, studies addressing peripheral immunity in T1D can appear inconsistent due to variations in experimental design and methodology. For example, peripheral islet-reactive $CD4^+$ T cells from individuals with T1D had a Th1-biased effector memory phenotype compared with healthy controls (HC) despite that the frequency of islet-reactive cells was similar (14, 15). Elevated serum levels of interferon (IFN)-γ, CCL2, and CXCL10 after seroconversion and before diagnosis emphasise the involvement of Th1- and pro-inflammatory responses (16, 17). Moreover, Th17 cells and Th1/Th17 plasticity have been associated with β-cell autoimmunity prior to and after diagnosis of T1D (18, 19).

Autoimmune thyroid diseases

Hypothyroidism is caused by a reduced production of thyroxine (T4) and conversion into triiodothyronine, which leads to decelerated basal cell metabolism. In contrast, hyperthyroidism defines a state of increased thyroidal T4 production. Both conditions are commonly caused by autoimmunity in iodine-sufficient populations (20), where Hashimoto's thyroiditis (HT) and Graves' disease (GD) are archetypes of hypo- and hyperthyroidism, respectively. Autoimmune thyroid diseases are partly diagnosed based on serum levels of thyroid-stimulating hormone (TSH) and free T4, which have a negative non-linear relationship in addition to age- and sex-dependent reference intervals (21). Women and older individuals are predominantly diagnosed with HT and GD (20), whereas T1D is more common amongst children and adolescents (2). Genetic risk factors for developing autoantibodies and abnormal thyroid function include polymorphisms in HLA- (HLA-DR3 haplotype), immune-related (*BACH2, IL4*), and thyroid-specific (*TPO, TSHR*) genes (22-24). The strongest environmental risk factors for autoimmune thyroid diseases are exposure to radiation and excessive iodine prophylaxis (25, 26), although infections have also been moderately associated with disease progression in predisposed

individuals (27, 28). Like T1D, autoantibodies targeted against thyroidal proteins can be detected before and after diagnosis. Autoantibodies against thyroid peroxidase (TPO) and thyroglobulin can be detected for both diseases, although anti-TPO is more specific for HT (29, 30). Thyroid-stimulating immunoglobulins are specific diagnostic markers for GD, as they bind the TSH receptor and thereby stimulate excessive T4 production (31).

Efforts have been made to characterise HT and GD according to the Th1/Th2 paradigm, but peripheral immune responses are multifaceted and do not necessarily reflect the pathogenesis *in situ*. Autoimmune processes associated with either HT or GD can also exist in parallel, leading to transitions between hypo- and hyperthyroidism (32). Increased serum levels of interleukin (IL)-12, IFN-γ, and CXCL10 indicate that HT is driven by Th1-type cytokines (33, 34), whereas both Th1- and Th2-type cytokines are elevated in GD (34, 35). Studies addressing the phenotype of peripheral $CD4^+$ T cells from patients with autoimmune thyroid diseases have reported contradictory results. One study found an increased frequency of IFN-γ^+ Th1 cells in patients with HT, whereas individuals with GD had a higher frequency of IL-4^+ Th2 cells (36). There are also indications that Th17 cells might be involved in the pathogenesis of autoimmune thyroid diseases (36, 37). However, a more recent study reported no alterations of Th1-, Th2-, and Th17 cell subsets in HT (38). In contrast to T1D, thyroidal biopsies can be excised from living individuals to investigate tissue-resident immune responses. Both thyroidal and immune cells from individuals with HT and GD seem to contribute to a Th1-biased and pro-inflammatory environment, as shown by the expression of CXCR3, CCR2, CXCL10, and CXCL9 in these cell types (35, 39). Individuals with autoimmune thyroid diseases, especially with HT, have well-developed intrathyroidal lymphoid follicles containing thyroid antigen-specific B cells that are committed to antibody production (40). Loss of thyroidal cells in HT has been attributed to three distinct mechanisms, namely antibody-mediated cytotoxicity, $CD4^+$ and $CD8^+$ T cell-mediated cytotoxicity, and death receptor-mediated apoptosis (41). In GD, survival of thyroidal cells is instead promoted by IL-4 mediated up-regulation of anti-apoptotic genes.

Autoimmune Addison's disease

Autoimmune Addison's disease (AD) is caused by immune responses against proteins in zona glomerulosa and zona fasciculate within the adrenal cortex. Destruction of these layers leads to deficiencies of aldosterone and cortisol, which are important hormones for salt balance and metabolic stress responses. AD is clinically manifested when > 90 % of the cortical cells are destroyed or non-functional (42, 43). The risk of developing AD is increased with the presence of the DR3-DQ2 and DR4-DQ8 haplotypes (44, 45), which are the same genetic risk factors as for T1D. Like the abovementioned autoimmune endocrine diseases, autoantibodies against adrenal proteins are detected at pre- and

clinical stages of AD. The most specific autoantibody for AD is targeted against the steroidogenic cytochrome P450 enzyme 21-α hydroxylase (21OH), but patients can also develop autoantibodies against 17-hydroxylase and Side Chain Cleavage enzyme (44, 46, 47).

Studies investigating immune responses in patients with AD are scarce, as this is a rare condition and often accompanied by other autoimmune endocrine diseases (48). Autoantibodies and chemokines in serum have been extensively studied, whereas few studies have focused on the phenotype and function of immune cells. For example, 21OH-antibodies are mainly of the IgG1 isotype that is characteristic for Th1-biased immune responses (46). The hypothesis that AD is a Th1-driven disease is further supported by elevated serum levels of CXCL9 (49, 50), CXCL10 (49-53), and CXCL11 (52) compared with HC. Most patients with AD also have $CD4^+$ and $CD8^+$ T cells that secrete IFN-γ upon stimulation with 21OH (49, 54, 55). Antigen-presenting cells (APC) and binding of anti-21OH antibodies appear to influence T cell responses during AD (54).

Autoimmune tautology

Despite that autoantigens and symptoms vary between diseases, the deduction from abovementioned studies is that T1D, HT, GD, and AD share immunological features. This idea is not novel, as "mosaic of autoimmunity" and "autoimmune tautology" are established concepts (56, 57). These concepts postulate that several factors are common between autoimmune diseases, although their numbers, variety, and temporal occurrence contribute to the diversity and heterogeneity of diseases. The described factors are categorised as 1) genetic and epigenetic, 2) hormonal, 3) environmental, and 4) uniform immunopathology. Genetic risk factors can be shared between several diseases, such as allele combinations within HLA class II genes, or specific for a certain disease (57). It has also been hypothesised that a skewed X-chromosome inactivation predisposes women for autoimmune diseases, as irregular expression of self-antigens on the X-chromosome could lead to inadequate induction of central or peripheral tolerance (58, 59). The second category of factors comprises endogenous hormones that can modulate the activation and function of immune cells. For example, the high proportion of women amongst patients with autoimmune diseases is explained by the stimulatory effects of prolactin (PRL) and 17-β-oestradiol (E2) on B and T cells (60, 61). Environmental risk factors include any external agent that can modify the structure of self-antigens, exposure of self-antigens to the immune system, or immune cell functions. In summary, autoimmune tautology postulates that genetic and epigenetic factors predispose individuals to fail central and peripheral tolerance mechanisms (56, 57). Autoimmune responses can later be triggered by inauspicious hormonal background and environmental factors, leading to overt disease.

Individuals previously diagnosed with T1D, HT, GD, or AD have a predisposition to develop other autoimmune endocrine diseases, namely poly-autoimmunity or Autoimmune Polyendocrine Syndrome. In contrast to a prevalence of 1-7 % of HT in the general population (20, 62), the frequency of thyroid autoimmunity is up to 25 % in patients with T1D (63, 64). Development of adrenal autoimmunity is slightly more common amongst patients with T1D than HC (65), whereas 50-80 % of patients with AD have other autoimmune endocrine diseases (44, 66). A study reported that the prevalence of poly-autoimmunity in patients with HT and GD was 15 % and 10 %, respectively, with significant relative risks for additional T1D and AD (67). An issue with all abovementioned studies is that no or few simultaneous comparisons between diseases have been made. Secondly, immune deviations associated with T1D, HT, GD, and AD have been examined for a limited number of immune cell lineages and analytes due to methodological limitations. Modern high-dimensional and high-throughput technologies should therefore be employed more often to simultaneously assess immunological parameters between patients with autoimmune endocrine diseases.

Pregnancy in healthy women

Metabolic and pancreatic adaptions

Pregnancy is an event that entails several physiological adaptions, which are essential to prevent rejection of the conceptus and ensure successful foetal development. For example, the fat and glucose homeostasis in healthy women is continuously adjusted during gestation. Catalano and colleagues demonstrated that there is a gradual increase in insulin secretory response throughout gestation, whilst there is a decline in peripheral insulin sensitivity only during the last trimester (68, 69). The first half of pregnancy is consequently associated with maternal anabolism, whereas nutrients are later prioritised for foetal growth. Augmented insulin secretion appears to be independent of insulin sensitivity (69), indicating that the pancreas undergoes intrinsic adaptions during pregnancy. In 1978, van Assche and colleagues discovered that the fraction of endocrine tissue within the pancreas and the percentage of β-cells within islets increase in pregnant women (70). A more recent study revealed that a greater fractional β-cell area is attributed to an increased density of small islets and dispersed β-cells amongst exocrine tissue (71).

Placental and peripheral immunomodulation

Another striking feature of pregnancy is the extensive immunomodulation that aims to protect the semi-allogenic foetus from rejection. Most changes occur

within the placenta, a chimera of maternal decidual stroma and foetal trophoblasts, as women must maintain the ability to eliminate infections (72). Maternal immune cells are recruited to the placental compartment, where they differentiate into antigen-specific immunomodulatory effector cells during placentation and gestation. Stroma cells, APC, decidual NK cells, and suppressive regulatory T lymphocytes then employ various mechanisms to create an immune-privileged site for the foetus (72). The importance of these tolerance mechanisms is emphasised by the fact that women affected by spontaneous abortion have a placental expansion of activated effector memory $CD4^+$ and $CD8^+$ T cells (73). As it is hypothesised that the placental milieu is mirrored by alterations in blood, peripheral immune cell subsets and protein profiles have been examined. For example, it has been reported that a placental and systemic Th2-biased cytokine profile is connected to a successful pregnancy (74, 75). High-dimensional technologies and unbiased multivariate statistical analyses have recently improved our understanding of complex immune networks during pregnancy and after parturition. In healthy women, peripheral levels of cytokines (especially pro-inflammatory), chemokines, and growth hormones generally decrease through gestation until the postpartum period (76, 77). Serum protein profiles appear nevertheless to be distinct between gestational ages, and there is a significant inter-regulation between analytes (77, 78). Apps and colleagues also demonstrated that 32 subsets of peripheral $CD45^+$ immune cells are either affected by transient or persistent gestation-associated perturbations (78).

