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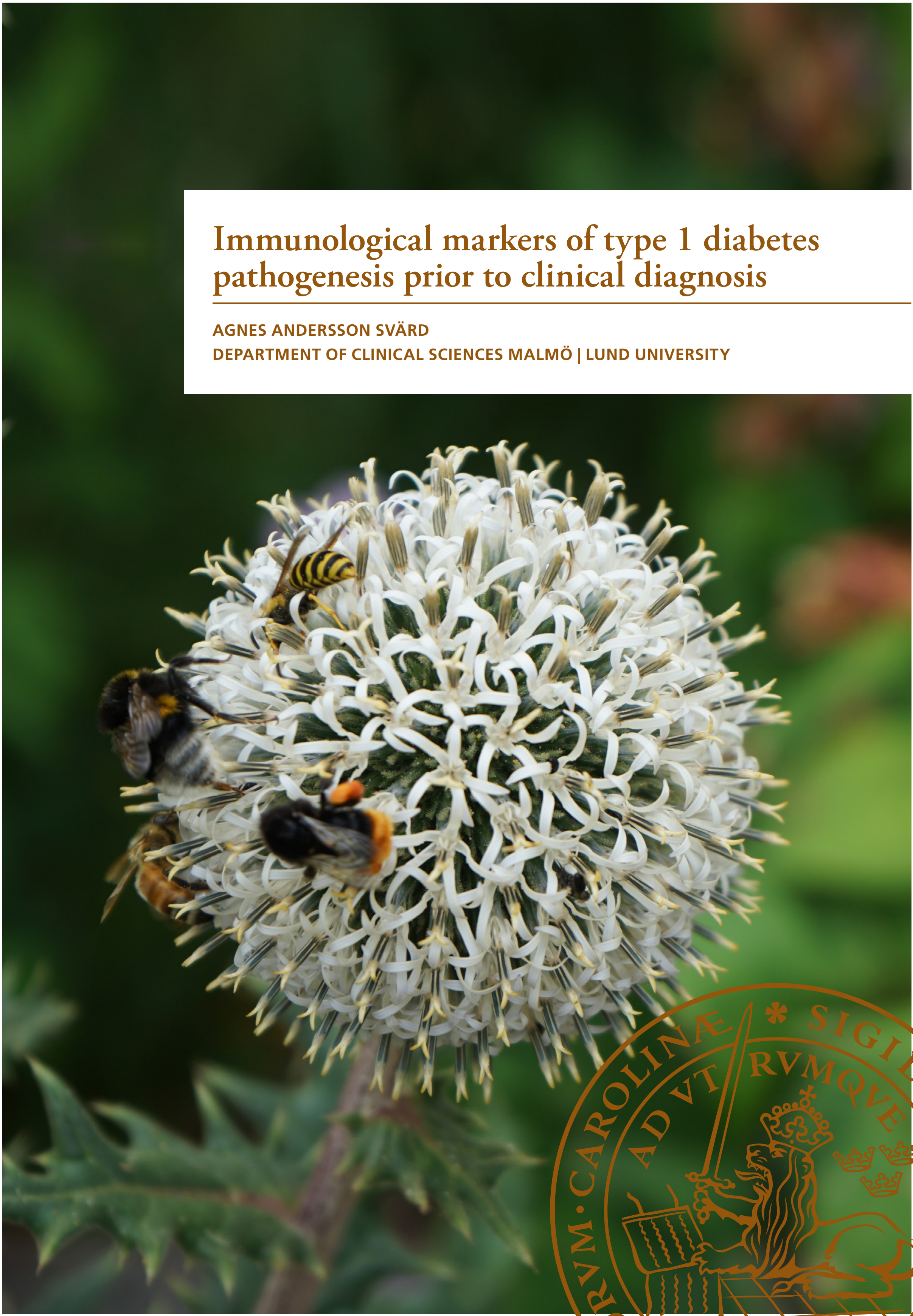
Immunological markers of type 1 diabetes pathogenesis prior to clinical diagnosis

AGNES ANDERSSON SVÄRD

DEPARTMENT OF CLINICAL SCIENCES MALMÖ | LUND UNIVERSITY



Immunological markers of type 1 diabetes pathogenesis prior to clinical diagnosis

Agnes Andersson Svärd

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DOCTORAL DISSERTATION

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To be defended on September 23rd 2022, at 9 am,
Lilla Aulan Medical Research Centre, Jan Waldenströms gata 1, Skåne University Hospital Malmö, Sweden.

Faculty opponent
Professor Kristina Lejon
Umeå University, Umeå, Sweden

Organization LUND UNIVERSITY	**Document name:** Doctoral dissertation
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Author: Agnes Andersson Svärd	Sponsoring organization

Title and subtitle: Immunological markers of type 1 diabetes pathogenesis prior to clinical diagnosis

Background: Type 1 diabetes is an autoimmune disease characterized by insulin deficiency due to pancreatic islet beta cell function loss, resulting in increased blood glucose levels. Beta-cell autoantibodies are markers of the autoimmune that might be present months to years before clinical diagnosis. HLA-DR-DQ is strongly associated with the risk of type 1 diabetes. The HLA-DR3/4-DQ2/8 genotype confers the highest risk. However, only one in 15 (7%) of individuals with this human leukocyte antigen (HLA) genotype develop the disease over a life-time. **Aims:** The overall aim of this thesis was to investigate immunological markers and survey type 1 diabetes pathogenesis. Specifically, we aimed to investigate if there is an association between antigen-presenting cells' ability to present antigen to immune cells, HLA, and autoantibodies. In addition, we aimed to investigate plasma lipid profiles in relation to HLA and to identify and examine novel type 1 diabetes susceptibility loci. **Methods:** Participants (n = 67) in the Swedish Diabetes Prediction in Skåne (DiPiS) study donated a blood sample for cross-sectional analysis of white blood cells. Isolated peripheral white blood cells ($CD16^+CD66^+$, $CD19^+$, $CD16^+$, $CD14^+CD16^-$, $CD4^+$, and $CD8^+$) were investigated by flow cytometry to examine the cell surface median fluorescence intensity (MFI) of Class II HLA-DQ. Information on follow-up in the DiPiS study was obtained from the DiPiS database. HLA high-resolution sequencing of HLA-DRB1, -DRB345, -DQA1, -DQB1, -DPA1, and -DPB1 was performed. Type 1 diabetes-associated autoantibodies to insulin (IAA), glutamic acid decarboxylase 65 (GADA), insulinoma-associated protein-2 (IA-2A), and all three variants of zinc transporter 8 (ZnT8A) were analysed in standardized radio-binding assays (papers I, II and IV). In Paper I, HLA-DQ cell surface MFI on isolated blood cell subtypes was investigated in relation to HLA and autoantibodies. In Paper II, lipidomic profiles were determined using ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS). Lipid profiles were investigated in relation to HLA and autoantibodies. In Paper III, molecular inversion probe sequencing (MIP) technology was used to identify and examine additional type 1 diabetes susceptibility loci in HLA-DR3 homozygous type 1 diabetes patients (n = 365) and control subjects (n = 668), originating from four large type 1 diabetes studies. In Paper IV, TaqMan® SNP Genotyping Assays were utilized to identify a risk haplotype of three single nucleotide polymorphisms (tri-SNP) in intron 1 of HLA-DRA1. The tri-SNP was investigated in relation to HLA, autoantibodies, and HLA-DQ MFI on isolated cell types. **Results:** In Paper I, a pattern of decreasing HLA-DQ cell surface MFI was observed with increasing autoimmunity burden, the burden of autoantibodies at cross-sectional sampling (sAB), or over time during follow-up in DiPiS (cAB), on $CD16^+$, $CD14^+CD16^-$, $CD4^+$, and $CD8^+$ cells. HLA-DQ cell surface MFI was associated with HLA-DQ2/8 in $CD4^+$ T lymphocytes, marginally in $CD14^+CD16^-$ monocytes and $CD8^+$ T lymphocytes. These associations appeared to be related to autoimmunity burden. In Paper II, levels of several specific phospholipid species varied with the level of autoimmunity but not the development of type 1 diabetes. Five glycosylated ceramides were increased in IAA-positive subjects compared to subjects without this autoantibody. Long-chain triacylglycerol levels seemed to be associated with HLA genotypes. Paper III describes a new tri-SNP haplotype in intron 1 of the HLA-DRA1 gene found to modify the risk of type 1 diabetes in HLA-DR3 homozygous subjects. In Paper IV, four tri-SNPs (ACA, ACG, AGG, and GCA) were identified. HLA-DQ cell surface MFI decreased with increasing autoimmunity burden on $CD16^+$, $CD14^+CD16^-$, $CD4^+$, and $CD8^+$ cells in subjects with the AGG haplotype compared to GCA.
Conclusion: HLA-DQ cell surface MFI may be related to the degree of autoimmunity burden. Lipidomic profiles may improve the sub-phenotyping of subjects with a high risk of type 1 diabetes. The tri-SNP could help clarify the role of HLA in type 1 diabetes susceptibility. These parameters may increase the precision of predicting type 1 diabetes in subjects with increased genetic risk for type 1 diabetes who are followed longitudinally.

Key words: Type 1 Diabetes, HLA, Autoantibodies, PBMC, Flow cytometry, Lipids, SNP, Genotyping

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To all children with type 1 diabetes

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Abbreviations

APC	antigen-presenting cells
BDD	Better Diabetes Diagnosis
cAB	cumulative autoimmunity burden
Cer	ceramide
DBS	dried blood spot
DiPiS	diabetes Prediction in Skåne
eQTL	expression quantitative trait locus
FDR	first-degree relative
GAD65	glutamic acid decarboxylase 65
GADA	glutamic acid decarboxylase 65 autoantibody
GlcCer	glycosylated ceramide
GWAS	genome-wide association study
HbA1c	glycated hemoglobin A1c
HLA	human leukocyte antigen
IA	islet autoimmunity
IA-2	insulinoma-associated protein-2
IA-2A	insulinoma-associated protein-2 autoantibody
IAA	insulin autoantibody
IASP	islet autoantibody standardization program
LC	liquid chromatography
LD	linkage disequilibrium
LMSD	lipid maps structure database
LPC	lysophosphatidylcholine
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MS	mass spectrometry
NGS	next-generation sequencing
OR	odds ratio

OGTT	oral glucose tolerance test
PC	phosphatidylcholine
PC(O)	alkylphosphatidylcholine
PCR	polymerase chain reaction
RBA	radiobinding assay
sAB	sample autoimmunity burden
SM	sphingomyelin
SNP	single nucleotide polymorphism
T1DGC	Type 1 Diabetes Genetics Consortium
TEDDY	The Environmental Determinants of Diabetes in the Young
TG	triacylglycerol
Tri-SNP	haplotype of three SNPs (rs3135394, rs9268645, and rs3129877) in intron-1 of the HLA-DRA1 gene
UHPLC	ultra-high-performance liquid chromatography
UHPLC-QTOF-MS	ultra-high-performance liquid chromatography squadrupole time-of flight mass spectrometry
Q-TOF	quadrupole time-of flight mass spectrometry
WHO	World Health Organization
ZnT8RA	arginine 325 zinc transporter 8 autoantibody
ZnT8WA	tryptophan 325 zinc transporter 8 autoantibody
ZnT8QA	glutamine 325 zinc transporter 8 autoantibody
ZnT8A	zinc transporter 8 autoantibodies to any of the amino acid variants at position 325

Abstract

Background: Type 1 diabetes is an autoimmune disease characterized by insulin deficiency due to pancreatic islet beta cell function loss, resulting in increased blood glucose levels. Beta-cell autoantibodies are markers of the autoimmune that might be present months to years before clinical diagnosis. HLA-DR-DQ is strongly associated with the risk of type 1 diabetes. The HLA-DR3/4-DQ2/8 genotype confers the highest risk. However, only one in 15 (7%) of individuals with this human leukocyte antigen (HLA) genotype develop the disease over a life-time.

Aims: The overall aim of this thesis was to investigate immunological markers and survey type 1 diabetes pathogenesis. Specifically, we aimed to investigate if there is an association between antigen-presenting cells' ability to present antigen to immune cells, HLA, and autoantibodies. In addition, we aimed to investigate plasma lipid profiles in relation to HLA and to identify and examine novel type 1 diabetes susceptibility loci.

Methods: Participants (n = 67) in the Swedish Diabetes Prediction in Skåne (DiPiS) study donated a blood sample for cross-sectional analysis of white blood cells. Isolated peripheral white blood cells (CD16^{+}CD66^{+}, CD19^{+}, CD16^{+}, CD14^{+}CD16^{-}, CD4^{+}, and CD8^{+}) were investigated by flow cytometry to examine the cell surface median fluorescence intensity (MFI) of Class II HLA-DQ. Information on follow-up in the DiPiS study was obtained from the DiPiS database. HLA high-resolution sequencing of HLA-DRB1, -DRB345, -DQA1, -DQB1, -DPA1, and -DPB1 was performed. Type 1 diabetes-associated autoantibodies to insulin (IAA), glutamic acid decarboxylase 65 (GADA), insulinoma-associated protein-2 (IA-2A), and all three variants of zinc transporter 8 (ZnT8A) were analysed in standardized radio-binding assays (papers I, II and IV). In Paper I, HLA-DQ cell surface MFI on isolated blood cell subtypes was investigated in relation to HLA and autoantibodies. In Paper II, lipidomic profiles were determined using ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS). Lipid profiles were investigated in relation to HLA and autoantibodies. In Paper III, molecular inversion probe sequencing (MIP) technology was used to identify and examine additional type 1 diabetes susceptibility loci in HLA-DR3 homozygous type 1 diabetes patients (n = 365) and control subjects (n = 668), originating from four large type 1 diabetes studies. In Paper IV, TaqMan® SNP Genotyping Assays were utilized to identify a risk haplotype of three single nucleotide polymorphisms (tri-SNP) in intron 1 of HLA-DRA1. The tri-SNP was investigated in relation to HLA, autoantibodies, and HLA-DQ MFI on isolated cell types.