Hormonal regulation of pregnancy

It is still debated whether improved function, hyperplasia, reduced apoptosis, or β-cell neogenesis leads to augmented insulin secretion, but it is evident that metabolic alterations coincide with elevated serum levels of PRL, human placental lactogen (hPL), E2, and progesterone (PG) (79). Indeed, several studies have shown that these hormones directly affect β-cell function and expansion. A comprehensive *in vitro* study from 1993 showed that stimulation of human islets with either PRL or hPL increases the cumulative insulin secretion, although hPL had a greater potency than PRL (80). Besides enhanced secretory function, hPL can induce proliferation of β-cells from female donors (80) and protect primary human islets from induced apoptosis (81). In pregnant mice lacking *Prlr* specifically in the pancreas, glucose-dependent insulin secretion and glucose tolerance were impaired compared with pregnant wild type-mice (82). These *Prlr*-deficient mice failed to expand the β-cell mass due to a reduced expression of genes linked to β-cell differentiation (*Pdx1*) and function (*Ins2*). In healthy women, lower serum levels of PRL during early pregnancy were an independent predictor of reduced β-cell function, whole-body insulin sensitivity, and glucose tolerance throughout the gestation period and after

parturition (83). E2 can protect β-cells against inflammation-induced apoptosis and enhance glucose-induced insulin secretion (79).

Immunological adaptions during pregnancy and at parturition also coincide with augmented production or functional withdrawal of steroid hormones. There is ample evidence from *in vitro* and *in vivo* studies that E2 and PG affect placental and peripheral immunity [reviewed in (84, 85)]. For instance, E2 can induce differentiation of peripheral regulatory T and B lymphocytes in addition to tolerogenic DC (85). PG has a potent effect on T cell differentiation, as it can specifically promote the secretion of IL-4 and -5 from human antigen-specific Th0-, Th1-, and Th2 clones (86). Medroxyprogesterone acetate, a synthetic PG analogue that is used clinically to prevent preterm labour, can inhibit Th1- and Th17 responses whilst maintaining IL-4 secretion from Th2 cells and enhancing Th22 responses (87).

Pregnancy and type 1 diabetes

Obstetric and neonatal complications

The effects of pregnancy on T1D remain equivocal, as metabolic and hormonal alterations can affect the disease management. As reduced or even suboptimal glycaemic control is associated with obstetric complications and neonatal morbidity (88-90), it is essential that individuals with T1D continue with a strict insulin regimen prior to conception and during the gestation period. However, *ex vivo* studies have demonstrated that oxidative stress and inflammatory responses are activated in placental tissue from women with T1D irrespective of glycaemic control (91, 92). Consequently, the aim to approximate a HbA1c of < 6.5 % (48 mmol/mol) might not reduce the incidence of complications, and a strict regimen can also trigger severe hypoglycaemic events. Indeed, up to 45 % of women with T1D experience a higher rate of severe hypoglycaemia during pregnancy than before conception (93, 94).

Metabolic and pancreatic adaptions

Interestingly, most hypoglycaemic events occur during the first trimester despite that the daily insulin requirement is generally lower than before conception and during subsequent trimesters (94, 95). Severe hypoglycaemia associated with early gestation is therefore not caused by excessive insulin dosages. In 2000 and 2009, two independent studies reported that fasting or random C-peptide levels continuously increased in women with L-T1D during the gestation period (96, 97). These findings imply that a gradual recovery of β-cell function can be attained despite low C-peptide detectability prior to pregnancy (97). These studies have been recently questioned by a more controlled study that employed a mixed meal tolerance test, which could not demonstrate any

gestational alteration in plasma C-peptide levels (98). As the abovementioned studies neither included control cohorts nor used ultrasensitive C-peptide assays, it must be clarified whether individuals with T1D can attain a β-cell recovery during pregnancy. Moreover, women with L-T1D and good glycaemic control have a surge of gestation-related hormones equivalent to HC (99-101), which suggests that PRL, hPL, E2, and PG might enhance insulin secretion from dormant β-cells or induce *de novo* β-cell neogenesis (9, 102). There is nevertheless a lack of studies investigating the correlation between improved β-cell function and placental hormones in individuals with L-T1D.

Placental and peripheral immunomodulation

There are few comparative immunological studies between healthy women and patients with L-T1D during pregnancy. Such studies have often focused on a small number of immune cell populations or analytes, and not in a longitudinal approach. For instance, one study reported that women with L-T1D had lower peripheral levels of CCL5 during the first trimester than healthy women (103). Another study addressing pre-eclampsia in women with T1D found that CCL2 levels during the last trimester and the longitudinal increase of CCL5 were greater in the normotensive T1D-group than in HC (104). Individuals with L-T1D who suffered from obstetric complications had a more pro-inflammatory serum profile than patients with uncomplicated pregnancies.

Aims

The overall aim for this doctoral thesis was to compare the peripheral immune system in individuals with different autoimmune endocrine diseases, and to investigate how physiological adaptions during pregnancy affect T1D.

The specific objectives for this thesis were:

- To characterise the phenotype of peripheral blood mononuclear cells (PBMC) to explore similarities and divergences between T1D, HT, GD, and AD (**paper I**)
- To evaluate whether immunological profiles in plasma and PBMC supernatants vary between individuals with different autoimmune endocrine diseases (**paper II**)
- To examine longitudinal effects of pregnancy on β-cell function and the plasma proteome, with focus on proteins related to cell regulatory and immunological processes, in women with L-T1D (**paper III**)

”Det värsta vi kan göra är att fly. Det är värre än att förlora striden, för nederlaget kan alltid lära oss något, men det enda vi uppnår med att fly är att utropa vår fiende till segrare”

Ur Pilgrimsresan av Paulo Coelho

Method and Materials

Study design and cohorts

Two separate studies were conducted in order to achieve the abovementioned aim for this thesis. A cross-sectional design was applied to characterise the peripheral immunity in T1D, HT, GD, and AD, whereas effects of pregnancy on T1D were evaluated in a prospective cohort study.

Paper I and II

Individuals with four different autoimmune endocrine diseases in addition to HC were recruited at Uppsala University Hospital between January 2017 and June 2018. All participants were 18-50 years old and had no signs of infections, malignancies, or other serious diseases at the time of inclusion. Patients had only one known autoimmune endocrine disease and did not present derangements in hormone production and autoantibodies for any of the other studied diseases. HC had no first-degree relatives with autoimmune endocrine diseases, no derangements in hormonal function, and were negative for the tested disease-specific autoantibodies. All patients were actively treated for their respective diseases: intensive insulin therapy for T1D, levothyroxine for HT, block-replace therapy (thiamazole with addition of levothyroxine after four weeks) for GD, and hydrocortisone for AD. Patients with GD had not been previously treated with radioiodine or ablative surgery. Medicine doses might have varied between individuals within each disease group, as treatments are individually adjusted to normalise derangements in glucose control, thyroid status, or cortisol levels and electrolytes. Only individuals with optimised hormonal and metabolic control at the time of sampling were included in the study.

Although **paper I** and **II** examined the same cohort, the number of patients with GD and HC differed due to sample loss during acquisition (**Figure 1**). Individuals with T1D were divided into N-T1D and L-T1D groups based on elapsed time since diagnosis, as it was deemed important to investigate whether the peripheral immunity alters as a consequence of the natural history of T1D (105). Naturally, clinical manifestation, progression, tissue degeneration, and diagnosis differ between autoimmune endocrine diseases. HT is commonly diagnosed later than the onset of symptoms and immune responses, whereas GD is usually transient over a period of two years. It was therefore

unfeasible to recruit individuals with autoimmune thyroid diseases during the same phase as described for T1D. Moreover, isolated AD is not highly prevalent in Sweden and younger individuals with AD tend to develop poly-autoimmunity more frequently than older patients (57, 66, 106). Due to these circumstances, individuals with N-T1D were younger than the AD group (**paper I**) or the L-T1D and AD groups (**paper II**). The cross-sectional study was approved by the Regional Research Ethical Committee in Uppsala (Dnr 2014/485) and was consistent with The Declaration of Helsinki. All participants gave their written informed consent prior to inclusion in the study.

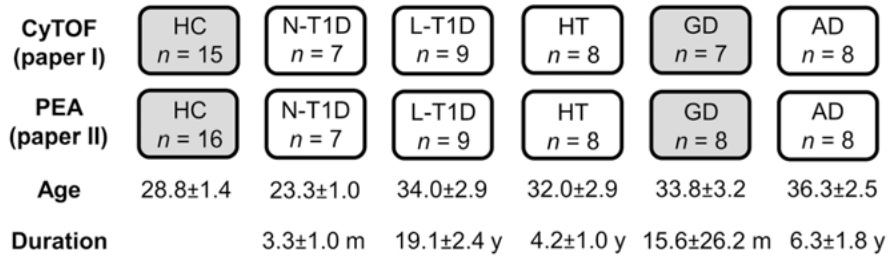

Figure 1. Study of peripheral immunity in individuals with autoimmune endocrine diseases. Patients with type 1 diabetes (T1D), Hashimoto´s thyroiditis (HT), Graves´ disease (GD), and autoimmune Addison´s disease (AD) in addition to healthy controls (HC) were recruited to a cross-sectional study. Participants with T1D were further divided based on the time since diagnosis: new-onset (N-T1D) and long-standing (L-T1D). Cytometry by Time-Of-Flight (CyTOF) and proximity extension assay (PEA) were employed to analyse $CD45^+$ immune cells and immune-related analytes, respectively. Sample sizes of each group are described, and grey boxes indicate divergent sample sizes. Mean age (±SEM) of the participants and time of sampling since diagnosis (duration; mean ± SEM) are described. m; months, y; years.

Paper III

Pregnant women with L-T1D (n = 15) were recruited at Uppsala and Örebro University Hospitals, Sweden. Pregnant patients were eligible for the study if they had been diagnosed with T1D for > 10 years at conception and were < 12 weeks of gestation. Two women had a consecutive pregnancy shortly after the first delivery and were followed once again according to the study protocol. Therefore, they were considered as two new biological samples (total sample size, n = 17). Except for visits in the current study, the women had scheduled visits every second week with a diabetes specialist at the maternity wards. These appointments were performed according to clinical routines, which comprised HbA1c monitoring and treatment adjustments to achieve optimal glycaemic control. The pregnant women performed research visits at Uppsala or Örebro University Hospitals on three occasions (**Figure 2**). As previous studies assessing β-cell function in healthy individuals and women with T1D have used similar timepoints during first and third trimesters (69,

96, 97), visits as shown in **Figure 2** were chosen for reproducibility purposes. However, comparisons with the pre-pregnancy period could not be made in this study due to infrequent clinical appointments and inconsistent sampling routines. Samples were instead obtained approximately two months after delivery, as sufficient time would have elapsed for pregnancy-related adaptions to wane and estimate trends in β-cell function.

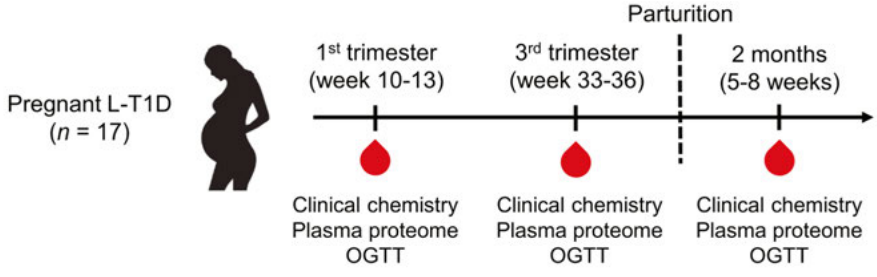

Figure 2. Visits for pregnancy study. Women with long-standing type 1 diabetes (L-T1D) were recruited if gestation had not passed 12 weeks. After overnight fasting, peripheral venous blood was collected for clinical chemistry and plasma proteome analyses. An oral glucose tolerance test (OGTT) was then performed to assess glucose-induced β-cell function. These analyses were made during three visits [mean week]: 1) first trimester [11.6], 2) third trimester [34.4], and 3) two months postpartum [6.2]. Time intervals for each occasion are described within parentheses.

Plasma and serum samples from non-pregnant women with L-T1D (n = 30) were used as a control cohort to compare β-cell function and the plasma proteome. This cohort was selected from a previous cross-sectional study in which individuals were characterised as C-peptide negative or positive (107). Non-pregnant women were in average 3.2 years younger than the pregnant group (mean [intervals], non-pregnant: 25.9 [23.9-27.9], pregnant: 29.1 [27.4-30.7], $p < 0.05$). This study was approved by the Regional Research Ethical Committee in Uppsala (Dnr 2013/273) and was consistent with The Declaration of Helsinki. All participants gave their written informed consent prior to inclusion in the study.

Blood sampling, preparation of PBMC, and cell culture

According to recommendations issued by European Federation of Clinical Chemistry and Laboratory Medicine (108), peripheral venous blood was collected in the morning (8-10 AM) after overnight fasting since 10 PM. These pre-analytical conditions are necessary to avoid biological variability in chemistry and immunological analyses (108-110). Blood samples for **paper I** and **II** were collected in sodium-heparin tubes, which were transported at room-temperature and processed within 27 h after sample collection. Plasma was

separated from heparinised blood by ultracentrifugation and stored at -70°C prior to analysis (**paper II**). For **paper III**, peripheral venous blood collected in EDTA-, citrate-, lithium heparin-, and serum gel tubes was immediately analysed for HbA1c, glucose, triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, creatinine, anti-GAD, anti-IA2, and PRL. Blood in additional EDTA-tubes were centrifuged to obtain plasma, which was stored at -70°C prior to protein assays and C-peptide measurements. All samples from both pregnant and non-pregnant women were stored at -70°C for < 12 months prior to C-peptide measurements.

PBMC were isolated from heparinised blood by performing density-gradient centrifugation with Ficoll-Paque™ PLUS (density 1.077 ± 0.001 g/mL; GE Healthcare; UK). PBMC were then used for 1) phenotypic characterisation by mass cytometry and 2) secretion assay, where the former was prioritised if cell counts were not sufficient for both experiments. For **paper I**, cells were incubated overnight prior to mass cytometry staining. For **paper II**, PBMC were cultured with and without Dynabeads® Human T-Activator CD3/CD28 (Gibco; US) immediately after isolation. Dynabeads® is commonly used for T cell activation and expansion, as it gives primary and co-stimulatory signals without the need of APC or antigen. Although T cells are specifically stimulated by Dynabeads®, final analyte concentrations in supernatants from PBMC cultures might result from bystander activation of other $CD45^+$ cells. Isolated cell populations could have been used to investigate lineage-specific secretomes, but this procedure was not applicable due to 1) the limited blood volume and 2) high cell demand for mass cytometry. A bead/cell-ratio of 1:20 was chosen to avoid over-stimulation, high proliferation and activity, and cell death. After seven days of culture, cell supernatants were retrieved and stored at -70°C prior to analysis.

Mass cytometry (paper I)

Flow cytometry is a common method to examine expression of extra- and intracellular proteins at a single-cell level with high specificity. However, emission bands of fluorophores can easily overlap, so called spectral spill-over (**Figure 3**), which can cause misinterpretations of marker expression. As the demand to simultaneously analyse a growing number of markers in heterogenous cell populations has increased, spectral spill-over prevents further multiplexing of flow cytometry panels (111). Mass cytometry or Cytometry by Time-Of-Flight (CyTOF®) combines flow cytometry and mass spectrometry (MS) to overcome the abovementioned issue. Instead of fluorescent dyes, cells are stained with monoclonal antibodies conjugated to lanthanide (rare earth metal) isotopes. Atomic masses of these isotopes have a narrow range enabling high resolution, almost absent interference, and no background (111-

113). CyTOF® also avoids background signals from biological material, corresponding to autofluorescence, as lanthanides are not natural constituents in cellular proteins. There are nevertheless few disadvantages with mass cytometry. Despite that isotopes are specifically enriched and inspected prior to antibody conjugation, impurities (normally ≤ 1 %) and oxidation might shift atomic mass units (112, 113). Due to the low cell recovery, CyTOF® might not be an optimal method for phenotyping if sample volume is a limiting factor.

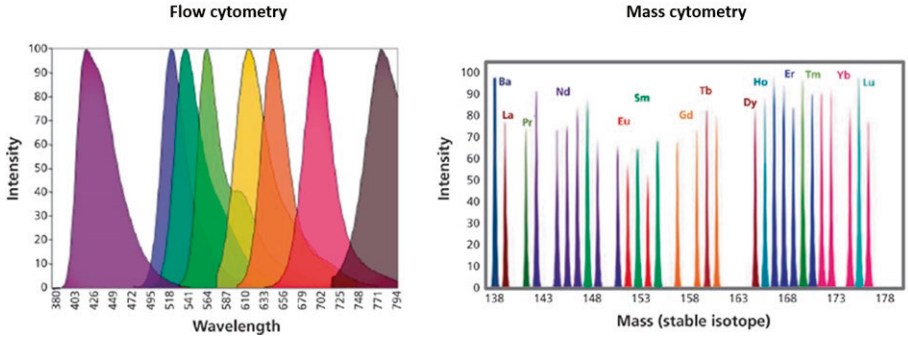

Figure 3. Comparison of flow and mass cytometry. Fluorophores have emission spectra that can extend over many wavelengths. This creates overlaps between different fluorescent dyes, which means that signals can be measured in the wrong detector and cause misinterpretations of marker expression. Mass cytometry has a high ion resolution, as lanthanide isotopes have different atomic mass units. Isotopes are detected in separate channels, leading to improved sensitivity and accuracy. Figure modified with permission from Virani F, Tanner S. Mass Cytometry: An Evolution in ICP-MS Enabling Novel Insights in Single-Cell Biology. *Spectroscopy*. 2015;30(5).

This technology employs an inductively coupled plasma (ICP)-MS instrument, which comprises four main parts (**Figure 4**). Firstly, stained cells in suspension are injected into a heated pneumatic nebuliser, where addition of argon gas creates a turbulent flow, dries cells, and creates aerosols (111, 112). Single cells are subsequently atomised and ionised in the ICP, producing ion clouds that represent the original cell structure. Ion clouds contain both reporter-specific ions, low mass ions from biological components, and non-charged particles. The latter components are selectively removed by using a quadrupole before ionised metal isotopes can flow into the TOF chamber. As reporter ions have unique m/z ratios, the acceleration towards the time-resolved detector differs between ions. Consequently, a mass spectrum for each cell is acquired that corresponds to reporter-specific identities and quantities.

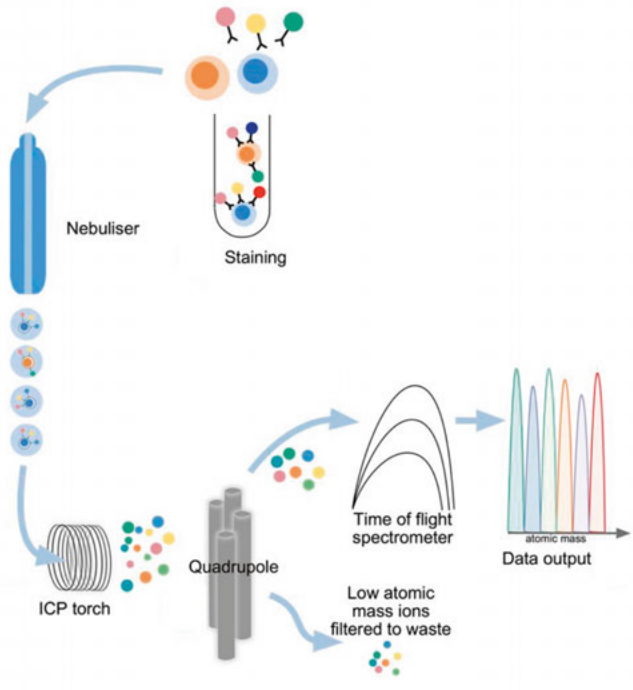

Figure 4. Workflow and instrumentation during mass cytometry. Figure modified from Stern L, McGuire H, Avdic S, Rizzetto S, Fazekas de St Groth B, Luciani F, Slobedman B, Blyth E. *Front Immunol*. 2018;9:1672 and used in accordance with the Creative Commons Attribution License.

Panel design and CyTOF® protocol

A panel with 32 metal-conjugated antibodies was designed to phenotypically characterise all lineages within peripheral $CD45^+$ cells (**Table I**). The panel was divided between two cocktails for staining of extra- and intracellular markers, respectively. Lineage markers were used to identify major cell populations, whereas remaining markers were included to examine activation and function (for example CD25 and CD69), differentiation (such as CD27 and CD45RA), cytokines (IL-2, IL-4, IL-10, IL-17A, and IFN-γ), chemokine receptors (for example CXCR3), and transcription factors (Tbet, GATA3, and FOXP3).

Table I. In-house mass cytometry panel. A panel was designed to phenotypically characterise peripheral blood mononuclear cells by including markers for lineage, activation and function, differentiation, cytokines, chemokine receptors, and transcription factors. Thirty-two lanthanide-conjugated antibodies were divided between extra- (Extra) and intracellular (Intra) staining cocktails. Clones of the monoclonal antibodies are described. Monoclonal antibodies from Biolegend were conjugated with corresponding isotope at the Flow Cytometry Unit, Linköping University (*).