Results: In Paper I, a pattern of decreasing HLA-DQ cell surface MFI was observed with increasing autoimmunity burden, the burden of autoantibodies at cross-sectional sampling (sAB), or over time during follow-up in DiPiS (cAB), on CD16^{+}, CD14$^+$CD16$^-$, CD4$^+$, and CD8$^+$ cells. HLA-DQ cell surface MFI was associated with HLA-DQ2/8 in CD4$^+$ T lymphocytes, marginally in CD14$^+$CD16$^-$ monocytes and CD8$^+$ T lymphocytes. These associations appeared to be related to autoimmunity burden. In Paper II, levels of several specific phospholipid species varied with the level of autoimmunity but not the development of type 1 diabetes. Five glycosylated ceramides were increased in IAA-positive subjects compared to subjects without this autoantibody. Long-chain triacylglycerol levels seemed to be associated with HLA genotypes. Paper III describes a new tri-SNP haplotype in intron 1 of the HLA-DRA1 gene found to modify the risk of type 1 diabetes in HLA-DR3 homozygous subjects. In Paper IV, four tri-SNPs (ACA, ACG, AGG, and GCA) were identified. HLA-DQ cell surface MFI decreased with increasing autoimmunity burden on CD16$^+$, CD14$^+$CD16$^-$, CD4$^+$, and CD8$^+$ cells in subjects with the AGG haplotype compared to GCA.

Conclusion: HLA-DQ cell surface MFI may be related to the degree of autoimmunity burden. Lipidomic profiles may improve the sub-phenotyping of subjects with a high risk of type 1 diabetes. The tri-SNP could help clarify the role of HLA in type 1 diabetes susceptibility. These parameters may increase the precision of predicting type 1 diabetes in subjects with increased genetic risk for type 1 diabetes who are followed longitudinally.

Thesis at a glance

Paper	Aim	Methods	Results	Conclusion
I	To investigate if there is an association between HLA-DQ cell surface expression on isolated peripheral blood cells. HLA and autoantibodies.	Subjects (n = 67, aged 10-15 years) in DiPiS follow-up donated a blood sample from which peripheral white blood cells CD16$^+$CD66$^+$, CD19$^+$, CD16$^+$, CD14$^+$CD16$^-$, CD4$^+$ and CD8$^+$) were isolated. Class II HLA-DQ cell surface median fluorescence intensity (MFI) was investigated by flow cytometry. Autoantibodies were analysed in-house using a radio-binding assay. High-resolution sequencing was used for Class II HLA typing.	HLA-DQ cell surface MFI decreased with increasing autoimmunity burden, defined by autoantibodies at cross-sectional sampling or during follow-up in DiPIS, was observed on CD16$^+$, CD14$^+$CD16$^-$, CD4$^+$ and CD8$^+$. HLA-DQ cell surface MFI was associated with HLA-DQ2/8 in CD4$^+$ T lymphocytes, marginally in CD14$^+$CD16$^-$ monocytes and CD8$^+$ T lymphocytes.	HLA-DQ cell surface expression appears to be related to autoimmunity burden.
II	To investigate lipidomic profiles in relation to HLA and autoantibodies.	Samples and data were obtained from the cohort presented in Paper I. Lipidomic profiles were determined in plasma using ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry. Lipidomics data were analysed according to genotype. Lipid profiles were investigated forrelative HLA and autoantibodies.	Levels of several specific phospholipid species varied in relation to the level of autoimmunity but not the development of type 1 diabetes. Five glycosylated ceramides were increased in IAA-positive subjects compared to subjects without this autoantibody. Long chain TG seemed to be influenced by HLA genotypes.	Identifying lipid profiles in subjects with a high risk of type 1 diabetes may improve sub-phenotyping in high-risk populations.
III	To identify new type 1 diabetes susceptibility loci using molecular inversion probe sequencing technology.	Molecular inversion probe (MIP) sequencing technology was used to identify and examine identify and examine additional type 1 diabetes susceptibility loci in HLA-DR3 homozygous type 1 diabetes patients (n = 365) and control subjects (n = 668), originating from four large type 1 diabetes studies.	A three SNPs (tri-SNP) haplotype in intron 1 of HLA-DRA1 was found to modify the risk of type 1 diabetes in HLA-DR3 homozygous subjects.	The DRA tri-SNP could help clarify the role of HLA in type 1 diabetes susceptibility.
IV	To investigate a new risk haplotype of tri-SNP in intron 1 of HLA-DRA1 in relation to HLA, autoantibodies and HLA-DQ MFI on isolated peripheral cell types	Samples and data were obtained from the cohort presented in Paper I. TaqMan® SNP Genotyping Assays were utilized to identify a risk haplotype of three single nucleotide polymorphisms (tri-SNPs) in intron 1 of HLA-DRA1. The tri-SNP was investigated in relation to HLA, autoantibodies and HLA-DQ MFI on isolated cell types.	Four tri-SNPs (ACA, ACG, AGG and GCA) were identified. HLA-DQ cell surface MFI decreased with increasing autoimmunity burden on CD16$^+$, CD14$^+$CD16$^-$, CD4$^+$ and CD8$^+$ cells in subjects with the AGG haplotype compared to GCA.	The DRA tri-SNP could be related to autoimmunity burden and HLA-DQ cell surface MFI.

List of papers

This thesis is based on the following papers, which will hereafter be referred to by their Roman numerals. Paper I, II and VI are published under the Creative Commons Attribution 4.0 International (CC BY 4.0). Permission to reprint Paper III has been granted by the American Diabetes Association.

Paper I: Decreased HLA-DQ expression on peripheral blood cells in children with varying number of beta cell autoantibodies.

Andersson Svärd, A., Maziarz, M., Ramelius, A., Lundgren, M., Lernmark, Å., Elding Larsson, H. & DiPiS Study Group.

Journal of Translational Autoimmunity. 2020; 9(3): 100052.

Paper II: Characterization of plasma lipidomics in adolescent subjects with increased risk for type 1 diabetes in the DiPiS cohort**.**

Andersson Svärd, A., Lernmark, Å., Maziarz, M. & DiPiS Study Group.

Metabolomics. 2020; 16(19):109.

Paper III: Genetic Variation Within the HLA-DRA1 Gene Modulates Susceptibility to Type 1 Diabetes in HLA-DR3 Homozygotes.

Aydemir, Ö., Noble, J. A., Bailey, J. A., Lernmark, Å., Marsh, P., **Andersson Svärd, A.**, Bearoff, F., Blankenhorn, E. P., Mordes, J. P. & Better Diabetes Diagnosis (BDD) Study Group.

Diabetes. 2019; 68, 7, s. 1523-1527 5 s.

Paper IV: Possible relationship between the HLA-DRA1 intron haplotype of three single-nucleotide polymorphisms in intron 1 of the HLA-DRA1 gene and autoantibodies in children at increased genetic risk for autoimmune type 1 diabetes.

Andersson Svärd, A., E. Benatti, M. Lundgren, Å. Lernmark, M. Maziarz, H. Elding Larsson, and the Diabetes Prediction in Skåne Study Group.

ImmunoHorizons. 2022; 6: 614–629.

Introduction

The immune system

The immune system provides a layered defence of increasing specificity to defend the body against infection. A fine-tuned signalling system is important for the multitude of organs, cells, and proteins of the immune system to work together to fight infection. Pathogens that enter the body are recognized by immune system mechanisms that help clear the infection.

HLA

The human leukocyte antigen (HLA) system is a group of genes that encode proteins on all cells except red blood cells and particularly on immune cells in humans. HLA is analogous to the major histocompatibility complex (MHC) in other animals, such as the mouse. HLA partly determines the response of an individual to infections and influences the response of immune cells to pathogens. The HLA family comprises more than 200 genes, and more than 40% encode proteins involved in immune functions that play critical roles in defence against infectious diseases, cancer, and susceptibility and resistance to autoimmune diseases. The nomenclature of HLA alleles (Figure 1) is set by the WHO Nomenclature Committee[1].

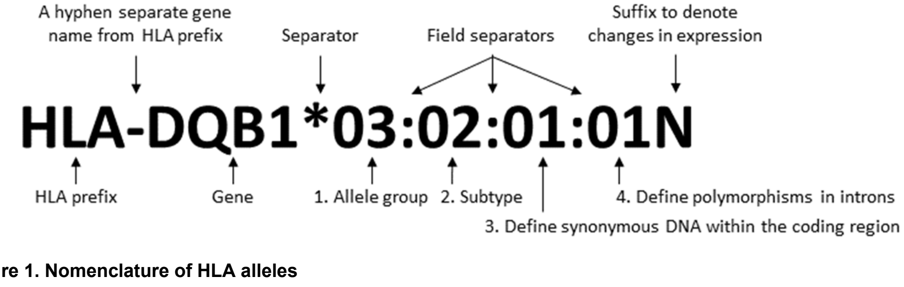

A hyphen separate gene name from HLA prefix
Separator
Field separators
Suffix to denote changes in expression
HLA-DQB1*03:02:01:01N
HLA prefix
Gene
1. Allele group
2. Subtype
4. Define polymorphisms in introns
3. Define synonymous DNA within the coding region

Figure 1. Nomenclature of HLA alleles
The name of an HLA allele has a unique number separated by colons describing allele group (1), specific HLA protein (2), define synonymous DNA within the coding region (3), and define differences (polymorphisms) in a non-coding region (4). Additional suffixes may be added to indicate expression status: 'null alleles' (N), 'low' (L) cell surface expression compared to normal levels, soluble 'secreted' (S) proteins or proteins present in the 'cytoplasm' (C) not expressed on the cell surface, 'aberrant' (A) expression if it is unclear if a protein is expressed, or 'questionable' (Q) if a mutation in the allele has been seen to affect expression levels in other alleles. The nomenclature of HLA alleles is set by the WHO Nomenclature Committee[1]. Adapted from http://hla.alleles.org/nomenclature/naming.html.

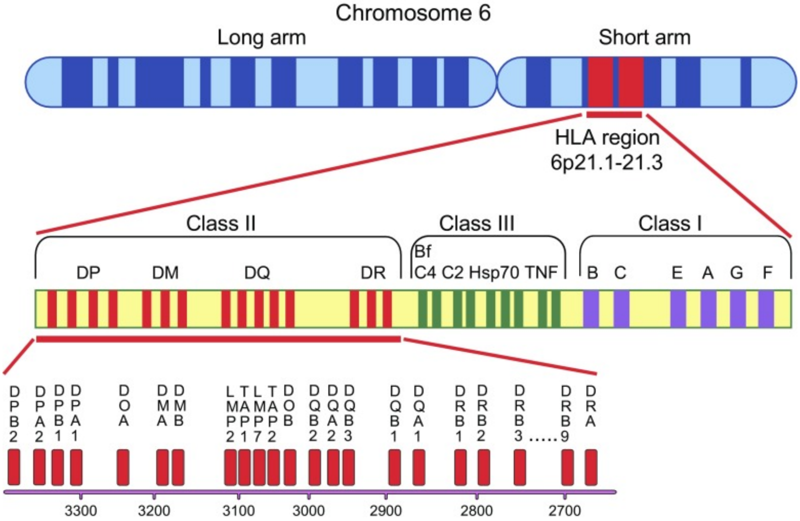

Chromosome 6
Long arm
Short arm
HLA region
6p21.1-21.3
Class II
Class III
Class I
DP
DM
DQ
DR
Bf
C4 C2 Hsp70 TNF
B
C
E
A
G
F
DPB2
DPA2
DPB1
DPA1
DOA
DMA
DMB
LMP2
TAP1
LMP7
TAP2
DOB
DQB2
DQA2
DQB3
DQB1
DQA1
DRB1
DRB2
DRB3.....9
DRA
3300
3200
3100
3000
2900
2800
2700

Figure 2. The HLA gene complex on chromosome 6.
Positions of the Class II HLA-DR-DQ-DP genes are presented as a close-up. Reproduced from Regnell et. al[2].

The HLA gene complex is the most polymorphic region in the human genome, with close to 18,000 different HLA alleles characterized thus far. New alleles continue to be identified and sequenced. The most updated list can be found in the IMGT/HLA database[3]. The HLA gene complex is divided into three regions, Class I, II, and III (Figure 2). Class I and II genes encode molecules that bind and present peptide fragments to T lymphocytes via the antigen-binding groove of the mature HLA cell surface protein. Class I molecules are found on most cell types and present endogenous peptides derived from the intracellular environment of infected or damaged cells. The major function of the Class I molecule is to present antigens to cytotoxic T (T_C) lymphocytes, which kill the infected or damaged cells. Class II molecules are primarily found on antigen-presenting cells (APCs) and present antigens to the helper T (T_H) lymphocytes to elicit an immune response. The Class II molecules sample the extracellular environment and thus present peptides, such as bacteria or viruses, derived from outside the cell. Both Class I and Class II HLA genes are fundamental to the body's recognition of self and non-self[4,5]. The Class III region contains genes for cytokines and the complement system components that have an important role in shaping an adapted immune response[4]. The complement system is tasked with activation inflammation, labelling pathogens and cells for clearance by immune cells and enhancing phagocytosis by innate immune cells.

HLA has been widely studied for its role in transplantation biology and is important roles in transplantation immunology. Certain highly polymorphic HLA genes function as histocompatibility barriers and are critical markers for donor-recipient matching in bone marrow transplantation. A donor with an identical HLA type can donate tissue more successfully than a donor who is not matched[6].

Immune cells

The immune system cells develop from stem cells in the bone marrow and become different types of white blood cells (Figure 3). White blood cells can be found in the tissues and peripheral blood of the body.

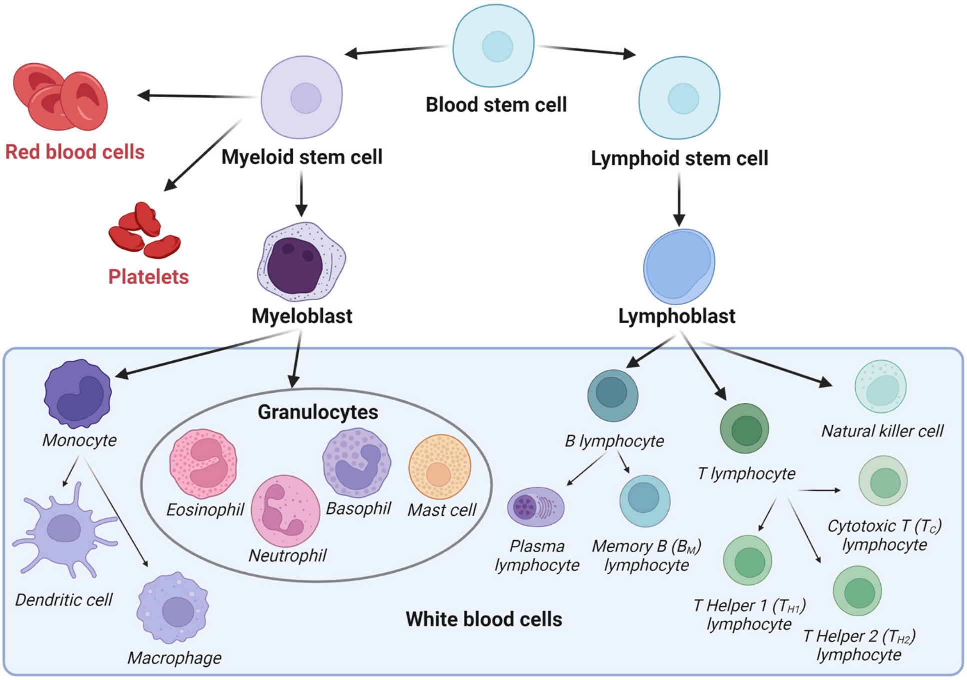

Blood stem cell
Red blood cells
Myeloid stem cell
Lymphoid stem cell
Platelets
Myeloblast
Lymphoblast
Monocyte
Granulocytes
Eosinophil
Basophil
Mast cell
Neutrophil
Dendritic cell
Macrophage
B lymphocyte
Natural killer cell
T lymphocyte
Plasma lymphocyte
Memory B (B_M) lymphocyte
Cytotoxic T (T_C) lymphocyte
T Helper 1 (T_{H1}) lymphocyte
T Helper 2 (T_{H2}) lymphocyte
White blood cells

Figure 3. White blood cells are part of the immune system and develop in the bone marrow. Granulocytes cells (eosinophils, neutrophils, basophils, and mast cells), monocytes, and lymphocytes (B lymphocytes, T lymphocytes and natural killer cells) are the three main types of white blood cells. The image was created using BioRender.com.

A general description of immune cells

There are three main types of white blood cells: granulocytes, monocytes, and lymphocytes. Granulocytes (eosinophils, neutrophils, basophils, and mast cells) contain granules with toxic material in their cytoplasm released in response to a pathogen. The granules contain antimicrobial agents, enzymes, and other proteins. Phagocytes include macrophages, neutrophils, dendritic cells, and eosinophils, and their function within the immune system is to identify and eliminate pathogens that might cause infection. Phagocytes engulf pathogens by attaching to and wrapping around them, trapping them in a phagosome (a compartment in the phagocyte). The pathogen is destroyed when the phagosome lyses with a lysosome or granule containing toxic material. Eosinophils are phagocytic cells that release toxins to kill parasites and bacteria and cause tissue damage. Neutrophils are granulocytes and phagocytes. Neutrophils are considered the first responders of the innate immune system that rapidly migrate to sites of inflammation. They specialize in engulfing and digesting cellular debris, pathogens, and other foreign substances[7,8]. Basophils are non-phagocytic cells responsible for defence against parasites. Mast cells are granulated tissue cells that degranulate in response to tissue injury and initiate inflammatory reactions through the vasoactive properties of histamine.

Any substance capable of eliciting an adaptive immune response is called to as an antigen (antibody generator). An antigen taken up by an APC is combined with HLA and presented on the cell surface of activated T and B lymphocytes. Antibodies are a type of protein created by plasma B lymphocytes in response to pathogens. Antibodies are pathogen-specific and attach to a pathogenic antigen[7,8]. Antigen-presenting cells (APCs) (dendritic cells, monocytes, macrophages, and regulatory T lymphocytes) process and present antigens on Class I and II MHC cell surface epitopes (Figure 4). Dendritic cells are professional APCs that link innate and adaptive immunity. Monocytes migrate to sites of inflammation and mature into tissue macrophages and dendritic cells. A macrophage specializes in engulfing and digesting cellular debris, pathogens, and other foreign substances, consequently stimulating other immune cells. Dendritic cells are professional APCs that display antigens to activate naïve T lymphocytes.

Lymphocytes (B lymphocytes, T lymphocytes, natural killer (NK) cells and cytotoxic T (T_C) lymphocytes) are cells of the adaptive immune system that carry out an immune function in response to a stimulus. Mounting an appropriate immune response depends on the careful regulation of lymphocyte activation. To this end, lymphocytes require two independent signals to become fully activated. The first, an antigen-specific signal is sent via the unique antigen receptor: T cell receptor (TCR) on T lymphocytes or surface immunoglobulin on B lymphocytes. A co-stimulatory signal is a secondary signal independent of the antigen receptor and is critical to allow full activation, sustain cell proliferation, prevent anergy and/or apoptosis, induce differentiation to effector and memory status, and allow cell-cell cooperation. The expression of inhibitory receptors, in turn, regulates co-stimulation upon lymphocyte activation. Lymphocytes stimulated through the antigen receptor alone fail to produce cytokines, are unable to sustain proliferation, and often undergo apoptosis or become nonresponsive to subsequent stimulation[9,10].

B lymphocytes develop in the bone marrow and can mature into plasma or memory B lymphocytes. Plasma lymphocytes produce large volumes of antibodies in response to pathogens, while memory B lymphocytes carry a memory of past infections. Memory B lymphocytes are long-lived and capable of responding to a particular antigen on its reintroduction long after the exposure that prompted its production.

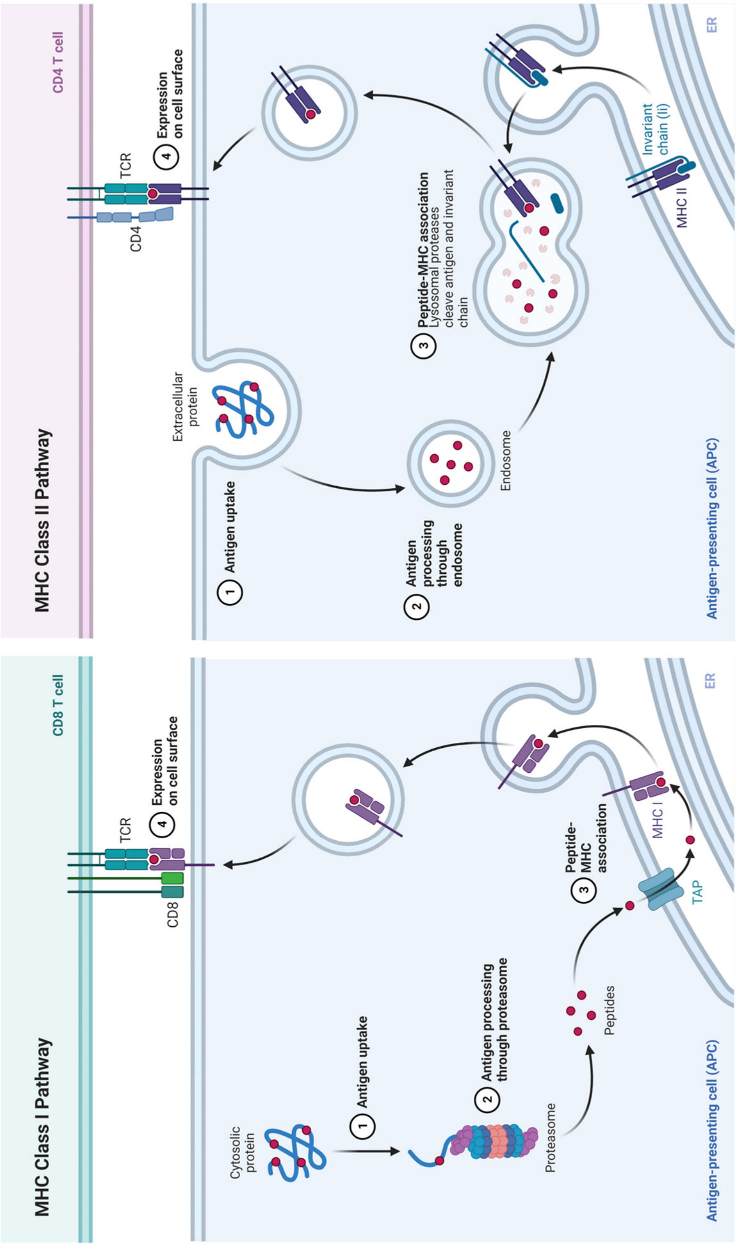

MHC Class I Pathway
CD8 T cell
TCR
CD8
4 Expression on cell surface
Cytosolic protein
1 Antigen uptake
2 Antigen processing through proteasome
Proteasome
Peptides
3 Peptide-MHC association
TAP
MHC I
ER
Antigen-presenting cell (APC)
MHC Class II Pathway
CD4 T cell
TCR
CD4
4 Expression on cell surface
Extracellular protein
1 Antigen uptake
2 Antigen processing through endosome
Endosome
3 Peptide-MHC association
Lysosomal proteases cleave antigen and invariant chain
Invariant chain (Ii)
MHC II
ER
Antigen-presenting cell (APC)

Figure 4. MHC class I and II pathways

Antigen-presenting cells process and present antigens on Class I and II MHC cell surface epitopes through different pathways. Class I MHC molecules present peptides derived from cytosolic proteins to cytotoxic T lymphocytes while Class II MHC present processed antigens derived from extracellular proteins to helper T lymphocytes. Reprinted from "MHC Class I and II Pathways", by BioRender.com (2022), retrieved from https://app.biorender.com/biorender-templates.

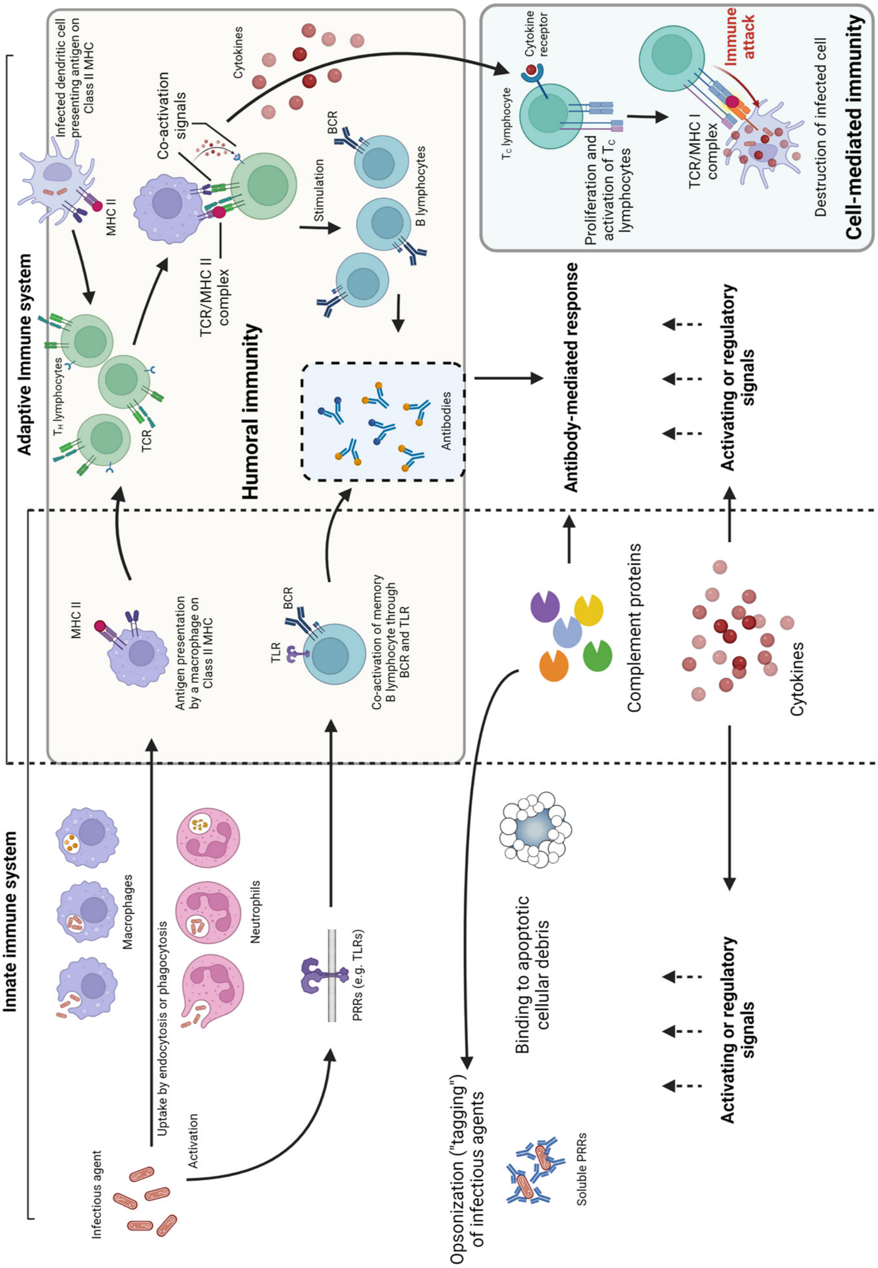
T lymphocytes develop in the thymus and enter the periphery as naïve T lymphocytes mature but are not activated. Naïve T lymphocytes become activated once they bind to an antigen presented on Class II MHC on a macrophage or dendritic cell surface and receive a co-stimulatory signal. T_H lymphocytes secrete cytokines to mediate inflammation signals and immunity to other immune cells and help B lymphocytes produce antibodies. Cytokines are a protein that impacts the immune system by either ramping it up or slowing it down. T_C lymphocytes and natural killer (NK) cells interact with signals from other cells to activate and inhibit innate, non-specific immunity. T_C lymphocytes are the primary effector cells of adaptive immunity and eliminate infected cells. Natural killer lymphocytes are related to T_C lymphocytes but lack antigen receptors. Memory T (T_M) lymphocytes are derived from activated T_C lymphocytes and are long-lived and antigen-experienced. After T_C lymphocytes attack a pathogen, T_M lymphocytes linger to stop any recurrence. T_M lymphocytes are quickly converted into large numbers of T lymphocytes upon re-exposure to a specific invading antigen, thus providing a rapid response to past infection. Regulatory T (T_{REG}) lymphocytes regulate the self-reactivity of T lymphocytes in the periphery to help suppress the immune system[7,8].

Immunity

The innate, adaptive, and passive immune systems mediate an immune response to pathogens. The innate and adaptive immune systems complement each other and interact to efficiently form an overall system of immune defence (Figure 5)[8,11]. The innate immune system is directed towards immediate responses to pathogens. Type I and II interferons are cytokines and signalling proteins that signal neighbouring cells to raise a barrier, signal infected cells to die or recruit white blood cells to stimulate long-lasting immunity[8,12].

The adaptive immune response can be either humoral or cell-mediated. A series of interactions between APCs and B and T lymphocyte cells in a humoral response result in an antibody response. APCs engulf and degrade infectious agents and present antigen fragments on the cell surface by Class II HLA molecules (Figure 5). T_H lymphocytes binding to Class II HLA on APCs activate the proliferation of T_H lymphocytes and release of cytokines. T_H lymphocytes then bind to the HLA complex on B lymphocytes, activating B lymphocyte proliferation and differentiation. B lymphocytes transform into plasma cells that secrete large quantities of antibodies directed against the specific antigen. Some B lymphocytes transform into memory cells that allow a faster antibody-mediated immune response upon future infection[8,12]. This process is utilized in vaccination strategies to generate immunity[13]. A cell-mediated response involves cytotoxic T lymphocytes binding MHC class I on APCs and results in the lysis of foreign or infected cells (Figure 5)[8,12].