Lanthanide	Marker	Clone	Supplier	Step
102Pd	Barcode 1		Fluidigm	Barcoding
104Pd	Barcode 2		Fluidigm	Barcoding
105Pd	Barcode 3		Fluidigm	Barcoding
106Pd	Barcode 4		Fluidigm	Barcoding
108Pd	Barcode 5		Fluidigm	Barcoding
110Pd	Barcode 6		Fluidigm	Barcoding
89Y	CD45	HI30	Fluidigm	Extra
141 Pr	CCR6	GO34E3	Fluidigm	Extra
142 Nd	CD19	HIB19	Fluidigm	Extra
144 Nd	IL-4	MP4-25D2	Fluidigm	Intra
145 Nd	CD4	RPA-T4	Fluidigm	Pre-stain
147 Sm	CD11c	Bu15	Fluidigm	Extra
148 Nd	CD16	3G8	Fluidigm	Extra
149 Sm	CD25	2A3	Fluidigm	Extra
150 Nd	CD8a	RPA-T8	Biolegend*	Extra
151 Eu	CD123	6H6	Fluidigm	Extra
152 Sm	CD56	HCD56	Biolegend*	Extra
153 Eu	CCR4	205410	Fluidigm	Extra
155 Gd	CD27	L128	Fluidigm	Extra
156 Gd	CD86	IT2.2	Fluidigm	Extra
158 Gd	IL-2	MQ1-17H12	Fluidigm	Intra
159 Tb	CCR7	GO43H7	Fluidigm	Extra
160 Gd	Tbet	4B10	Fluidigm	Intra
161 Dy	CD69	FN50	Biolegend*	Extra
162 Dy	FOXP3	PCH101	Fluidigm	Intra
163 Dy	CXCR3	G025H7	Fluidigm	Extra
164 Dy	IL-17A	N49-653	Fluidigm	Intra
165 Ho	CD45RO	UCHL1	Fluidigm	Extra
166 Er	IL-10	JES3-9D7	Fluidigm	Intra
167 Er	GATA3	TWAJ	Fluidigm	Intra
168 Er	IFNg	B27	Fluidigm	Intra
169 Tm	CD45RA	HI100	Fluidigm	Extra

170 Er	CD3	UCHT1	Fluidigm	Extra
171 Yb	CD20	2H7	Fluidigm	Extra
172 Yb	CD38	HIT2	Fluidigm	Extra
174 Yb	HLA-DR	L243	Fluidigm	Extra
175 Lu	CD14	M5E2	Fluidigm	Extra
176 Yb	CD127	A019D5	Fluidigm	Extra
191/193Ir	DNA		Fluidigm	Single cells
195Pt	Protein		Fluidigm	Live/dead

Ten million PBMC were first pre-incubated with anti-CD4-^{144}Nd to improve the anti-CD4 staining upon stimulation. In order to analyse intracellular cytokines, 5 x 10^6 cells were incubated with phorbol 12-myristate 13-acetate, ionomycin, and protein transport inhibitors (monensin and brefeldin) for 4 h. There are different methods to ensure that dead cells are excluded from analysis (112, 113). In this protocol, PBMC were pulsed with cisplatin that covalently binds proteins in permeable, dead cells. PBMC were then stained for surface markers, fixed with FOXP3 Fixation/Permeabilization buffer (Affymetrix eBioscience; US), and lastly stained for intracellular markers. After an overnight fixation in 2 % paraformaldehyde, each sample was barcoded with a specific combination of palladium isotopes. Barcoded samples can thus be pooled to reduce variability during data acquisition. Cells were further incubated with Ir191/193, a universal DNA intercalator, to exclude doublets from data analysis (111-113). After a wash in deionized water and prior to injection into the ICP-MS instrument, PBMC were resuspended with 0.1 % EQ four element calibration beads. To adjust for potential sensitivity changes in the instrument, these beads were used for data normalisation by using mass bead signals.

Proximity extension assay (paper II and III)

There are several technologies to analyse proteins in various sample matrices, which have distinct advantages, disadvantages, and applications. ELISA and Luminex® are common single- and multiplex immunoassays, respectively, which are based on binding of detection antibodies to proteins followed by absolute quantification through fluorescence or chemiluminescence. Proximity extension assay (PEA) is a novel technology that can measure up to 1,536 analytes in parallel, which has been developed and optimised by Assarsson, Lundberg, and colleagues (114, 115). Three different 92-plex panels (Olink Proteomics AB; Sweden) were used to measure well-known and exploratory

analytes associated with immunological responses and cell regulatory events (**Figure 5**).

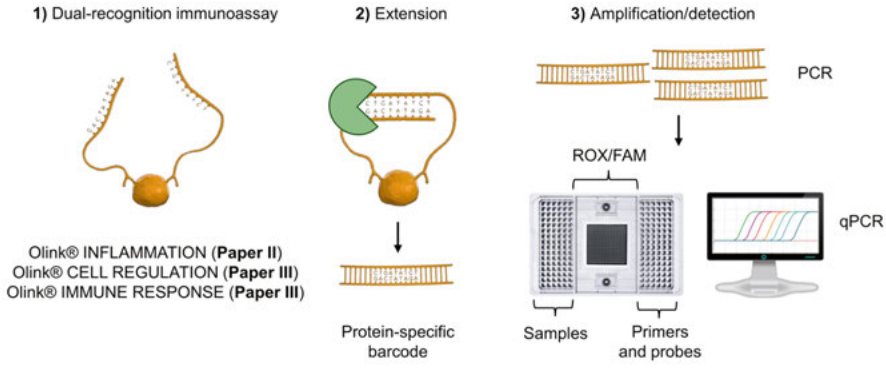

Figure 5. Schematic overview of proximity extension assay. This technology comprises three steps: 1) dual-recognition immunoassay, 2) extension phase, and 3) amplification and detection. Proteins (N = 92) in a sample are bound by pairs of monoclonal detection antibodies, which recognise two adjacent yet distinct epitopes on targeted analytes. The detection antibodies are conjugated to two complementary oligonucleotides, which associate with each other by proximity. A DNA polymerase (green) can subsequently create analyte-specific dsDNA barcodes during the extension step. This technology has a high sensitivity, as barcodes are multiplied by PCR (creating amplicons of the original barcode). Samples are then transferred to a 96.96 interchanged fluid circuit along with primers and probes for each analyte-specific amplicon. Each sample reacts with each probe during the quantitative real-time PCR (qPCR), where 5-carboxy-X-rhodamine (ROX) and fluorescein (FAM) are used as passive and reporter dyes, respectively. Figure adapted from ©Olink Proteomics AB and used under permission.

Frozen plasma and supernatants from PBMC cultures were undiluted during measurements, as determined by dilution experiments and due to the dynamic range of assays. In the first step of PEA, matched oligonucleotide-labelled detection antibodies bind to protein targets in solution (dual-recognition step). The complementary DNA oligonucleotides can only hybridise if they are juxta-positioned, which occurs when both monoclonal detection antibodies have bound to their target. Hybridised oligonucleotides subsequently serve as templates for DNA polymerase to synthesise dsDNA barcodes (extension step). In the last step, dsDNA barcodes are amplified and then quantified by high-throughput microfluidic qPCR using the BioMark™ HD System (Fluidigm; US). Primers, probes, and samples are transferred to a 96.96 Dynamic Array™ Integrated Fluidic Circuit (Fluidigm; US), where detection is based on the 5'-nuclease activity principle (**Figure 6**). In contrast to conventional multiplex immunoassays (116), cross-reactivity contributing to signal readout is efficiently avoided in PEA due to the dual-recognition immunoassay and

the protein-to-DNA conversion. The signal amplification by PCR also improves assay sensitivity, reduces matrix interferences, and decreases required sample volume. The latter is especially important for clinical samples, as the volume might be a limiting factor (**paper II** and **III**). As PEA is a homogenous assay (114), meaning that there are no intermittent washes between procedures (**Figure 5**), targeted proteins remain during the entire assay.

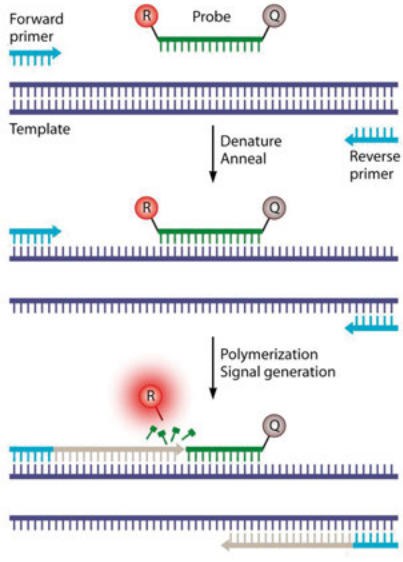

Figure 6. Principle for TaqMan® gene expression assay. A reaction mixture containing forward and reverse primers in addition to a probe is used in quantitative PCR to detect the presence of cDNA templates. cDNA templates are equivalent to amplicons in the proximity extension assay. A TaqMan® probe comprises a reporter dye (R), such as fluorescein, at the 5'-end and a non-fluorescent quencher (Q) at the 3'-end. The reporter dye remains non-fluorescent if the quencher is attached to the probe. After denaturation of templates, the PCR temperature is reduced that enables primers and probes to anneal onto complementary DNA sequences. A Taq DNA polymerase then cleaves probes during the polymerisation step, which results in increased fluorescence signal as the reporter is separated from the quencher. The figure is used in accordance with terms of the Creative Commons Attribution License.

PEA is a discovery-oriented technology, as both well-known and exploratory proteins can be investigated. Olink® panels are designed based on computational predictions, knowledge transfer between organisms, and aggregated data from previous experiments, which entails some issues. Firstly, several analytes that were detected in plasma are secreted or expressed within several tissues and cell types. Secondly, all analytes have different expression patterns that span from ubiquitous to stimuli-induced. These features make it complicated to draw conclusions about origin and functional consequences of divergent plasma proteins. It is also important to query ascribed protein annotations in the panels, as a significant amount of bioinformatic data is based on gene

analyses. Therefore, it must be considered whether PEA is appropriate or not for a specific experimental design. It could be sufficient to employ PEA on a limited number of samples, for "biomarker discovery", and continue with more targeted assays such as Luminex® and electrochemiluminescence (117). The low detectability for certain proteins of interest in **paper I**, such as IFN-γ, further evokes reassessments regarding targeted assays. Another proteomic approach would have been a combination of gel electrophoresis and MS. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis is an efficient technology to separate protein isoforms in various sample matrices, which provides a semi-quantitative measurement of visually varying proteins (118). Identities of diverging protein isoforms are subsequently confirmed by MS, which is a very sensitive technology and gives information about protein structure (119). However, this proteomic approach is laborious, involves extensive sample preparation, requires more sample volume than PEA, and is sensitive to high-abundant plasma proteins (118-120).

Oral glucose tolerance test and C-peptide measurements (paper III)

In healthy individuals, insulin secretion is initiated when glucose is passively transported into β-cells (**Figure 7**). Insulin secretion during and after food ingestion occurs in two phases: a rapid, sharp initial response followed by a slow yet sustained secretion into the blood (121). The first phase comprises fusion and exocytosis of pre-existing insulin vesicles that are adjacent to the cell membrane. The second phase represents an enhanced pre-proinsulin production, post-translational modifications, and vesicle translocation.

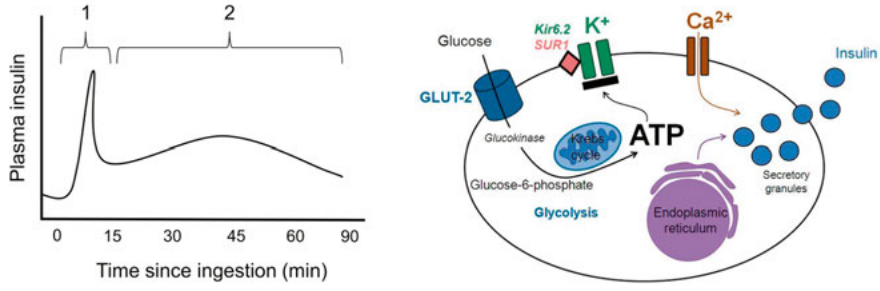

Figure 7. Mode of insulin secretion. Insulin is secreted by pancreatic β-cells into the bloodstream in a biphasic manner. The first phase comprises a rapid increase in plasma insulin concentration, whereas the second phase involves a sustained insulin production and secretion. These phases represent how β-cells respond to glucose during and after a meal. Glucose is passively transported over the plasma membrane via glucose transporter 2 (GLUT-2), leading to increased adenosine triphosphate (ATP)-production through oxidative phosphorylation in mitochondria. Due to an increased intracellular ATP/ADP-ratio, potassium channels (Kir6.2/SUR1) close and induce a membrane depolarisation. This depolarisation causes β-cell specific calcium channels to open, which leads to an influx of Ca^{2+} that is required for vesicle translocation and fusion. Right image from Henzen C. *Swiss Med Wkly*. 2012;142:w13690 and used in accordance with terms of the Creative Commons Attribution License.

β-cells convert pre-proinsulin into mature 51 aa-long insulin and C-peptide molecules at a 1:1 ratio within the endoplasmatic reticulum and Golgi apparatus (**Figure 8**) (122).

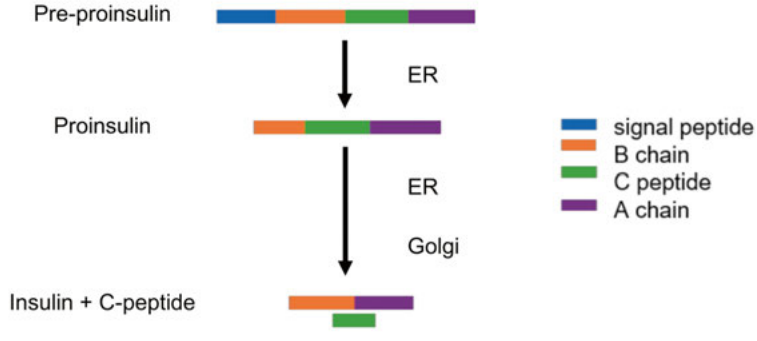

Figure 8. Pre-proinsulin conversion. The insulin gene is translated within the endoplasmatic reticulum (ER) into pre-proinsulin that contains four domains. The signal peptide is cleaved from pre-proinsulin, and proinsulin is further processed within the ER and Golgi apparatus. Post-translational modifications include folding, disulphide bonds between the A and B chains, and removal of the C-peptide by type I/II endoproteases. Secretory vesicles subsequently bud off from the Golgi apparatus, which contain mature insulin and functional C-peptide.

There are several methods to assess β-cell function, which provide different kinds of information and necessitate distinct resources (121). The OGTT is a reliable and less onerous method that is clinically applied for diagnosis of prediabetes, gestational diabetes, insulin resistance, and reactive hypoglycaemia. OGTT also accounts for potentiating factors secreted within the intestine in response to ingested glucose, which is more physiologically relevant than intravenous tolerance tests. After overnight fasting, OGTT comprises ingestion of a 75 g-glucose solution preferably within 10 min. Collection of peripheral venous blood then occurs at different timepoints, where sampling at baseline (before ingestion) and after two hours is performed in a clinical setting. Nevertheless, analysis of fasting or random plasma glucose is the prevalent diagnostic method despite that it does not reveal mechanisms behind derangements in glucose tolerance. For research, blood samples are customarily taken at baseline and at regular intervals within a 2-hour period to assess β-cell secretory responses. Measurement of mature insulin is an inaccurate approximation for functional secretion, as insulin undergoes hepatic first-phase metabolism (121, 122). As C-peptide is a stable molecule that is secreted at a 1:1 ratio (**Figure 8**), it serves as a surrogate marker for β-cell function during OGTT. It is important to emphasise that the biphasic insulin secretion cannot be reproduced by an OGTT, as opposed to an intravenous glucose challenge, due to the prolonged glucose absorption within the intestine (121).

In **paper III**, the glucose load was reduced to 50 g to diminish hyperglycaemic effects in the pregnant women with L-T1D. Peripheral venous blood was collected at baseline in addition to 10 min, 30 min, 60 min, 90 min, and 120 min after glucose ingestion. As individuals with L-T1D have low, if any, residual β-cell function, an ultrasensitive ELISA (Mercodia; Sweden) was used to measure both fasting and stimulated C-peptide in plasma samples from the study participants. The detection limit for the assay was 2.5 pmol/L (0.0076 μg/L).

Data analysis

Dimensionality reduction analysis, unsupervised clustering, and statistical analysis (paper I)

Data of single, live $CD45^+$ cells from each sample were manually gated using Cytobank (123). Data were first hyperbolic ArcSinh-transformed with a cofactor of 5 and then a 5-level hierarchical stochastic neighbour embedding (HSNE) analysis was performed in Cytosplore^{+HSNE} with default settings (124). All markers were selected in the HSNE analysis except cytokines. Major cell lineages were identified at the overview level and analysed separately in a data-driven manner (**Figure 9**) (124). To facilitate subset identification and reduce computing time, $CD4^+$ and $CD8^+$ T cells were further divided into

naïve and memory cells in subsequent levels according to the expression of CD45RA, CD45RO, CCR7, and CD27. Clustering was performed by Gaussian mean-shift (GMS) in Cytosplore. Clusters showing similar phenotype were manually merged. Further exploration of cluster frequencies and median ArcSinh5-transformed marker expression was performed using Cytofast (125). For cytokine expression, differential median ArcSinh5-expression between clusters with similar phenotype in unstimulated and stimulated samples was calculated. Shapio Wilk's normality test was used to evaluate data distribution. The χ^2-test was used to assess differences in sex distribution between the study groups. One-way ANOVA and Tukey's test for multiple comparisons were applied for statistical analysis of data ($\alpha = 0.05$). All statistical analyses were performed in GraphPad Prism 8 software.

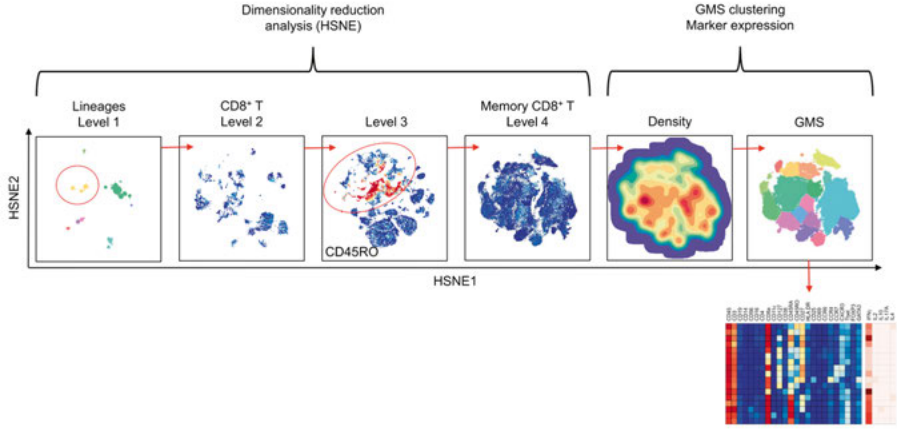

Figure 9. Example of hierarchical data exploration. Hierarchical stochastic neighbour embedding (HSNE) analysis is a dimensionality reduction technique that efficiently maintains the non-linear local data structure, whilst enabling analysis of a large number of cells. The $CD8^+$ T cell lineage from the overview level was selected and embedded at more detailed levels. Memory $CD8^+$ T cells (red circle) were selected at level 3 based on CD45RO expression. At level 4, unsupervised Gaussian mean-shift (GMS) clustering used the local probability density of HSNE-embedded cells (density map) to identify phenotypically distinct clusters (colour partitions). Median ArcSinh5-transformed marker expression for all identified clusters was then visualised in heatmaps in Cytofast.

Data processing for proximity extension assay (paper II and III)

PEA includes a substantial quality control system to ensure that technical variations in assays and samples are reduced, and that systematic errors are removed by normalisation. Four internal controls were spiked at a pre-determined concentration into all samples. These internal controls consisted of two non-human proteins (incubation controls), an antibody conjugated to two complementary oligonucleotides (extension control), and a synthetic dsDNA

amplicon (detection control). Moreover, three external controls were included to each plate. A negative control was used to determine the detection limit for each analyte, whilst an inter-plate control was added for data normalisation. External sample controls were added to calculate intra- and inter-assay coefficients of variance.

Quantitation cycle (Cq)-values were acquired, and assay runs were verified in Fluidigm® Real-Time PCR analysis software. Subsequently, data processing was performed in Olink® NPX Manager. Cq-values for each analyte were adjusted against the Cq-value for the extension control to reduce technical variation within samples. Adjusted Cq-values were then normalised against the inter-plate control to improve inter-assay repeatability. As samples were randomised within the plates, an intensity normalisation was applied to reduce technical bias. This step involved a global adjustment in order to centre values for each assay around their medians and across plates. By using predetermined correction factors for each analyte, adjusted Cq-values were inverted and transformed into a relative arbitrary unit called Normalised Protein eXpression (NPX). NPX is expressed on a logarithmic scale (log2), meaning that a difference of +1 NPX corresponds to a concentration doubling. Standard deviations for both incubation controls and the detection control were then calculated to evaluate variation over entire plates (threshold of ±0.2 NPX). To assess sample quality, median values and standard deviations for one incubation control and the detection control were calculated in each sample before comparing against the median of all samples. Samples failed the quality control if either internal control diverged more than ±0.3 NPX.