Passive immunity is provided when antibodies to pathogens are given instead of producing them through the own immune system. Passive immunity can occur naturally during pregnancy when maternal antibodies are transferred through the placenta or during breastfeeding and protect the offspring from infection[8,12].



Innate immune system
Adaptive Immune system
Infectious agent
Uptake by endocytosis or phagocytosis
Activation
Macrophages
Neutrophils
PRRs (e.g. TLRs)
MHC II
Antigen presentation by a macrophage on Class II MHC
TLR
BCR
Co-activation of memory B lymphocyte through BCR and TLR
Infected dendritic cell presenting antigen on Class II MHC
MHC II
T_H lymphocytes
TCR
Co-activation signals
TCR/MHC II complex
Cytokines
Stimulation
B lymphocytes
BCR
Antibodies
Humoral immunity
T_C lymphocyte
Cytokine receptor
Proliferation and activation of T_C lymphocytes
TCR/MHC I complex
Immune attack
Destruction of infected cell
Cell-mediated immunity
Antibody-mediated response
Activating or regulatory signals
Complement proteins
Cytokines
Opsonization ("tagging") of infectious agents
Binding to apoptotic cellular debris
Soluble PRRs
Activating or regulatory signals

Figure 5. The innate and adaptive immune systems and their overlap.
In the innate immune system, responses to infectious agents are generally immediate and non-specific, such as phagocytosis and endocytosis by macrophages and neutrophils. Pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), recognize pathogen-associated molecular patterns on various microorganisms. Soluble PRRs, such as complement proteins, and acute phase reactants, such as C-reactive protein (CRP), also have a role in innate immunity as they label microorganisms and bind to apoptotic cellular debris in a non-specific manner. In the adaptive immune system, a specific immune response is achieved by engaging receptors with a selective reactivity with specific antigens (T-cell receptors (TCRs) and immunoglobulin receptors on B lymphocytes). An adaptive immune response includes expansion and differentiation of the specific responder cells, which establishes a memory for the specific antigen response. The relationship between the innate and adaptive immune systems has not yet been fully established. Macrophages endocytose or phagocytose antigens in a non-specific manner and present it to T lymphocytes, generating a highly specific immune response. Co-stimulation of B lymphocytes by TLRs can result in the production of specific antibodies to self-antigens. Cytokines may stimulate both an innate and adaptive immune response. Complement proteins also have a role in the innate and adaptive immune systems as they mediate the effector responses induced by antibodies. APC, antigen-presenting cell; BCR, B cell receptor; MHC I, Class I major histocompatibility complex; MHC II, Class II major histocompatibility complex; T_H lymphocyte, helper T lymphocyte; T_C lymphocyte, cytotoxic T lymphocyte. The image is adapted from Gregersen et. al.[11] using BioRender.com.

Immune tolerance

Tolerance is defined as specific unresponsiveness of the adaptive immune system to particular antigens and is characteristic of the normal immune system[11]. Tolerance is classified into central or peripheral tolerance depending on where the state is originally induced.

Central tolerance (negative selection) is the process of eliminating any developing autoreactive B or T lymphocytes reactive to the self-proteins[11,14]. During the maturation in the bone marrow, B lymphocytes undergo negative selection when they bind self-proteins. Autoreactive B lymphocytes either undergo apoptosis, induced an anergy (a state of on-reactivity), or receptor editing. The self-reactive B lymphocyte changes specificity by developing a new B lymphocyte receptor, achieved by rearranging genes. Receptor editing gives B lymphocytes a chance to edit the B lymphocyte receptor before apoptosis or to enter an anergic state[11,14].Thymocytes are immature T lymphocytes that mature in the thymus and undergo a positive and negative selection process determined by their interaction with HLA and self-peptides (Figure 6). T lymphocytes must be able to recognize Class I or II HLA with bound non-self-peptide. During positive selection, non-functional and strongly self-reactive thymocytes do not interact with the Class I or II HLA complexes and are eliminated by apoptosis. Thymocytes with a sufficient affinity for Class I or II HLA mature into cytotoxic or helper T lymphocytes, respectively. During negative selection, the thymocyte affinity to self-peptides is tested where self-reactive thymocytes undergo apoptosis.

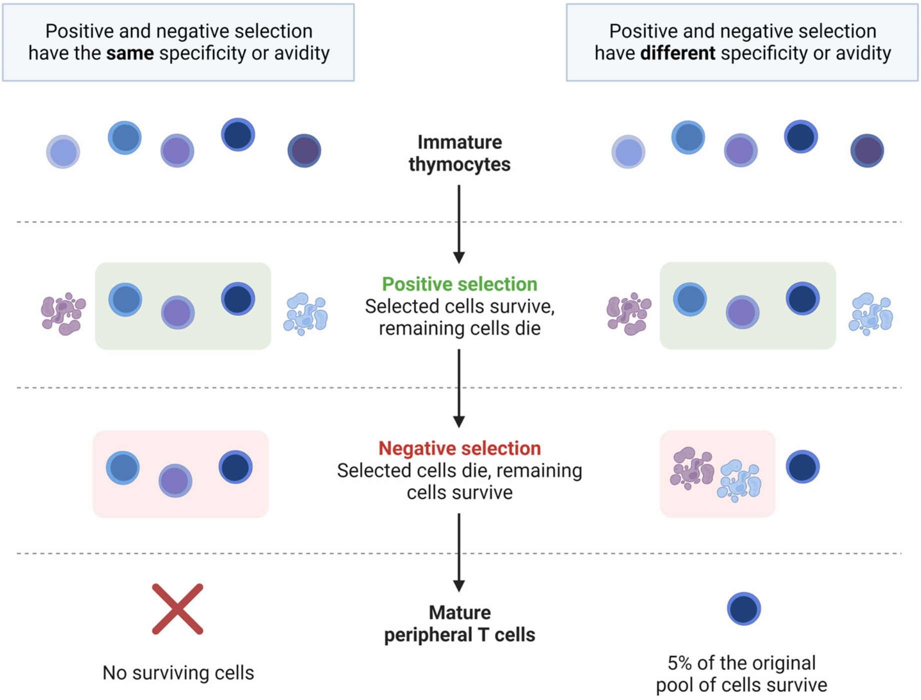

Positive and negative selection have the **same** specificity or avidity
Positive and negative selection have **different** specificity or avidity
Immature thymocytes
Positive selection
Selected cells survive, remaining cells die
Negative selection
Selected cells die, remaining cells survive
Mature peripheral T cells
No surviving cells
5% of the original pool of cells survive

Figure 6. Positive and negative selection of T lymphocytes.
A positive and negative selection process of interaction with HLA and self-peptides help thymocytes mature into T lymphocytes. During positive selection, non-functional and strongly self-reactive thymocytes are eliminated by apoptosis as they do not interact with the Class I or II HLA complexes. Self-reactive thymocytes undergo apoptosis during negative selection as their affinity to self-peptides is tested. Reprinted from "Positive and Negative Selection of T Cells", by BioRender.com (2022), retrieved from https://app.biorender.com/biorender-templates.

Thymocytes can migrate into the periphery as naïve T lymphocytes and are activated by HLA and self-peptides below a certain threshold. Most T lymphocytes develop into effector cytotoxic or helper T lymphocytes that will mediate both humoral (antibody-mediated) and cellular immune responses[11,14] (Figure 7).

Peripheral tolerance is a secondary mechanism to ensure that B or T lymphocytes, leaving the bone marrow or thymus, do not become autoreactive. Peripheral tolerance is distinct from central tolerance and occurs as developing immune cells exit the thymus or bone marrow before their export into the periphery. T_{REG} lymphocytes are key mediators of peripheral tolerance and develop during the negative selection where the T lymphocyte receptor shows a high affinity for self-peptides. The mechanism of the regulatory T lymphocytes is not fully understood but includes managing effector immune response on many levels. Regulatory T lymphocytes may inhibit activation of T lymphocytes by APCs, inhibit differentiation of T lymphocytes into cytotoxic effector cells and/or prevent T lymphocytes from helping B lymphocytes in antibody production[11]. Peripheral tolerance is key to preventing over-reactivity of the immune system from autoreactivity to self-antigens or various environmental antigens (such as allergens)[11,14].

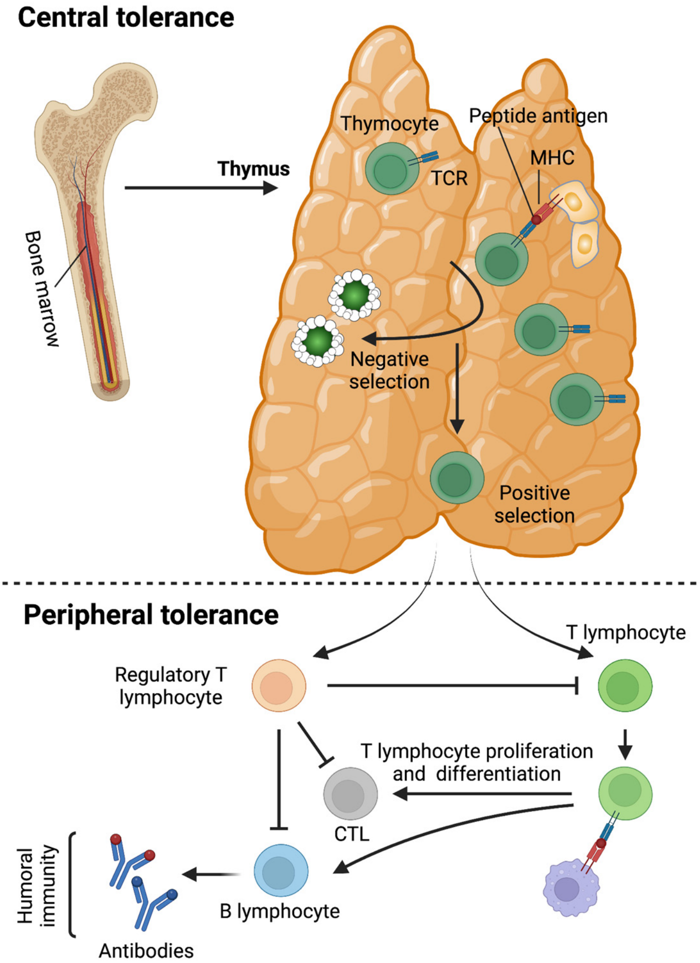

Central tolerance
Bone marrow
Thymus
Thymocyte
TCR
Peptide antigen
MHC
Negative selection
Positive selection
Peripheral tolerance
T lymphocyte
Regulatory T lymphocyte
T lymphocyte proliferation and differentiation
CTL
B lymphocyte
Humoral immunity
Antibodies

Figure 7. Central and peripheral tolerance.
Strongly self-reactive and non-functional thymocytes are eliminated through a positive and negative selection process. In the positive selection process, self-reactive thymocytes have self-peptides that interact with major histocompatibility complex (MHC) proteins. Non-functional thymocytes are prompted to apoptosis in the negative selection process. Thymocytes that are allowed to migrate into the periphery as mature T lymphocytes are positively selected and activated by self-peptide and MHC below a certain threshold. In the periphery, the thymocytes mature into T lymphocytes, that are key mediators of peripheral tolerance. Most thymocytes develop into helper and cytotoxic T lymphocytes that mediate both humoral (antibody-mediated) and cell-mediated immune responses, while some develop into regulatory T lymphocytes. Regulatory T lymphocytes regulate or suppress other cells in the immune system to maintain immune homeostasis and self-tolerance, dampen inflammation, and prevent autoimmunity. The image is adapted from Gregersen et. al.[11] using BioRender.com.

Autoimmunity

Autoimmunity is an abnormal response by the immune system against self-antigens. Deficits in central and peripheral tolerance allow autoreactive B and T lymphocytes into the periphery and cause autoimmune reactions against self-antigens[11]. The immune system does not randomly lose the ability to distinguish between self and non-self-antigens. Certain individuals have a genetic susceptibility to developing autoimmune diseases. The main sets of genes suspected in many autoimmune diseases are related to HLA. Type 1 diabetes, celiac disease, and autoimmune thyroiditis are examples of autoimmune diseases where variants of Class II HLA genes confer a risk of developing the disease[15]. However, only a small fraction of genetically predisposed individuals may develop an autoimmune disease. Autoimmunity may be present without any clinical symptoms. Autoantibodies are common biomarkers of autoimmunity; in some cases, their presence is a risk factor for autoimmune disease. Genetic risk factors also have a role in determining the progression of clinical disease, and identifying these factors offers a possibility to develop targeted preventive therapies[11].

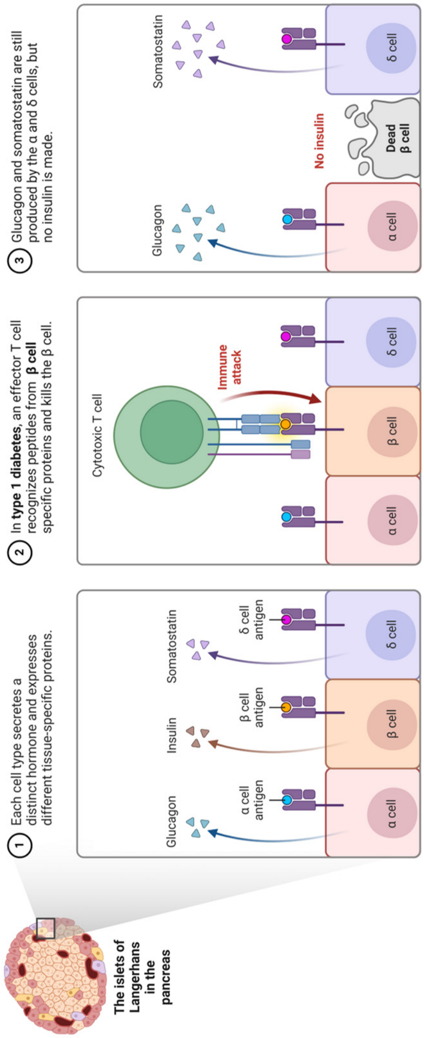
Type 1 Diabetes

Type 1 diabetes is an autoimmune disease characterized by insulin deficiency due to loss of the pancreatic islet beta cells (Figure 8), both in numbers and in function, resulting in increased blood glucose levels[16,17]. What triggers the immune-mediated insulitis destroying islet beta cells and ultimately type 1 diabetes is still unknown. When symptoms of diabetes appear, it has been estimated that only 10–20% of the beta cells are still functioning, and insulin therapy is necessary for survival[18,19]. Younger children often have a shorter asymptomatic period, months before clinical onset, whereas it may take years for the beta-cell destruction to prompt clinical symptoms in older individuals[20,21].