Statistical analysis (paper II)

Data for supernatants from unstimulated and stimulated PBMC cultures were analysed separately. The induced secretion was also assessed by calculating the pairwise difference of NPX between stimulated and unstimulated samples. Principal component analysis (PCA) was applied to visualise similarities and differences in multivariate datasets between plasma and supernatant samples, respectively. One-way ANOVA adjusted for multiple testing with the Benjamini-Hochberg method, followed by Tukey's post hoc test for significant assays, were applied for analysis of immunological data ($\alpha = 0.05$). One-way ANOVA with Tukey's test for multiple comparisons and the χ^2-test were used to analyse clinical data ($\alpha = 0.05$). Two-tailed non-parametric Spearman correlation was applied to investigate covariations between immunological and clinical data ($\alpha = 0.05$), along with linear regression to find the best-fit line.

Statistical analysis (paper III)

The ratio between triglycerides and HDL-cholesterol was used to calculate insulin resistance at each visit, where a cut-off value of 1.65 was set as previously described (126, 127). Patients were considered positive for autoantibodies if anti-GAD > 5 IU/mL and anti-IA2 > 7 kU/L. Area under the curve for C-peptide values acquired during 120 min-OGTTs was calculated by the trapezoid rule. Absolute differences between highest and fasting C-peptide concentrations during OGTT, as well as the percental change, were calculated. The ratio between peak and fasting C-peptide concentrations was also assessed. Statistical analyses were conducted in GraphPad Prism version 9 and IBM SPSS Statistics version 27. Sharpio-Wilk and Kolmogorov-Smirnov tests were used to determine the data distribution of clinical variables. C-peptide data were transformed to a 10-logarithmic scale when a Gaussian distribution was not followed, and normality tests were again performed to evaluate transformed data. Two-tailed unpaired Welch t-test or two-tailed unpaired Mann-Whitney test was used to compare data between pregnant and non-pregnant participants. A linear mixed effects model with the Benjamini-Hochberg method for false discovery rate were applied to compare longitudinal changes in clinical variables. For non-parametric longitudinal data, Friedman test followed by uncorrected Dunn´s post hoc test were used. The $\chi 2$-test was applied to compare frequencies of categorial variables, such as C-peptide detectability and autoantibody positivity. A significance level of $\alpha = 0.05$ was defined for all tests.

Statistical analyses for proteomic data were conducted in RStudio version 4.0.0. PCA was applied to identify outliers and clusters. A two-sided unpaired Welch t-test was used to compare protein levels between non-pregnant individuals and both trimesters, separately, with a significance level at $\alpha = 0.05$. A linear mixed effects model was applied to assess longitudinal changes in the proteome between trimesters and the postpartum period. A maximum likelihood approach was chosen for parameter estimation, where visits and individuals constituted fixed and random effects, respectively. An unstructured covariance matrix was assumed. The Benjamini-Hochberg method was applied for correction of multiple testing in the model, and a false discovery rate <0.05 was considered significant. A network analysis of the plasma proteome was performed on the online platform STRING version 11.0. A full network type was chosen to identify both functional and physical protein interactions, the interaction score was set at a high confidence level (0.700), and edges illustrate the strength of data support (confidence). A network was considered to have significantly more interactions than expected at a PPI enrichment p-value < 0.05. UniProt and The Human Protein Atlas version 19.3 were implemented to obtain in-depth information on protein function and tissue expression. Explorations were made using UniProt numbers specified in Olink® panels.

”Jag upptäckte att sökandet kan vara lika intressant som att finna. När man har tagit sig förbi rädslan”

Ur Brida av Paulo Coelho

Main findings and Discussion

Peripheral immunity in autoimmune endocrine diseases

The first part of this thesis aimed to characterise and compare peripheral $CD45^+$ cells from individuals with four different autoimmune endocrine diseases and HC by employing CyTOF® (**paper I**). The first level of HSNE analysis identified no differences in the frequency of major cell lineages between the groups. All eight cell lineages were subsequently selected and explored in detail by HSNE and GMS clustering, which visualised that PBMC are a heterogenous population of phenotypically distinct cell subsets. Statistical analysis of each cluster revealed alterations within subsets of B cells, monocytes (Mo), NK cells, and memory $CD8^+$ T cells in three patient groups (**Figure 10**). Frequencies of subsets identified within naïve and memory $CD4^+$ T cells, regulatory T cells, naïve $CD8^+$ T cells, and myeloid and plasmacytoid DC were similar between individuals with autoimmune endocrine diseases and HC.

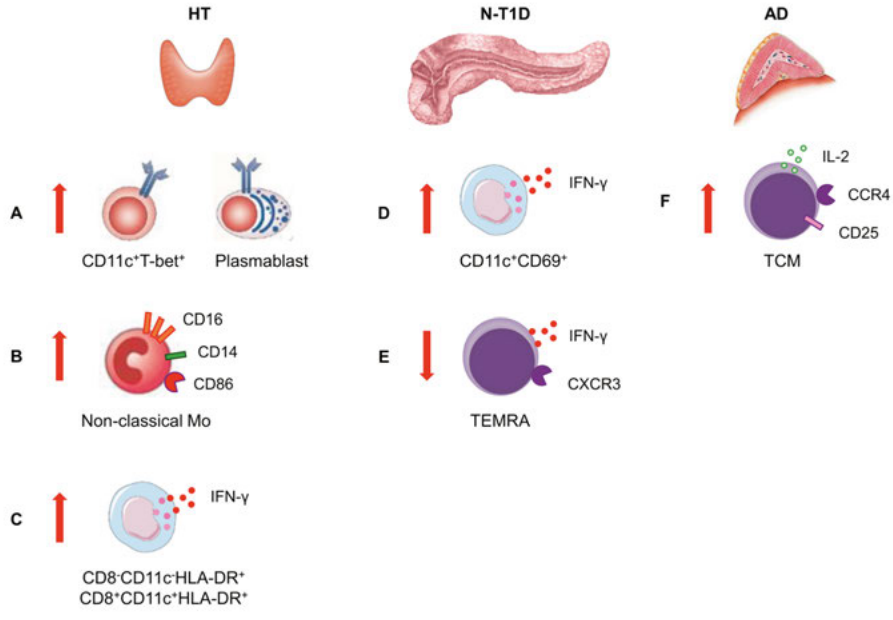

Figure 10. Alterations of peripheral CD45$^+$ cell subsets in patients with autoimmune endocrine diseases. Out of 125 phenotypically distinct PBMC subsets, seven clusters were altered in individuals with Hashimoto´s thyroiditis (HT), new-onset type 1 diabetes (N-T1D), and autoimmune Addison's disease (AD). Patients with HT were affected by changes in **(A)** two CD19$^+$ B cell subsets, **(B)** non-classical monocytes (Mo), and **(C)** two clusters of CD56^{dim} NK cells. Alterations in **(D)** activated NK cells and **(E)** CD45RA-expressing effector memory CD8$^+$ T cells (TEMRA) were detected within the N-T1D group. A rare subset of **(F)** central memory CD8$^+$ T cells (TCM) characterised individuals with AD. Red arrows indicate direction of change in peripheral blood. Titles below cells represent distinguishing marker expression or recognised definitions for each cluster. CCR; C-C motif chemokine receptor, CXCR; C-X-C motif chemokine receptor, IFN; interferon, IL; interleukin, TNF; tumour necrosis factor.

CD19$^+$ B cell and CD14$^+$ Mo subsets in patients with HT

An elevated frequency of CD20^{lo}CD27^{hi}CD38^{hi}HLA-DR^{int} plasmablasts was detected in patients with HT, which corroborates previous findings that HT is characterised by enhanced intrathyroidal B cell maturation and circulating antibody-producing B cells (38, 40). Peripheral expansion of plasmablasts in autoimmune diseases has been recently attributed to antigen-experienced CD11c$^+$T-bet$^+$ B cells (128-130). In **paper I**, a subset of CD11c$^+$CD27$^-$ CXCR3$^+$T-bet$^+$ B cells appeared to be more frequent in patients with HT, supporting the idea that these cells might promote a plasmablast expansion. Patients with HT were also characterised by an increased frequency of CD86$^+$CD14^{lo}CD16$^+$ non-classical Mo. Non-classical Mo generally express markers related to antigen-presentation and secrete IL-1β/TNF-α to a higher degree than classical Mo (131). A parallel expansion of this cell subset and

plasmablasts in the HT group is an interesting observation, as anti-TPO antibodies can activate $CD14^+$ Mo and thereby induce antibody-dependent cell-mediated cytotoxicity and cytokine secretion (132-134). Studies on peripheral and tissue-resident Mo from patients with T1D and GD have shown no alterations in frequency, consistent with our results, yet an altered cytokine secretion pattern (135, 136).

$CD56^{dim}$ NK cell subsets in patients with HT and N-T1D

Two subsets of $CD56^{dim}HLA\text{-}DR^+$ NK cells, a $CD8^-CD11c^-$ and a $CD8^+CD11c^+$ cluster, were more frequent in patients with HT. These two subsets expressed high levels of IFN-γ, which was comparable to the expression in $CD56^{hi}$ NK cells. It has been shown that $CD56^{dim}HLA\text{-}DR^+$ NK cells develop during pro-inflammatory conditions (137) and have better stimuli-induced secretion of IFN-γ than $HLA\text{-}DR^-$ NK cells (137, 138). Consequently, a high expression of IFN-γ in divergent $HLA\text{-}DR^+$ clusters suggests that these cells can be involved in the maintenance of inflammatory immune responses in patients with HT. Patients with N-T1D had an expansion of activated mature/effector $CD56^{dim}$ NK cells that expressed IFN-γ. The expression of CD11c and CD69 on this peripheral cluster is intriguing, as these markers are normally detected to a higher degree on tissue-resident NK cells (139, 140). In addition, $CD69^+$ NK cells seem to express IFN-γ to a higher degree than $CD69^-$ NK cells (141). As this alteration was not observed in samples from individuals with L-T1D, a higher frequency of activated NK cells might reflect an intensified pro-inflammatory process at an early stage of T1D.

Memory $CD8^+$ T cell subsets in patients with N-T1D and AD

The frequency of $CD45RA^+CD27^-CCR7^-CXCR3^+T\text{-}bet^+IFN\text{-}\gamma^+$ TEMRA cells was reduced in individuals with N-T1D. As $CD8^+$ T cells are recruited to insulin-positive islets to contribute to β-cell apoptosis and dysfunction (12, 142, 143), a reduction of TEMRA cells in peripheral blood from the N-T1D group might be explained by their migration into the pancreas. $CD25^+CCR4^+GATA3^+$ central memory $CD8^+$ T cells were more frequent in patients with AD than in the other groups. This cluster accounted for the elevated IL-2 expression in memory $CD8^+$ T cells from individuals with AD. A similar Th2-like central memory $CD8^+$ T cell subset has been previously described, which lacked regulatory functions despite CD25 expression (144, 145). An expansion of such rare and phenotypically distinct central memory T cells in patients with AD might be due to a hydrocortisone-induced $CD8^+$ T cell differentiation (146-148), which might not reflect the pro-inflammatory environment within the adrenal glands.