Blood glucose is regulated by cells in the islets of Langerhans. Insulin is produced and released by the beta cells. Insufficient insulin secretion from the beta cells results in hyperglycaemia and high blood glucose levels since insulin is needed to uptake glucose[22]. Low insulin levels also cause the inability to take up and convert glucose into glycogen, a source of energy mostly stored in the liver. Untreated lack of insulin or insufficient insulin release causes a life-threatening condition, ketoacidosis. Ketoacidosis is caused by a fast build-up of acids, ketones, in the blood from lipolysis (breakdown of fat) as an energy source due to glucose shortage in the cells but also due to uncontrolled lipolysis (normally controlled by insulin). Untreated type 1 diabetes is a life-threatening disease. Administration of insulin is essential for survival and must be continued indefinitely.

The history of type 1 diabetes

A condition that appears to have been type 1 diabetes was first described by the ancient Egyptians more than 3000 years ago. They described the condition as excessive urination, thirst, and weight loss. The sweet taste of the urine was identified as glucose in the early 1800s, and shortly after that, glucose was shown to be normally present in the blood. Hyperglycaemia in dogs after pancreatectomy was first reported in 1889 by Oscar Minkowski (1858-1913) and Josef von Mering (1849-1908)[23]. Before the 1920s, there were no treatments for the symptoms of diabetes, and the disease was ultimately deadly. Insulin was first discovered in 1921 by Banting, Best, Macleod, and Collip at the University of Toronto. Banting and Macleod earned the Nobel prize in medicine in 1922. The first patients with diabetes were treated with insulin derived from the bovine pancreas in 1922. The treatment resulted in lowered blood sugar and eliminated glycosuria and ketosis. Insulin derived from bovine the pancreas was available as treatment by 1923[24,25]. Complications were common in the first decades of insulin treatment as the crystallized insulin was impure and had to be injected several times daily with great pain.



① Each cell type secretes a distinct hormone and expresses different tissue-specific proteins.

② In **type 1 diabetes**, an effector T cell recognizes peptides from **β cell** specific proteins and kills the β cell.

③ Glucagon and somatostatin are still produced by the α and δ cells, but no insulin is made.

The islets of Langerhans in the pancreas
Glucagon
Insulin
Somatostatin
α cell antigen
β cell antigen
δ cell antigen
α cell
β cell
δ cell
Cytotoxic T cell
Immune attack
α cell
β cell
δ cell
Glucagon
Somatostatin
No insulin
Dead β cell
α cell
δ cell

Figure 8. Immune response in type 1 diabetes.

β cells secrete insulin to lower blood glucose and are one type of the hormone-secreting cells of the Langerhans islets in the pancreas. In type 1 diabetes, the β cells are targeted by an autoimmune process. T lymphocytes recognize peptides from β cell-specific proteins leading to the β cells being the elimination target. The destruction of the β cells leads to a loss of insulin secretion. Reprinted from "Immune Response in Type 1 Diabetes", by BioRender.com (2022), retrieved from https://app.biorender.com/biorender-templates.

Glycated haemoglobin (HbA1c) reflected the average glucose level in the last 8–12 weeks and was first reported in 1968 to represent an objective measurement of glycaemic control. Home blood glucose control became practically feasible with the introduction of blood glucose test strips in the late 1970s[26]. Insulin injection pens and later the portable insulin pump replaced glass and steel injection syringes in the early 1980s. Glycaemic control was established to prevent and delay the progression of complications in patients with type 1 diabetes with the Diabetes Control and Complications trial published in 1993[27].

Epidemiology

In the last few decades, the incidence of type 1 diabetes has more than doubled and increased 3–5% per year[28]. There is a dramatic variation in incidence between countries and within Europe. Except for on Sardinia where there is a north and south gradient with a high incidence in the northern latitude[29]. The annual incidence of type 1 diabetes is the highest in Finland, with 57.6 patients/100,000 people aged 1-15 years. The geographical location is less important than genetic risk as the incidence rate in Sardinia is close to that of Finland[30,31]; there is an increase in incidenced in Saudi Arabia [32,33], while type 1 diabetes is a rare disease in Asia[34].

Type 1 diabetes is generally assumed to be hereditary even though most patients lack a family history of diabetes; children with a first-degree relative (FDR) with diabetes only account for only about 10–12%[35,36]. In contrast to most autoimmune diseases, type 1 diabetes is slightly more common in males than females. There are small differences in incidence rates between the sexes. Generally, the incidence peak in children occurs earlier in girls, but the general differences between 0–15 years in the age group are small. After puberty, a male predominance of 1.3-2 to 1 is present in many populations[37,38]. Children whose parents have type 1 diabetes are at greater risk of developing type 1 diabetes than children whose parents do not have type 1 diabetes. Historically, the risk of diabetes in the offspring of fathers with type 1 diabetes has been reported as being higher than that in the offspring of mothers with type 1 diabetes or in those with siblings who have type 1 diabetes[38]. The studies consistently show that the risk of childhood-onset diabetes in the offspring of an affected father is two to three times greater than that of an affected mother. Equally, islet autoantibodies are more commonly found in the offspring of fathers with diabetes than mothers with diabetes [38]. The incidence of type 1 diabetes differs by seasonality as has been described by several studies[39]. More patients are diagnosed during the winter and autumn months, a phenomenon that is present with small variations between countries. This seasonal variation also seems more significant in the older age group, in children between 10 and 14 years at diagnosis[40].

Aetiology

The triggering event of the autoimmune process leading to type 1 diabetes is still unknown, but a possible virus-triggered autoimmune response has been suggested as the trigger[41-43]. Due to the immune system's complexity, there may be one or a combination of several factors that trigger type 1 diabetes in different individuals.

Genetic susceptibility

HLA has been implicated in the susceptibility to diseases and the development of autoimmunity. Type 1 diabetes is a disease of unidentified aetiology but is strongly associated with two different HLA haplotypes.

HLA and Risk for Type 1 Diabetes

Type 1 diabetes is strongly associated with HLA-DR-DQ; the genotype HLA-DR3/4-DQ2/8 confers the highest risk[44]. About 90% of children with type 1 diabetes have at least one of the DR3-DQ2 or DR4-DQ8 haplotypes, compared to about 20% of the general population of the western world[45,46]. Inheriting both haplotypes confers the highest risk of developing type 1 diabetes, and those children represent about 30% of all children developing the disease, compared to only 3% of the healthy population[45]. However, only one in 15 individuals (7%) with this HLA type develop the disease[47]. This suggests that factors other than or in addition to HLA are necessary to trigger the autoimmune process.

Non-HLA Genetic Risk Factors

The genetic component of type 1 diabetes cannot be explained by HLA-derived effects alone. More than 70 additional genetic loci have been associated with type 1 diabetes risk in genome-wide association studies (GWAS). The functions of many of these candidate genes and single nucleotide polymorphisms remain unclear[48]. However, improved assays and methods add to our current understanding of gene interactions in the context of type 1 diabetes risk[49]. The prediction of early type 1 diabetes risk can be increased to 10% by adding non-HLA genetic risk factors[50].

eQTL

In humans, a single nucleotide polymorphism (SNP) is a variation of a single nucleotide at a specific genome location. It is the most common type of genetic variation[51]. A SNP may replace the nucleotide cytosine (C) with the nucleotide thymine (G) in a certain stretch of DNA (Figure 9) and may or may not alter protein structure.

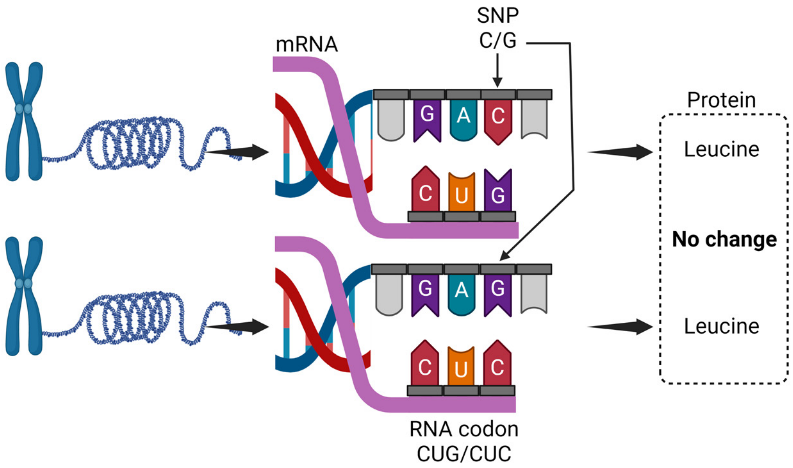


Figure 9. Single nucleotide polymorphism (SNP) may or may not alter protein structure.
A SNP is a change in a single nucleotide in a DNA sequence. When SNPs occur within a gene or in a regulatory region near a gene, they may play a more direct role in disease by affecting the gene's function. A SNP does not alter protein structure if the RNA codon encodes the same protein and if the protein folding is not altered. The image was created using BioRender.com.

Most SNPs do not affect health or development. However, some of these genetic differences, however, have proven important in studying human health. One way to determine if an SNP is a causal variant is to estimate its contribution to gene expression (expression quantitative trait locus, eQTL). An eQTL is a locus that explains a fraction of the genetic variance of a gene expression phenotype. Standard eQTL analysis involves a direct association test between markers of genetic variation with gene expression levels typically measured in tens or hundreds of individuals. The variant most associated with disease may be near a gene of interest; however, that variant may regulate the expression of a different, more distal effector gene. This association analysis can be performed proximally or distally to the gene. One of the major advantages of eQTL mapping using the GWAS approach is that it permits the identification of new functional loci without requiring any previous knowledge about specific cis or trans-regulatory regions[52]. Type 1 diabetes-associated eQTL mapping studies have focused on cell-specific effects in immune cell types[53,54]. This approach assumes that these tissues are critical to understanding the development and pathology of type 1 diabetes.

Linkage Disequilibrium

Linkage disequilibrium (LD) is defined as the non-random association of alleles at different loci in a population. In short, LD is the tendency for alleles at two genetic loci to be found together more often than expected. If the frequency of association of alleles in different loci is higher or lower than what would be expected, and if the loci were independent and associated randomly, the loci are said to be in LD. LD is strong in the HLA region, and specific combinations of allelic variants form haplotypes. HLA-DR-DQ haplotypes associated with type 1 diabetes include the HLA-DRB1*03:01 and HLA-DQB1*02:01 as well as HLA-DRB1*04:01 and HLA-DQB1*03:02 alleles, which confer high risk for type 1 diabetes. Although LD can help detect genetic regions involved in disease, it confounds attempts to identify the actual gene in the region involved in disease and to identify additional genes in the region contributing to disease[55].

Pathogenesis

Autoantibodies

Autoantibodies are currently theorized to be markers of the immune cell-mediated destruction of the pancreatic islet beta cells. Circulating beta cell autoantibodies increase risk for progression to clinical onset of type 1 diabetes. The presence of beta cell autoantibodies may precede clinical onset by months to years[20,21,56].

Autoantibodies against beta cell proteins

The appearance of diabetes-related autoantibodies to one or several of the autoantigens, insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma antigen 2 (IA-2), and Zinc transporter 8 (ZnT8), signal an autoimmune pathogenesis of beta cell destruction[20]. Therefore, it is possible to predict the appearance of type 1 diabetes before any hyperglycaemia arises. Within 12 months, 60% of children with a first autoantibody may develop a second autoantibody, perhaps followed by a third or fourth autoantibody. The number of positive islet autoantibodies correlates to the risk of progressing to clinical disease[56]. The cumulative risk of diabetes varies with age, younger age at seroconversion, multiple autoantibodies, high antibody levels, and persistent insulin autoantibodies (IAA)[57].

IAA

Insulin autoantibodies (IAA) are the first appearing islet autoantibodies and are associated with younger age at diagnosis[50]. IAA is more common as the first appearing autoantibody in children with HLA DR4-DQ8[50,58-60]. In longitudinal follow-up studies of children with genetic risk of type 1 diabetes, IAA as the first appearing autoantibody peaks at 1 year of age[61] and has been found in 80–100% of children diagnosed with type 1 diabetes before the age of 4 years[62]. The prevalence of IAA decreases with increasing age[61].

GADA

Glutamic acid decarboxylase autoantibodies (GADA) are directed against GAD65. GADA can be detected in 70–80% of newly diagnosed type 1 diabetes patients[62]. In longitudinal follow-up studies of children with genetic risk of type 1 diabetes, GADA as the first autoantibody is more common in children from 2 years of age, when it reaches a plateau[61]. GADA is more common as the first appearing autoantibody in children with HLA DR3-DQ2[50,58-60]. GADA can be found slightly more often in females[63].

IA-2A

Islet antigen-2 autoantibodies (IA-2A) are directed against IA-2, a plasma membrane protein. IA-2A is detected in 50–70% of patients with recent onset type 1 diabetes[62]. IA-2A is rarely the first appearing autoantibody but is more common together with one or multiple of the other type 1 diabetes-associated autoantibodies[50,64].

ZnT8A

Autoantibodies targeting Zinc Transporter 8 (ZnT8) have been identified as a major islet autoantigen by autoimmunity in type 1 diabetes. ZnT8 is considered an autoantigen with high specificity to islet B lymphocytes. By facilitating the cellular outflow of zinc, ZnT8 is a transporter protein proposed to be essential in the process of insulin crystallization and secretion[65-67]. There are three variants of the ZnT8 autoantibody (ZnT8A) that differ by tryptophan (W), arginine (R), or glutamine (Q) at position 325[68] . Subjects may develop ZnT8A, which is either specific to one, two, or all three variants. Additionally, ZnT8A were also detected among type 1 diabetes patients who were negative for conventional islet autoantibodies: IAA, GAD65A, and IA-2A[67]. ZnT8A are not commonly the first appearing autoantibody but more common together with one or multiple of the other type 1 diabetes-associated autoantibodies[63].

Lipids

Lipid biology is a major research target in understanding cellular physiology and pathology because of its many key biological functions. Lipids are a diverse and common group of compounds that act as structural components of cell membranes, a source of energy storage, and participate in signalling pathways[69,70]. A comprehensive classification, nomenclature, and chemical representation system is important to differentiate between the great diversity in lipid structure and function. The complete lipid profile within a cell is described as the "lipidome" and is a part of the "metabolome" that also includes sugars, nucleic acids and amino acids in genomics and proteomics. "Lipidomics" is a relatively new research field and a comprehensive analysis of lipid molecules[69,70]. This method can be used to study lipids in relation to metabolic diseases, such as obesity, atherosclerosis, stroke, hypertension, and diabetes, as it combines mass spectrometry (MS) and computational methods[71].

In the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study, an investigation of serum metabolites in children diagnosed with type 1 diabetes at 0.5–13.5 years of age showed that serum metabolites may mark progression to islet autoimmunity and from islet autoimmunity to diabetes, independent of the strength of HLA risk[72]. Another study investigated differences in serum from children followed from birth until clinical type 1 diabetes and healthy controls and found that a distinct cord blood lipidomic profile characterized type 1 diabetes progressors, a molecular signature of seven lipids predicted a high risk for progression to type 1 diabetes[73]. Additionally, specific lipids have previously been shown to be decreased in children participating in the DiPiS study who develop type 1 diabetes before 4 years of age[74].

Lipid structures

Many factors (e.g., variable chain length), biochemical transformations (e.g., oxidative, reductive, substitutional, ring-forming, etc.), and modifications (with functional groups of different biosynthetic origin) drive the structural diversity of lipids. Structural information and the related features are important to describe the biological functions of different lipids.

Lipid classification and nomenclature

The LIPID MAPS Structure Database[75] (LMSD) was developed in 2005 by the International Lipid Classification and Nomenclature Committee on the initiative of the LIPID MAPS Consortium. The LMSD is a comprehensive classification system for lipids based on well-defined chemical and biochemical principles and using a framework designed to be extensible, flexible, scalable, and compatible with modern informatics technology[76,77]. LMSD currently contains over 30,000 structures obtained from a variety of sources: LIPID MAPS Consortium's core laboratories and partners; lipids identified by LIPID MAPS experiments; computationally generated structures for appropriate lipid classes; biologically relevant lipids manually curated from Lipid Bank[78], LIPIDAT[79], Cyberlipids[80] and other public databases; peer-reviewed journals and book chapters describing lipid structures.

Lipids included in LMSD are classified following the LIPID MAPS classification scheme and assigned a unique LIPID MAPS identifier (LM ID). LMSD, comprises eight lipid categories, Fatty Acyls [FA], Glycerolipids [GL], Glycerophospholipids [GP], Sphingolipids [SP], Sterol Lipids [ST], Prenol Lipids [PR], Saccharolipids [SL], and Polyketides [PK]. The eight lipid categories all have their own subclassification hierarchy (main class, sub class (where applicable) and level 4 class (where applicable)). LMSD contains lipid structures and all relevant information for that molecule, such as common and systematic names, synonyms, molecular formula, exact mass, classification hierarchy and cross-references (if any) to other databases[71].

Staging type 1 diabetes

The pathogenesis of type 1 diabetes is currently divided into three stages (Figure 10). These stages relate to the autoimmunity level indicated by the detection of autoantibodies, the level of beta cell destruction, and the clinical symptoms of type 1 diabetes.

Pre-stage 1

Genetic susceptibility and genetic risk of type 1 diabetes are strongly associated with the HLA on chromosome 6, accounting for 30–50% of the risk[81]. A high risk of developing autoantibodies is associated with the HLA DRB1*0301-DQB1*0201 (DR3-DQ2) and DRB1*0401-DQB1*0302 (DR4-DQ8) haplotypes. The heterozygous HLA-DR3/4-DQ2/8 genotype is associated with the highest risk for developing autoantibodies and, subsequently, type 1 diabetes[82]. Other HLA haplotypes, such as HLA DRB1*04:03/04:07/04:10/1501 and DQA1*0102-DQB1*0602, are disease-resistant or protective haplotypes[15,83].

Stage 1

The appearance of autoantibodies is the first sign of the prodromal period of type 1 diabetes. Subjects in Stage 1 have developed autoimmunity with two or more islet autoantibodies but are still asymptomatic and normoglycemic[82]. Due to the slow progression to clinical onset in single autoantibody-positive subjects[56,84], two or more autoantibodies were selected to define Stage 1[82]. Children at this stage, screening for genetic risk at birth, have an approximate 44% and 70% risk of symptomatic disease in 5 and 10 years, respectively, and the lifetime risk approached 100%[56].

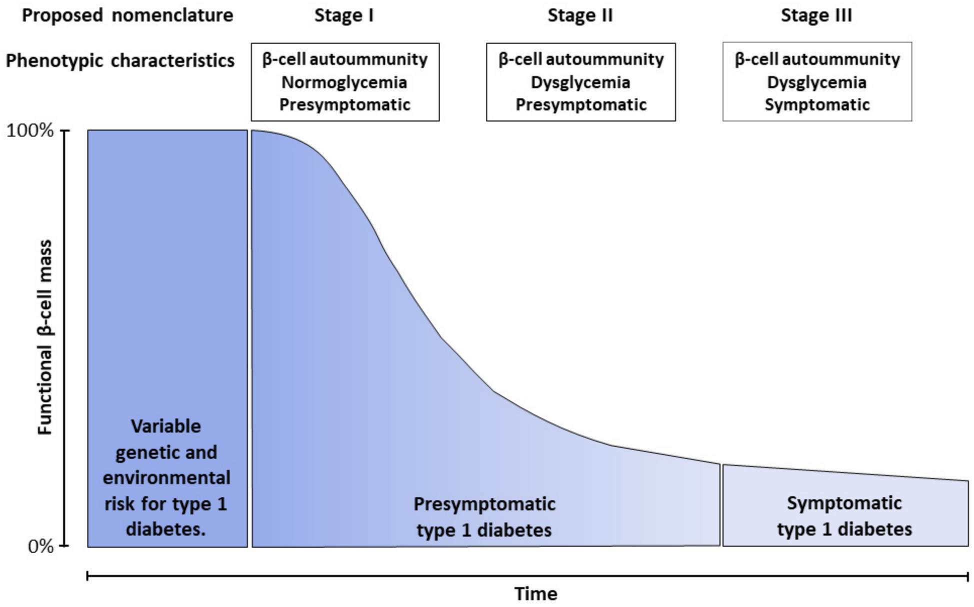

Proposed nomenclature
Stage I
Stage II
Stage III
Phenotypic characteristics
β-cell autoummunity
Normoglycemia
Presymptomatic
β-cell autoummunity
Dysglycemia
Presymptomatic
β-cell autoummunity
Dysglycemia
Symptomatic
100%
0%
Functional β-cell mass
Variable genetic and environmental risk for type 1 diabetes.
Presymptomatic type 1 diabetes
Symptomatic type 1 diabetes
Time

Figure 10. Staging of type 1 diabetes pathogenesis according to the 2015 statement of ADA, JDRF, and the Endocrine Society. Adapted from Insel et al.[82].

Stage 2

In this stage, subjects from Stage 1 have lost beta-cell function and have progressed to develop dysglycemia[82]. The subjects are still asymptomatic. An oral glucose tolerance test (OGTT) or continuous glucose monitoring at this stage often reveals rising glucose intolerance[85,86]. An approximate 75% risk of symptomatic disease in 5 years, and the lifetime risk approaches 100%[87].

Stage 3

At this stage, symptomatic disease arises due to loss of beta cell mass and function, resulting in insulin deficiency, dysglycemia, and symptoms of hyperglycemia. The clinical symptoms include polyuria, polydipsia, weight loss, fatigue, and in some ketoacidosis. Insulin treatment is now essential and lifelong[16,82].

The staging of type 1 diabetes allows earlier attempts at prevention and more efficient intermediate end points in clinical trials[87]. The rate of metabolic decompensation and ketoacidosis is significantly decreased during early diagnosis[88-90]. Further studies are necessary to address if patients and their families psychologically benefit from early diagnosis[91-93].

Diagnosis of type 1 diabetes

Recommendations (2021) for diagnosis of diabetes according to the WHO and the American Diabetes Association are fasting plasma glucose ≥7.0 mmol/L (≥126 mg/dL), where fasting is defined as no caloric intake for at least 8 h or oral glucose tolerance test (OGTT) resulting in plasma glucose ≥11.1 mmol/L (≥ 200 mg/dL) or HbA1c ≥48 mmol/mol (≥6.5%) or symptoms of hyperglycaemia of hyperglycaemic crisis combined with random plasma glucose ≥11.1 mmol/L (≥ 200 mg/dL)[94]. Beta-cell autoantibodies in combination with elevated blood glucose are necessary to properly classify type 1 diabetes[95]. The American Diabetes Association have updated the classification of type 1 diabetes to include the subclinical stages (Stage 1 and 2), and the clinical stage (Stage 3) already described above[82].

OGTT is a sensitive indicator of diabetes and early impaired glucose homeostasis and type 1 diabetes[96] and is commonly used in early diagnosis. The test should be performed using a glucose load containing anhydrous glucose (75 g or 1.75 g/kg up to 75 g for young children) dissolved in water. HbA1c has been proposed as an alternative criterion for diagnosis of diabetes and reflects an average of blood glucose concentrations over 90 days and should be performed in a laboratory setting. However, HbA1c is not an as good a measurement in children as it is in adults[94].

Prediction of type 1 diabetes

Screening for type 1 diabetes risk is important for selecting subjects to enrol in research studies. Many potential new-born and young children were previously missed when hereditary data from first-degree relatives were used to screen for type 1 diabetes risk. The majority, 85–90%, of newly diagnosed type 1 diabetes patients do not have a first-degree relative with the disease[97]. HLA genotyping is a cost-effective and fast screening process but misses a lot of information as only approximately 50% of genetic risk is conferred by HLA[98]. Although the highest risk of developing the first autoantibody and subsequently type 1 diabetes is conferred by HLA-DR3/4-DQ2/8, only 30% of all children that developing the disease have this genotype[45]. Next-generation sequencing for high-resolution HLA genotyping has recently been shown to identify 80% of individuals with a lifetime risk of type 1 diabetes, with 90% specificity and 90% sensitivity[99].

Children in follow-up studies have been hypothesized to be diagnosed with type 1 diabetes early in the clinical onset, often before symptoms arise and have been shown to have better metabolic control[64,94,100]. Studies have proven that it is possible, by using a risk score based on HLA and 30-40 SNPs, to identify children at very high risk of developing IAA and early type 1 diabetes[101,102]. Both risk scores were validated and reproduced in data from The Environmental Determinants of Diabetes in the Young (TEDDY) study. Children with no family history of type 1 diabetes who have the HLA DR3/DR4-DQ8 or HLA DR4/DR4-DQ8 genotype and a genetic risk score of >14.4 using the merged algorithm (corresponding to the upper 75th percentile of HLA DR3/DR4-DQ8 or HLA DR4/DR4-DQ8 TEDDY population) had a risk of 15.9% for developing beta-cell autoantibodies by age 5 years and 11.4% for developing multiple beta-cell autoantibodies by age 6 years.

Autoantibodies have been used repeatedly to study high-risk and general populations. Islet autoantibodies are the earliest sign of beta cell autoimmunity and significantly add accuracy to the prediction of type 1 diabetes. Although reversion of islet autoantibody positivity may occur, the risk of type 1 diabetes has increased from 0.4% to 14.5% in at-risk children that develop a single autoantibody[56,84]. The 10-year risk of at least 70% is conferred by developing of multiple autoantibodies[56]. Additionally, children with a higher number of autoantibodies have a significantly higher risk of developing type 1 diabetes[103-106]. Autoantibody titers have recently been investigated in attempts to create a risk score to increase the accuracy of predicting type 1 diabetes risk[107]. However, it is unclear what individual autoantibody titers would contribute[108]. Several studies have attempted to improve early type 1 diabetes prediction by using previously identified type 1 diabetes-related SNPs[109].

It is possible to predict progression to dysglycaemia and symptomatic type 1 diabetes by several factors related to glucose metabolism (such as an intravenous glucose tolerance test (IvGTT) or an OGTT). Loss of glucose tolerance occurs months before diagnosis[110].

Prevention of type 1 diabetes

Interventions can be targeted at three stages: before the development of autoimmunity (primary prevention) after autoimmunity is recognized (secondary prevention), or intervention after type 1 diabetes diagnosis when significant numbers of β cells remain (intervention).

Primary prevention studies are possible in children with high genetic risk, while subjects with an ongoing autoimmune process measured by islet autoimmunity can be included in secondary prevention studies. Secondary prevention studies aim to prevent the progression of islet destruction that will lead to type 1 diabetes. To carry out these studies, reliable prediction models are required. Current prediction models utilize combinations of HLA genotyping, autoantibodies, and measures of glucose tolerance to stratify risk.

Numerous studies have been performed to interrupt the type 1 diabetes process, both before and after a clinical diagnosis of type 1 diabetes, but there has been no successful treatment to prevent type 1 diabetes[111]. An immune tolerance treatment to re-achieving self-tolerance by administrating small doses of antigen under appropriate conditions could be one way to prevent disease as type 1 diabetes is associated with an autoimmune process with circulating beta-cell autoantibodies and lack of self-tolerance. In the TEDDY study, introducing probiotics early in life decreased the risk of islet autoimmunity compared with later probiotic supplementation or no probiotic supplementation[112].

After initiation of insulin treatment, there is a remission period of diabetes lasting weeks, months, and sometimes years after diagnosis. Reduced insulin requirements, increasing C-peptide concentrations, and relatively easily controlled blood glucose levels are believed to be the result of the recovery of β cells[113,114]. Changes in insulin sensitivity could play a role in the expression of the remission period, with decreased sensitivity at the time of diagnosis due to the hyperglycaemia with improvement after the establishment of metabolic control[115].

The ability to measure C-peptide concentrations in those receiving insulin therapy allows an accurate assessment of residual β cell function and can be used as a marker of the efficacy of therapeutic intervention[116]. Prolonging the honeymoon period can potentially to have significant beneficial effects in those with type 1 diabetes, as metabolic control is easier to establish in the presence of some residual insulin secretion. As a result, long-term diabetes-related complications are significantly less prevalent in those with residual insulin secretion. This was well demonstrated in the Diabetes Control and Complications Trial, in which those subjects with sustained C-peptide production were found to have rates of nephropathy, retinopathy, and hypoglycaemia that were half of those found in subjects without any residual insulin[117]. The population of newly diagnosed type 1 diabetes patients, therefore, also represents an important group for intervention. Therapies that can safely maintain endogenous insulin secretion in the longer term would represent an important clinical advance.

Aims of the thesis

This thesis investigates immunological markers and risk factors for type 1 diabetes pathogenesis.