Low-abundant plasma proteins in individuals with T1D, HT, and GD

After the phenotypic characterisation of peripheral $CD45^+$ immune cells, PEA was applied to interrogate the protein composition in plasma and cell culture

supernatants from patients with autoimmune endocrine diseases (**paper II**). PCA showed that patients could not be distinguished by the overall protein composition in either sample matrix, but two low-abundant analytes diverged in plasma samples. CUB domain-containing protein (CDCP)1 was more abundant in the N-T1D, L-T1D, HT, and GD groups than in HC. In addition, individuals with L-T1D and HT had elevated plasma levels of signalling lymphocytic activation molecule (SLAMF)1 compared with HC. Plasma levels of CDCP1 in patients with N-T1D were inversely correlated with fasting C-peptide concentrations ($r = -0.81$, $p = 0.048$). In contrast, increased levels of CDCP1 in the N-T1D group appeared to be associated with increased HbA1c ($r = 0.75$, $p = 0.07$). For the L-T1D group, a positive covariation between CDCP1 levels and body mass index was found ($r = 0.93$, $p = 0.0007$).

This is the first study that reveals an association between CDCP1, HT, and GD, whereas elevated CDCP1 levels in patients with L-T1D have been previously demonstrated (149). As CDCP1 interacts with the co-stimulatory receptor CD6 on T cells (150-152), it is interesting that aberrant CDCP1-singalling has been previously associated with autoimmunity and Th1/Th17 responses (150, 152). The inverse correlation between soluble CDCP1 and fasting C-peptide in patients with N-T1D implies that a reduced insulin production is connected to inflammation, possibly within the pancreas, at disease onset. Decreased pancreatic insulin secretion consequently entails a challenge for patients to maintain glycaemic control, as indicated by the positive correlation between CDCP1 and HbA1c. As CDCP1 was only associated with body mass index in the L-T1D group, it seems that the ability to maintain an optimised metabolic control long after diagnosis has a substantial effect on whole-body inflammation (153, 154). SLAMF1 is a co-stimulatory receptor that co-localises with CD3 upon T cell activation and affects downstream signalling (155). Membrane-bound and soluble SLAMF1 are induced upon activation and then constitutively expressed on memory T and B cells (156, 157). SLAMF1-ligation seems to preferentially stimulate IFN-γ secretion from $CD4^+$ T cells in addition to antibody production from B cells. As patients with HT had elevated frequencies of plasmablasts and $CD11c^+$T-bet^+ B cells (**Figure 10**), a higher abundance of soluble SLAMF1 suggests that these memory B cell subsets are activated and continuously stimulated to produce autoantibodies in HT. One previous study has detected an increased frequency of peripheral $SLAMF1^+CD4^+$ T cells in patients with autoimmune thyroid diseases (158).

Pancreatic, hormonal, and immunological adaptions in pregnant women with T1D

The last study aimed to examine longitudinal effects on β-cell function and the plasma proteome in pregnant women with L-T1D (**paper III**), as there is

a lack of studies that simultaneously investigate metabolic, hormonal, and immunological adaptions in this population.

β-cell function and glycaemic control

Detection and absolute concentrations of fasting C-peptide were higher in women with L-T1D during gestation and two months after parturition than in the non-pregnant group. Importantly, total insulin secretion, peak C-peptide concentrations, and absolute change in C-peptide levels during a 2-hour OGTT increased during pregnancy. Although the glucose-induced insulin secretion declined after parturition, it did not differ compared to first trimester values. This study demonstrates thus that pregnancy induces a long-standing recovery of β-cell function in individuals with T1D. The results are in line with previous studies investigating only fasting C-peptide (96, 97), but contradicts a study mainly evaluating stimulated C-peptide (98). A recovery of β-cell function might explain the improvement of HbA1c during pregnancy and after parturition, which was better than the glycaemic control of non-pregnant women with L-T1D. Both the insulin resistance and daily insulin doses were elevated during the third trimester, most likely due to anabolic processes (159), but insulin doses were subsequently lower for women two months after parturition than in the non-pregnant group despite no differences in insulin resistance. In parallel with an improved glucose-induced insulin secretion, PRL increased during pregnancy prior to a slow decline after parturition. As indicated by others, PRL might have modulated insulin secretory pathways within residual β-cells without inducing proliferation (80, 160).

Effects of pregnancy on the plasma proteome in T1D

By employing PEA, 184 analytes associated with processes such as lymphocyte activation, cytokine-mediated signalling, apoptosis, and differentiation were measured in plasma. Unsupervised PCA did not identify outliers or clusters amongst the samples, meaning that the plasma proteome at a global level was similar between pregnant and non-pregnant women as well as between study visits. However, several analytes diverged between study cohorts and timepoints when applying an independent sample t-test and linear mixed model, respectively (**Table II**).

Table II. Cell regulatory and immunological proteins differed between pregnant and non-pregnant patients as well as during and after pregnancy. Peripheral venous blood was collected at three visits during pregnancy: first trimester (1T), third trimester (3T), and two months postpartum (2PP). For comparison, frozen plasma samples from non-pregnant women with diabetes (NP) were used. A two-sided unpaired Welch t-test was applied and adjusted for multiple testing to compare protein levels between the NP group and both trimesters. A linear mixed effects model was used to assess longitudinal variations in the proteome of pregnant women. The model found that 79 analytes in total had changed between visits. The Benjamini-Hochberg method was then applied to correct for multiple testing across all proteins and fixed effects (false discovery rate, q-value).

Welch t-test		
Comparison	**Significant (adjusted $p < 0.05$)**	**Non-significant**
1T vs NP	16	168
3T vs NP	47	137
Linear mixed model		
Comparison	**Significant ($q < 0.05$)**	**Non-significant**
1T vs 3T	47	32
1T vs 2PP	50	29
3T vs 2PP	63	16

Two separate supervised network analyses were then performed to investigate whether there were functional or physical associations between diverging analytes. Both networks visualised that only 35 % of all nodes were associated with maternal immune processes. For changes between pregnant and non-pregnant women, the network analysis showed that only 16 of 47 proteins were functionally related. Nineteen of 79 analytes were connected when computing proteins that were affected by longitudinal changes between the first trimester and two months postpartum. All clusters in both network analyses except one could be similarly classified (**Figure 11**), where analytes that could be related to maternal β-cell biology and immunology were discussed in detail. The cluster termed "enzyme activity" was not discussed in **paper III**, as the enzymes are ubiquitous and related to protein glycosylation (GCNT1 and GALNT2), connective tissue development (GLB1 and HS3ST3B1), and central nervous system development (ARSB).

1) IL-6 governed	2) Endocine modification	3) Antigen-presentation	4) Enzyme activity
IL-6	GCG	LY75	**GLB1**
CCL11	PROK-1	CLEC4A	**ARSB**
FGF-21	*PTH1R*	*CD83*	*GALNT2*
CKAP4		*LAMP3*	*GCNT1*
AREG			*HS3ST3B1*
CLEC4C			
CLEC4D			
CLEC7A			
CD28			
ITGA11			
ITGB6			
IL12RB			

Figure 11. Four functional clusters were identified *in silico*. A supervised network analysis of diverging analytes between plasma samples was performed on the STRING platform. A full network type was chosen to identify both functional and physical protein interactions. A high confidence level was applied to only visualise protein interactions that had a high strength of data support. Functional enrichments within networks were computed, and protein databases were implemented to obtain in-depth information on protein function and tissue expression. Connected nodes were classified based on functional enrichments and database explorations: 1) IL-6 governed pathways, 2) Endocrine modification, 3) Antigen-presentation pathways, and 4) Enzyme activity. Analytes that only differed between pregnant and non-pregnant women with diabetes are written in bold. Analytes that were only affected by longitudinal changes during gestation and the postpartum period are written in italics.

Interesting correlations between improved β-cell function and proteomic alterations could be revealed, although **paper III** does not provide mechanisms for either reduced apoptosis, proliferation, or neogenesis of β-cells in pregnant women with T1D. Despite that IL-6 and glucagon (GCG) were not functionally connected (**Figure 11**), it is noteworthy that their plasma levels were higher during third trimester than during early-gestation and after parturition as well as compared with non-pregnant women. IL-6 can stimulate enzymatic processing of GCG into glucagon-like peptide (GLP)-1, leading to increased insulin secretion and glycaemic control (161). GLP-1 is the paracrine factor that stimulates β-cell proliferation, mass expansion, and insulin production in pregnant mice (162). Moreover, prokineticin (PROK)-1 was constantly more abundant during pregnancy than after parturition and the non-pregnant group. The network analysis also revealed that PROK-1 is associated to GCG by function or tissue location. Indeed, PROK-1 is secreted by cells in the placenta that promotes angiogenesis and capillary fenestration only in endocrine glands (163, 164). It has been demonstrated that PROK-1 and its receptor are present within the pancreas, which can stimulate proliferation and reduce apoptosis of endothelial cells (165, 166). This implies that pancreatic angiogenesis might have been induced in pregnant individuals with T1D, leading to increased oxygenation of residual β-cells and enhanced functional capacity.

Most immunomodulatory processes occur within the placenta during pregnancy (72), but the peripheral immunity is also affected. Both CCL11 and soluble CD28 were continuously low during pregnancy, but both analytes increased again after parturition. In addition, plasma levels of CCL11 were less abundant in pregnant women with L-T1D than in the non-pregnant group. CCL11 and other pro-inflammatory chemokines are reduced throughout gestation in healthy women before rebounding after parturition (76, 77). In accordance with the functional enrichment, women with L-T1D can also acquire a shift from leukocyte migration and humoral immune responses. Soluble CD28 has recently been investigated in autoimmune diseases, where increased peripheral concentrations are often connected to elevated disease activity (167-169). Moreover, healthy women who experienced spontaneous abortions had increased CD28 expression in decidual tissues in parallel with an elevated frequency of IFN-γ^+ T cells (170). These studies imply that T cell activation, survival, or function is impaired in women with T1D during pregnancy, which might have a beneficial effect on non-placental tissues such as pancreatic islets. Proteins related to endocytosis, processing, and presentation of antigens were separated into two functional clusters in the network analysis, but they could be similarly classified (**Figure 11**). Lymphocyte antigen (LY)75, CLEC4A, and CLEC4C were reduced in plasma from women with L-T1D at third trimester than in samples from non-pregnant individuals. LY75, CLEC4A, CD83, and lysosomal associated membrane protein (LAMP)3 had similar longitudinal changes in women with L-T1D: decreased during pregnancy before a rebound after parturition. According to the Uniprot database, expression of abovementioned proteins defines the maturation or activation of Mo, myeloid DC, and plasmacytoid DC. APC appear to play a critical role for a successful pregnancy, as shifts in ratios of APC subsets have been associated with obstetric complications in healthy women (171-175). Thus, reduced plasma levels of LY75, CLEC4A, CLEC4C, CD83, and LAMP3 might reflect a suppression of APC maturation, function (affecting downstream lymphocyte activation), or recruitment to tissues such as the placenta and pancreas.