The specific aims of this thesis were to:

- Investigate if there is an association between antigen-presenting cells' ability to present antigen to immune cells as peripheral blood cell HLA-DQ cell surface expression and HLA and autoantibodies. (Paper I)
- Investigate lipidomic profiles in adolescent subjects with a high-risk HLA genotype who have not progressed to type 1 diabetes, if lipid patterns could be tied to the HLA genotypes, and if there are any differences in plasma lipidomic composition in relation to several islet autoantibodies known to be associated with an increased risk for type 1 diabetes. (Paper II)
- Investigate SNPs for type 1 diabetes susceptibility loci using molecular inversion probe sequencing technology on samples from patients and controls from multiple large type 1 diabetes studies homozygous for the HLA-DR3 high-risk haplotype. (Paper III)
- Investigate HLA-DRA1 tri-SNP haplotypes as an additional risk element for type 1 diabetes relative to HLA-DQ cell surface expression on white blood cells, autoimmunity burden, and lipid profiles. (Paper IV)

Study populations

Study cohorts

DiPiS

In the Diabetes Prediction in Skåne (DiPiS) study, children with an increased genetic risk of type 1 diabetes were followed until 15 years old. The aim was to investigate the genetic and environmental factors that might contribute to or trigger the development of type 1 diabetes. This study cohort's subjects were included in papers I, II, and IV.

Between September 2000 and August 2004, 48,058 children were born in Skåne, the southernmost part of Sweden. Following oral consent from the mothers, umbilical cord blood samples (n = 35,683) were collected for screening of HLA and cord blood autoantibody analyses. The parents were asked to complete a questionnaire when their child was 2 months old to collect demographic data, family medical history of interest, and data in relation to events and illnesses from pregnancy until 2 years of age. Parents of 25,378 children completed the questionnaire and gave written consent to participate in the study. A basic risk score was constructed based on HLA-DQ genotype (DQ2/8, DQ2/2, DQ2/X, DQ8/8, DQ8/X or DQX/X (X is neither 2 nor 8)), FDR with insulin-dependent diabetes, autoantibodies detected in cord blood, high or low relative birth weight, and infection or gestational diabetes during pregnancy. Based on the risk score, 7,826 children were invited to participate in an annual follow-up with a questionnaire and blood sampling for islet autoantibodies. At the start of follow-up, 3,889 children were followed from 2 years of age. The DiPiS study timeline is outlined in Figure 11.

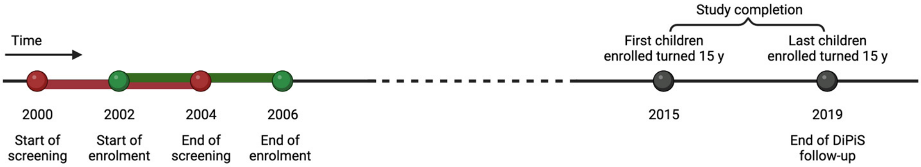


Figure 11. Timeline of key events in the Diabetes Prediction in Skåne (DiPiS) study.
Screening of high risk for type 1 diabetes was carried out from 2000 until 2004. Children with increased risk of type 1 diabetes were enrolled at 2 years old between 2002 and 2004 and followed either annually (negative or single autoantibody) or every 3 months (if multiple autoantibodies were detected at any earlier visit) until 15 years old or diagnosis of type 1 diabetes.

The follow-up of the children started at 2 years of age as very few were presumed to develop diabetes during the first years of life. At the annual follow-up visits, a blood sample for islet autoantibodies and a questionnaire covering the last year in the child's life was collected. Children who seroconverted to two or multiple islet autoantibodies were offered more intense follow-up every 3 months, which included islet autoantibodies, random plasma glucose, HbA1c, growth parameters, and a yearly OGTT. Children who developed type 1 diabetes during their participation in DiPiS dropped out of the study; many of them were diagnosed early in the disease progression. All samples are kept in a central repository at the study coordination centre in Malmö, Sweden.

The subjects included in our study had participated in annual to quarterly visits in the DiPiS follow-up, donating a blood sample at 10–15 years of age. Autoantibodies were analysed in blood samples from each visit. Autoantibody profiles for the subjects at birth and during follow-up are presented in Figure 12.

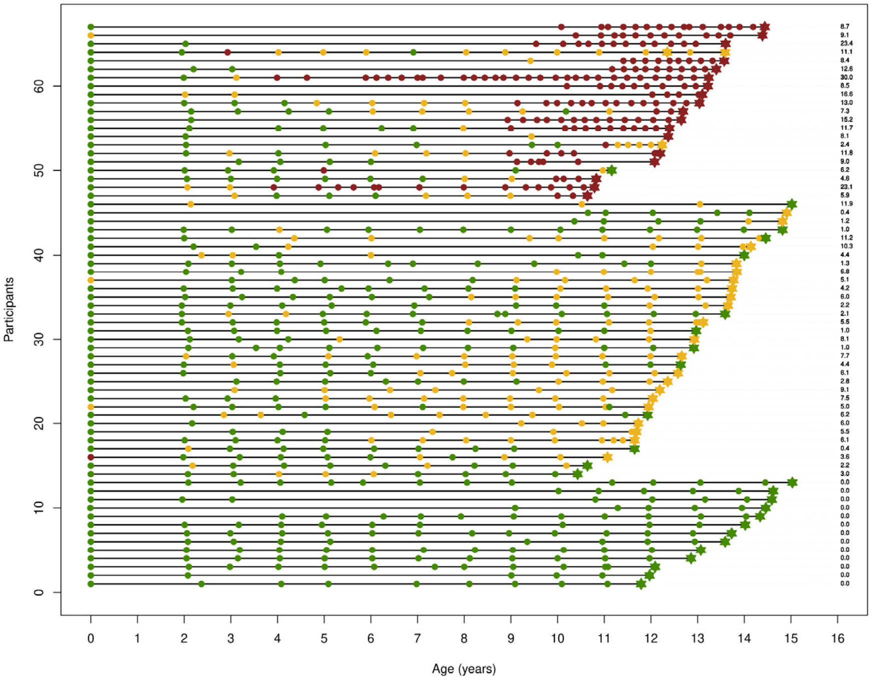


Figure 12. Timeline of the 67 subjects' participation in Diabetes Prediction in Skåne (DiPiS) and the sampling into our cross-sectional study cohort.
Autoantibody profiles of the n = 67 children in our study during follow-up as part of the DiPiS study and at the time of sampling into our study. Visits (circles for visits as part of DiPiS follow-up, stars for the time of sampling into our study) are coloured according to the autoantibody count (negative = green, single = yellow, multiple = red) at each visit. Autoantibodies measured were GADA, IA2A, IAA, and any of the three variants of ZnT8A against arginine, tryptophan, or glutamine at position 325 (R/W/Q, respectively). Autoimmunity burden was calculated as the area under the trajectory of autoantibodies over time is presented to the right.

TEDDY

The Environmental Determinants of Diabetes in the Young (TEDDY) study is a prospective cohort study that aims to identify environmental factors that influence the development of autoantibodies and type 1 diabetes in children. The study includes six clinical research centres, three in the US (Colorado, Georgia/Florida and Washington) and three in Europe (Finland, Germany and Sweden). Children screened but not enrolled in the TEDDY study were included in Paper III.

From September 2004 to February 2010, TEDDY screened 434,620 children, of whom 21,321 were eligible for follow-up. The screening was performed at TEDDY's six clinical centres. Eligibility was determined using separate genetic HLA-DQ criteria for children from the general population and children with an FDR with type 1 diabetes. Among the eligible children, 8676 children were enrolled in intensive follow-up. Follow-up started at 3 months of age and included site visits every 3 months until 4 years of age. After that, visits were performed every 6 months for autoantibody negative children and every 3 months for children with autoantibodies. A wide range of data is collected according to the study plan, including blood samples for PBMC, DNA/mRNA, HbA1c, and storage, urine, nasal swabs, nail clippings, tap water, salivary cortisol, accelerometer data, and body composition data, among others. Interviews were performed at each visit to account for infection, medication, immunization data, food records, negative life events, parental anxiety, depression, and physical activity[118]. All samples are kept in a central repository, and all data are kept in the study data coordinating centre in Tampa, Florida.

BDD (Better Diabetes Diagnosis)

The Better Diabetes Diagnosis (BDD) study is a nationwide project in Sweden that started in May 2005. The overall aim of the BDD study is to facilitate a precise classification of childhood diabetes. BDD also explores the heterogeneity of type 1 diabetes to enable adequate treatment and reduce risks of diabetes-related complications, and increase the knowledge of the aetiology of the disease. Between 2005 and 2010, the BDD study was performed within a research setting (BDD1). Since 2011, BDD has partly become a clinical routine in Sweden (BDD2)[119]. Subjects from the BDD1 study cohort were included in Paper III.

BDD1 includes children and adolescents (0–18 years old) with a diagnosis of diabetes according to the criteria of the American Diabetes Association[120,121]. In Sweden, diabetes teams in paediatric in-patient clinics care for these patients. All but two of the 42 paediatric clinics in Sweden participated in the study from the start in 2005, and since 2011, all the clinics have been included. All newly diagnosed patients, approximately 800 children and adolescents every year, and their caregivers are informed about the BDD study and are asked to give informed consent to participate in the study, and more than 99% of the patients give their consent.

At the clinical onset, blood samples are collected at admission to the hospital, in the majority of cases before insulin is given, and sent to the BDD laboratory for analyses of HLA-DQ genotype, autoantibodies against glutamate decarboxylase (GADA), insulin (IAA), insulinoma associated protein-2 (IA-2A), zinc transporter 8 (ZnT8A), and levels of serum C peptide (a by-product of insulin production). Results of these analyses are forwarded to the referring clinics. Blood samples from all patients are stored in the Region Skåne Biobank. Information on patient characteristics, ethnicity, heredity, and clinical symptoms at the onset of diabetes are recorded in the Swedish Paediatric Diabetes Register; Swediabkids. As a clinical routine, all children are followed longitudinally after diagnosis in the Swedish Paediatric Diabetes Register, Swediabkids (www.swediabkids.se) and the National Diabetes Register (NDR, https://www.ndr.nu/#/). Diagnosis of which type of diabetes is based on medical history, signs and symptoms at admission, and results of HLA profile, levels of autoantibodies, and C peptide. The initial diagnosis and classification are confirmed 1year after admission[119].

In BDD 1, all analyses were performed within a research setting. Analyses of HLA-DQ genotypes and autoantibodies were performed at the Clinical Research Centre (CRC), Malmö, Skåne University Hospital while analyses of C-peptide were performed at the Paediatric Research Laboratory, Linköping University. Results from analyses performed within BDD1 (i.e., HLA genotype, autoantibodies, and levels of C peptide) have shown benefits for correctly classifying diabetes in children and adolescents. Consequently, the National Diabetes Society in Sweden decided in December 2010 that many of the analyses performed within BDD1 were to be incorporated as part of the clinical routine in managing patients with new onset of diabetes. Thus, the BDD study continues (BDD2)[119].

T1DGC (Type 1 Diabetes Genetics Consortium)

The Type 1 Diabetes Genetics Consortium (T1DGC) was an international, multicenter program organized to promote research to identify genes and alleles that determining an individual's risk for type 1 diabetes. The goals of the T1DGC included identifying genomic regions and candidate genes whose variants modify an individual’s risk of type 1 diabetes and help explain the clustering of the disease in families, making research data available to the research community, and establishing resources that the research communitycan use[122].

The T1DGC assembled a resource of affected sib-pair families, parent-child trios, trio families from ethnic groups with a lower prevalence of type 1 diabetes, and case-control collections with banks of DNA, serum, plasma, and EBV-transformed cell lines. The desired ASP family structure was two affected siblings, both biological parents, and up to two unaffected siblings. The minimum family structure was two affected siblings. The eligibility criteria to participate in the T1DGC included: siblings with a diagnosis of type 1 diabetes, diagnosis before 35 years of age, use of insulin within 6 months of diagnosis, continuous use of insulin (without stopping for 6 months or more), and informed consent to collect blood, perform genetic analysis and exam (i.e., family history, other autoimmune diseases). Subjects from this study cohort were included in Paper III.

The T1DGC has assembled renewable genetic materials for family-based linkage and association studies and made research data available to the research community. Phenotype and genotype data from study participants have been used in research studies concerning the genetic origins of type 1 diabetes risk in families and the general population[122].

The 1000 Genomes Project

The 1000 Genomes Project was planned during a meeting at The Welcome Genome Campus in September 2007. The goal of the 1000 Genomes Project was to find common genetic variants with frequencies of at least 1% in the populations studied[123].

The 1000 Genomes Project took advantage of developments in sequencing technology, which sharply reduced the cost of sequencing. It was the first project to sequence the genomes of a large number of people to provide a comprehensive resource on human genetic variation. Data from the 1000 Genomes Project was quickly made available to the worldwide scientific community through freely accessible public databases[123]. Data from this study cohort was included in Paper III.

The 1000 Genomes Project has elucidated the properties and distribution of common and rare variations, provided insights into the processes that shape genetic diversity, and advanced an understanding of disease biology. This resource provides a benchmark for surveys of human genetic variation and constitutes a key component for human genetic studies by enabling array design, genotype imputation, cataloguing variants in regions of interest, and filtering of likely neutral variants[124].

Papers I, II, and IV

A subset of 67 healthy subjects from the DiPiS study cohort constitutes the study population in Paper I, II, and IV. From the subset of DiPiS children still under follow-up in 2015 and 2016, not diagnosed with type 1 diabetes, 98 children between ages 10 and 15 were selected randomly and asked to participate in a sub-study to DiPiS. The 67 subjects who agreed to participate donated 30–50 mL of peripheral blood. Because of random selection, autoantibody-negative children with high-risk HLA were overrepresented in this cohort, as children with high-risk HLA and single or multiple autoantibodies were likely to have already been diagnosed with type 1 diabetes. The study population was investigated by complete blood count, autoantibodies, and HLA high-resolution sequencing. The subjects were without or with type 1 diabetes-related autoantibodies, but all had increased genetic risk of type 1 diabetes.

Data for the follow-up in the DiPiS study was obtained from the DiPiS database in Malmö. The children have provided capillary blood samples in DiPiS, often from home, for autoantibody analyses at least four times during follow-up and as many as 33 times until the venous blood sample into this sub study to DiPiS. Blood glucose and HbA1c were available for all children in intense follow-up (2 or more autoantibodies simultaneously); the measurements were normal (random p-glucose < 11.1 mmol/L and HbA1c ranged between 27 and 40[94]), and these children would therefore be classified as stage 1 type 1 diabetes according to the current nomenclature[82]. After sampling into this study cohort, seven children developed type 1 diabetes. These children were diagnosed between 6 - 50 months after sampling.