Conclusions and Future perspectives

Based on the defined aim and objectives, this thesis concludes that:

- The majority of peripheral $CD45^+$ cell subsets were similar between the different autoimmune endocrine diseases and HC. However, patients with N-T1D, HT, and AD had altered frequencies of phenotypically distinct and occasionally rare subsets within B cell, Mo, NK cell, and cytotoxic T cell lineages

- The immunological profiles in plasma and supernatants from PBMC cultures were similar between individuals with autoimmune endocrine diseases and HC. However, an increased abundance of CDCP1 and SLAMF1 in plasma from patients with T1D, HT, and GD might reflect a higher degree of inflammation and lymphocyte activation

- Women with L-T1D regain endogenous insulin production during gestation that slowly wanes after parturition. Elevated plasma levels of PRL, PROK-1, or GCG might have affected residual β-cells through distinct mechanisms. Pregnant individuals with L-T1D also acquired a peripheral immunoprofile characterised by reduced leukocyte migration, T cell activation, and APC activity. Thus, enhanced β-cell function might be further protected by an immunomodulatory shift

This thesis pertains the concept “autoimmune tautology” and might therefore contribute to a better understanding of shared and diverging immunological features between autoimmune endocrine diseases. By applying CyTOF® and PEA, this thesis also demonstrates that multi-dimensional and high-throughput technologies facilitate more efficient research when more information can be obtained simultaneously from clinical samples. Increased knowledge on “autoimmune tautology” paves the way to understand how immunomodulation affects pre-existing deviant immune responses. Exogenous immunotherapies, such as monoclonal antibodies, mesenchymal stromal cell transplantation, and tolerance-inducing vaccination, have been regularly evaluated for rescuing residual β-cell function in patients recently diagnosed with T1D. This thesis reveals that pregnancy has a potent and multifaceted effect on women

with L-T1D, including improved β-cell function and immunomodulation, which improves the glycaemic control before and after parturition. By having investigated physiological effects of pregnancy on women with T1D, it might be possible to imitate a β-cell trophic environment and develop new treatments for all individuals with T1D irrespective of disease onset. Nevertheless, it is important to emphasise that this thesis comprises purely descriptive studies. Functional studies are consequently required to address mechanistic roles of 1) divergent $CD45^{+}$ cell clusters and analytes for the pathology of autoimmune endocrine diseases, and 2) hormonal and immunological factors during gestation for the improvement of β-cell function.

> ”Vägen verkar ofta vilja leda fel, för att uppmuntra den resande att upptäcka vad som döljer sig bakom nästa kurva”
>
> Ur Krönika: Mellan Jekaterinburg och Novosibirsk av Paulo Coelho

Populärvetenskaplig sammanfattning

Autoimmuna sjukdomar är ett samlingsnamn för tillstånd som orsakas av att immunförsvaret attackerar kroppens egna vävnader. Immunceller kan reagera på strukturer (proteiner) som antingen finns i ett enda organ eller är spridda i kroppen, vilket ger upphov till organspecifika respektive systemiska sjukdomar. En kategori av organspecifik autoimmunitet riktas mot hormonproducerande organ eller celler, vilket inkluderar bukspottkörteln, sköldkörteln och binjurarna. Detta kallas det endokrina systemet. Majoriteten av de hormon som produceras reglerar kroppens metabolism, till exempel hur energi lagras och förbränns samt hur salt- och vätskebalansen justeras. Autoimmuna sjukdomar som påverkar endokrina organ är således allvarliga och i många fall livshotande.

Det är fortfarande oklart hur de autoimmuna sjukdomarna uppkommer samt vad som avgör vilket eller vilka organ som drabbas. En teori är att drabbade individer har en genetisk bakgrund som leder till ett mer aktivt/mindre selektivt immunförsvar, vilket tillsammans med utlösande faktorer (*triggers*) i miljön kan framkalla sjukdom. Avvikande egenskaper och reaktioner hos immunförsvaret har historiskt sett ansetts vara likartade mellan de autoimmuna endokrina sjukdomarna. Risken att insjukna i en autoimmun endokrin sjukdom ökar dessutom om en individ redan har en annan sådan sjukdom. Tidigare metoder och tekniker har dock inte varit tillräckligt avancerade för att analysera flera parametrar parallellt i olika patientgrupper. Det finns därför ett behov av att undersöka och jämföra immunförsvarets reaktioner mellan olika autoimmuna sjukdomar.

Den första delen av avhandlingen omfattar två arbeten, där det perifera (blodbundna) immunsystemet jämfördes mellan patienter med typ 1 diabetes (T1D, insulinbrist), Hashimotos tyreoidit (HT, underskott av sköldkörtelhormonet tyroxin), Graves sjukdom (GD, överskott av tyroxin) och Addisons sjukdom (AD, kortisol- och aldosteronbrist i binjurarna). Immunceller från blod karaktäriserades i det första arbetet genom att undersöka uttryck av yt- och intracellulära markörer, vilka återspeglar cellers funktion, aktivering och differentiering. Merparten av det perifera immunförsvaret var identiskt mellan patientgrupperna och i jämförelse med friska individer. Det fanns dock ett fåtal celltyper med specifika egenskaper som skiljde sig åt mellan de autoimmuna endokrina sjukdomarna. Andelen antikroppsproducerande B-celler och en effektiv typ av monocyter var högre hos individer med HT. Monocyter är en outvecklad immuncell som tar upp strukturer från till exempel bakterier

och parasiter i syfte att presentera dessa för B- och T-celler, det specifika immunförsvaret. Patienter med HT hade dessutom en högre andel av proinflammatoriska NK-celler (”mördarceller”) i det ospecifika immunförsvaret jämfört med de andra grupperna. Individer med nydiagnostiserad T1D hade en högre frekvens av aktiverade NK-celler i blodet, men en lägre andel av långlivade T-celler med proinflammatoriska funktioner. Sådana T-celler ”minns” specifika strukturer som de har reagerat på tidigare och kan självständigt starta nya reaktioner snabbt. Patienter med AD hade en hög andel av en annan typ av ”minnes-T-celler” med ett ovanligt markörruttryck, vilka har en okänd funktion i immunsystemet. Slutsatsen av detta arbete är att de avvikande celltyperna speglar en skillnad i immunologiska mekanismer mellan HT, T1D respektive AD.

Signalsubstanser i blodplasma och odlingsmedium från immunceller analyserades i det andra arbetet. Resultaten visade att individer med autoimmuna endokrina sjukdomar inte har en perifer proinflammatorisk profil samt att profilen inte avvek avsevärt från friska individer. Patienter med T1D, HT och GD hade dock högre koncentrationer av två signalsubstanser i plasma än kontrollgruppen: CUB domain-containing protein-1 (CDCP1) och signalling lymphocytic activation molecule-1 (SLAMF1). Ökade mängder av CDCP1 och SLAMF1 indikerar att det finns en inflammation i kroppen samt att B- och T-celler är aktiverade i högre grad.

Den sista delen av avhandlingen handlar om hur graviditet påverkar kvinnor som har haft T1D i mer än 10 år. Kroppen genomgår flera fysiologiska förändringar under en graviditet, vilka ska främja fosterutvecklingen och skydda fostret från moderns immunförsvar. Bukspottkörteln hos friska kvinnor producerar mer insulin tidigt under graviditeten i syfte att ta upp och lagra energi till kommande fostertillväxt. Immunsystemets aktivering och funktion förändras också, eftersom modern måste utveckla en tolerans mot det från modern genetiskt avvikande fostret och således förhindra missfall. Immunsystemet moduleras framförallt i moderkakan, men immunologiska förändringar kan också detekteras i blodet. Det är för närvarande oklart om kvinnor med T1D kan uppnå samma graviditetsrelaterade förändringar som friska kvinnor, eftersom immunförsvaret redan är reaktivt och har attackerat de insulinproducerande cellerna i bukspottkörteln. Här undersöktes patienters förmåga att producera insulin under en glukosbelastning vid tre tidpunkter: graviditetsvecka 10–13, graviditetsvecka 33–36 samt två månader efter förlossning. Dessutom undersöktes hormoner och signalsubstanser relaterade till cellregulatoriska och immunologiska processer i plasma vid ovannämnda tidpunkter. Studien visar att gravida kvinnor med T1D faktiskt ökar sin insulinproduktion i fastande tillstånd och vid glukosbelastning, vilket förbättrade deras blodsockerkontroll. Dessa förändringar bibehölls efter förlossningen även om insulinproduktionen tycks minska långsamt därefter. En förbättrad funktion hos de insulinproducerande cellerna kunde relateras till ökade koncentrationer av prolaktin (reglerar laktation, steroidhormonproduktion och energiomsättning),

prokineticin-1 (utsöndras av moderkakan och stimulerar blodkärlsbildning inom det endokrina systemet) och glukagon (ökar frisättning av glukos till blodet) i plasma under graviditeten. Dessa hormoner har i tidigare studier visat sig påverka funktionen och antalet insulinproducerande celler positivt. En lägre koncentration av substanser relaterade till immuncellers rörelseförmåga, T-cellsaktivering och presentation av främmande eller kroppsegna strukturer kan också ha bidragit till en återhämtning av de insulinproducerande cellernas funktion.

Autoimmuna sjukdomar har oftast i tidigare studier behandlats som av varandra isolerade tillstånd trots att de alla har ett gemensamt ursprung: en avvikande reaktion i immunförsvaret. Avhandlingen är därför ett viktigt bidrag till grundforskningen om autoimmunitet, eftersom den präglas av ett jämförande helhetsperspektiv på immunsystemet. Det sista arbetet är unikt då det visar att en ökad funktion hos insulinproducerande celler medför en bättre blodsockerkontroll hos gravida kvinnor med typ 1 diabetes. De hormonella och immunologiska förändringarna i samband med denna återhämtning kan utnyttjas i framtida studier för att utveckla en kompletterande eller botande behandling för alla individer med typ 1 diabetes. En behandling som återhämtar bukspottkörtelns insulinproduktion, reducerar insulindoser och minskar variationer i blodsockerkontrollen skulle innebära en avsevärd ökad livskvalitet och kanske livslängd för patienter. Avhandlingen visar dessutom att användandet av nya tekniker leder till att mer komplex information om immunförsvaret kan fås från samma mängd provmaterial, vilket kan effektivisera framtida forskning.

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> ”Kärleken räcker för att ge värde åt ett helt liv”
>
> Ur Häxan från Portobello av Paulo Coelho

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