Paper III

The study was planned to investigate TCR gene SNPs. The study cohort included samples from case (n = 365) subjects with type 1 diabetes and control (n = 668) subjects without diabetes, but all were DR3/3 homozygous. The T1DGC samples (case = 222, controls = 195) represent the DR3/3 homozygotes of European ancestry available from 16,000 samples[125,126]. All DiPiS (case = 0, controls = 3), TEDDY (case = 0, controls = 470), and BDD (case = 143, controls = 0) samples were obtained in Sweden. None of the children screened for the TEDDY study who provided DNA for this study were subsequently enrolled in TEDDY.

In addition, an *in silico* analysis was performed using data from the 1000 Genomes Project database. Expression of Class II HLA genes in defined tissues and cell types was extracted from published databases. Effects of each high-risk SNP allele on gene expression were identified using the database at https://www.gtexportal.org/. The 1000 Genomes Project[124] database was interrogated for subjects expressing a three-SNP risk haplotype discovered in this study. Class II gene expression data were collected from experiments using Epstein-Barr virus (EBV)-transformed lymphocytes available for 483 individuals in the 1000 Genomes Project.

Ethical considerations

The procedures performed in this thesis were following with the appropriate ethical standards of the institutional and/or national research committee.

The Regional Ethics Board in Lund approved the Diabetes Prediction in Skåne (DiPiS) study (Dnr 2009/244). The Regional Ethics Board in Lund approved amendments (Dnr 2014/196, 2015/861) to study children with increased genetic risk for type 1 diabetes, who were followed longitudinally since birth without or with the development of beta cell autoantibodies.

Children screened but not enrolled in the TEDDY study were included in Paper III. Thus no TEDDY data is included in this thesis. The TEDDY study was approved by local institutional review or ethics boards at each site (University of Washington, Seattle; University of Colorado; Medical College of Georgia, Augusta; University of South Florida, Tampa; University of Turku, Finland; Technische Universitat, Munich, Germany; Lund University, Malmö, Sweden) and is monitored by an External Evaluation Committee formed by the National Institutes of Health. In Sweden, the TEDDY study was approved by the Regional Ethics Board in Lund (Dnr 2004/217, 2017/667, 2019/04405).

The Ethics Committee at Karolinska Institutet approved the BDD study (Dnr 04-826/1 with amendments 2006/108-32/1 and 2007/1383-32/1, 2009/1684/32 and 2011/1069-32).

This thesis includes resources provided by the T1DGC, a collaborative clinical study sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Allergy and Infectious Diseases, National Human Genome Research Institute, National Institute of Child Health and Human Development, and JDRF and supported by grant U01 DK062418. All subjects have consented to the study. Furthermore, the analysis is carried out on de-identified samples.

This thesis also includes resources provided by the 1000 Genomes Project. The 1000 Genomes Project developed guidelines on ethical considerations for investigators doing sampling, outlined in an Informed Consent Background Document and an Informed Consent Form Template used for sample collection. The analysis is carried out on de-identified samples.

Methods

Complete blood count

Complete blood count (CBC) was utilized in Paper I to count white blood cells, red blood cells, platelets and the concentration of haemoglobin and haematocrit. CBC was performed using a multiparameter automated haematology analyser (CELL-DYN®, Ruby Haematology Analyser, Abbott Laboratories, Diagnostics Division, Abbot Park, IL, US)[127,128]. The analysis was performed using 300 µl of whole blood (Paper I). The basic principle of the analysis includes a laser light scatter analysis for enumeration and differentiation of white blood cells, red blood cells and platelets. Analysed parameters include white blood cells (count cells × 10^9/L), lymphocytes (count cells × 10^9/L), neutrophils (count cells × 10^9/L), monocytes (count cells × 10^9/L), basophils (count cells × 10^9/L), eosinophils (count cells × 10^9/L), red blood cells (count cells × 10^{12}/L), haemoglobin (g/L), haematocrit (the volume percentage of red blood cells in the blood (L/L)), mean corpuscular volume (the average volume of red cells (fL), mean corpuscular haemoglobin (the average amount of haemoglobin per red blood cell (pg)), red blood cell distribution width (% coefficient of variation), platelets (10^9/L), and mean platelet volume (fL).

Magnetic-activated cell separation

In Papers I and IV, magnetic associated cell separation (MACS) was used to isolate six peripheral blood cell types from a blood sample obtained from the 67 DiPiS subjects. A thorough description of the cell isolation can be found in Paper I and IV; a brief description is given below.

MACS is based on antibodies coupled to magnetic beads, and there are multiple cell separation strategies using MACS. Here, positive cell selection targeting cell isolation based on one or multiple markers was utilized to isolate $CD16^+CD66^+$ neutrophils, $CD19^+$ B lymphocytes, $CD16^+$ cells, $CD14^+CD16^-$ monocytes, and $CD4^+$ and $CD8^+$ T lymphocytes. The antibody/bead complex binds to specific cells expressing the corresponding cluster of differentiation (CD) epitope. Magnetically labelled cells are retained when the cell suspension is placed into a magnetic field, magnetically labelled cells are retained, while unlabelled cells can be removed (Figure 13). The sample is removed from the magnetic field to recover labelled cells.

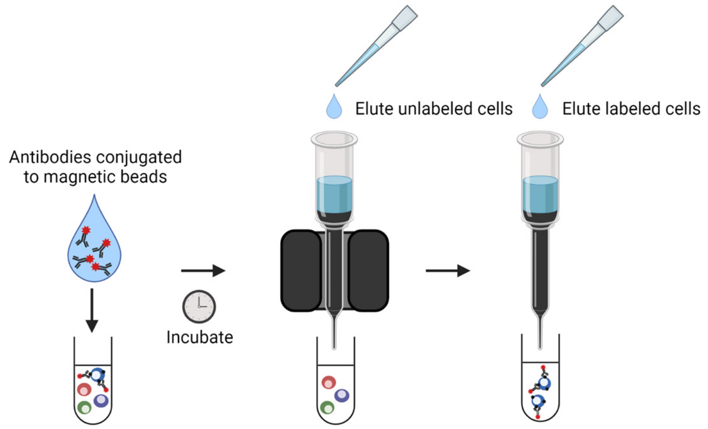


Figure 13. Magnetic-activated cell separation (MACS) by positive selection of a target cell type. Positive selection means that a particular target cell type is magnetically labelled. Specific MACS MicroBeads with a magnetic core are added, washed, and pelleted PBMC resuspended in small aliquots of the buffer. The mixture is incubated for a short while. A MACS column containing a matrix of superparamagnetic spheres, covered with a cell-friendly coating, is placed in a MACS Separator where the spheres amplify the magnetic field by 10,000-fold. During separation, the column is placed in the magnetic field of the MACS Separator. Magnetically labelled cells are retained within the column, whereas unlabelled cells flow through. After a washing step, the column is removed from the magnetic field, and the target cells are eluted from the column. Specific MACS MicroBeads are available to select numerous cell types and cell subsets positively. The image was created using BioRender.com.

Flow cytometry

In Papers I and IV, flow cytometry was utilized to examine HLA-DQ cell surface expression on six peripheral white blood cells, previously isolated by MACS. The principle for flow cytometry is outlined as follows (Figure 14). Traditional flow cytometers consist of three systems: fluidics, optics, and electronics. Flow cytometers utilize lasers as light sources to produce both scattered and fluorescent light signals that detectors, such as photodiodes or photomultiplier tubes, read. Cells are stained using monoclonal antibodies conjugated to fluorochromes.

The monoclonal antibodies are conjugated to specific clusters of differentiation (CD), proteins expressed on the cell surface, and are conjugated to fluorophores. The fluorochromes emit light of different wavelengths upon excitation by a specific laser. Flow cytometers rapidly analyse single cells or particles as they flow past single or multiple lasers while suspended in a buffered salt-based solution. Each particle is analysed for visible light scatter and one or multiple fluorescence parameters. Visible light scatter is measured in two directions, the forward direction (Forward Scatter or FSC), which can indicate the relative size of the cell and at 90°

(Side Scatter or SSC), which indicates the internal complexity or granularity of the cell. Light scatters independently of fluorescence. These signals are converted into electronic signals analysed by a computer and written to a standardized format (.fcs) data file. Cell populations can be analysed and/or purified based on their fluorescent or light scattering characteristics. Common characteristics measured in a flow cytometry experiment are cell size, relative granularity, and relative fluorescence.

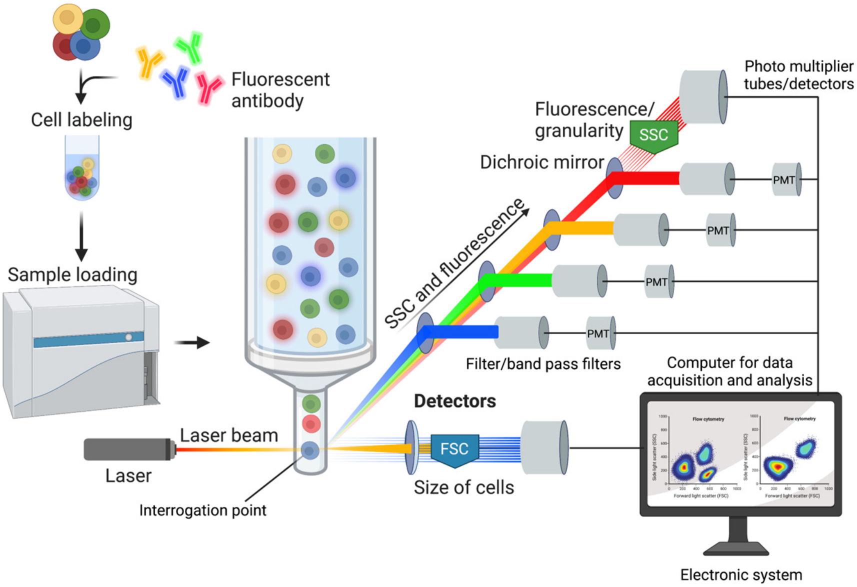

Cell labeling
Fluorescent antibody
Sample loading
Laser beam
Laser
Interrogation point
SSC and fluorescence
Dichroic mirror
Fluorescence/ granularity
SSC
Photo multiplier tubes/detectors
PMT
PMT
PMT
PMT
Filter/band pass filters
Detectors
FSC
Size of cells
Computer for data acquisition and analysis
Flow cytometry
Side light scatter (SSC)
Forward light scatter (FSC)
0
200
400
600
800
1000
Flow cytometry
Side light scatter (SSC)
Forward light scatter (FSC)
Electronic system

Figure 14. Schematics of the mechanisms of a flow cytometer.
A target cell type within a mixture of cells is fluorescently labelled. The cell mixture leaves the nozzle in droplets, and a laser beam strikes each droplet. FSC detector identifies cell size. SCC detector identifies fluorescence/granularity. Bandpass filters are positioned in front of the detectors and determine what collection of wavelengths and, ultimately, which fluorophores will be measured by each detector. The image was created using BioRender.com.

A thorough description of the monoclonal antibodies and the gating strategy can be found in papers I and IV; a brief description will follow. The isolated peripheral blood cell subsets were stained with titrated monoclonal antibodies and phenotyped by their CD markers using a CyAnADP® (Beckman Coulter, Brea, California, USA) flow cytometer with the Summit v4.3 software (DAKO, Copenhagen, Denmark). Quality control of the flow cytometer was performed once per week. Compensation beads were utilized to calibrate the flow cytometer before each run. The purpose of the calibration is to compensate for "spillover" when staining with multiple fluorescent dyes and emitted light from different fluorochromes overlap. At excitation, each fluorophore may emit light with higher wavelengths than own emission spectrum, which, to some extent, may overlap with other fluorochrome spectrums. A compensation process is performed by eliminating spectral overlap between different channels for a specific fluorophore by mathematic deconvolution programmed in the flow cytometer.

Acquired data were analysed with Kaluza Analysis Software 1.5a (Beckman Coulter) with a gating strategy adapted from Dang et.al[129] (Figure 15). Unstained and unsorted PBMC or erythrocyte pellets were used as negative controls. Stained and unstained PBMC and stained and unstained erythrocytes were used as positive and negative controls in the flow analyses for each subject. First, fluorescent minus one (FMO) controls were used for more accurate gating when multiple monoclonal antibodies were used to stain a cell type. In addition to the unstained cell negative gate, FMO could help to distinguish the real negative from the positive population by staining all the fluorophores minus the one in question. FMO controls are useful to put the correct gate when the expression level of a specific marker is low and to exclude background signals from spectral overlap. Second, duplicate events were removed using forward scatter height (FSC-H) plotted against forward scatter area (FSC-A). Third, cell populations were identified in the initial gate using side scatter (SSC) and forward scatter (FSC). The cell populations were plotted in separate histograms using the appropriate cell specific for HLA-DQ antibody for identification of cells and HLA-DQ median fluorescence intensity (MFI), respectively.

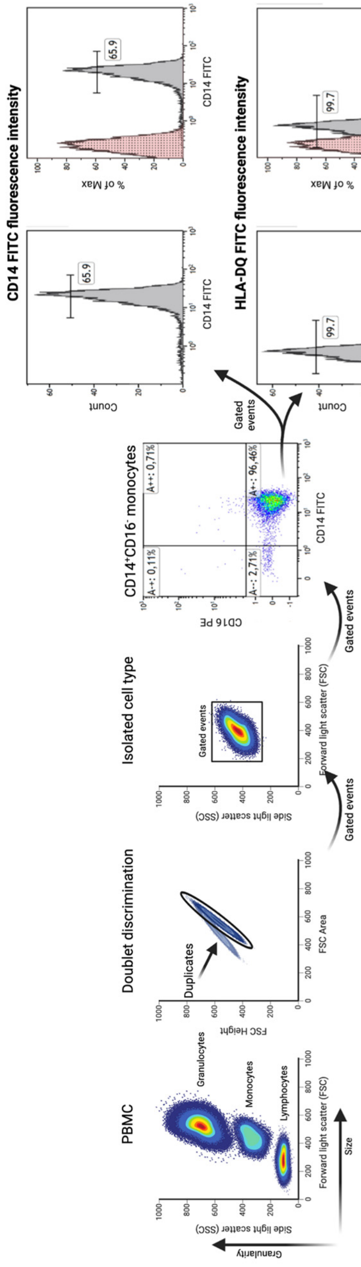


Figure 15. Schematic of the flow cytometry gating strategy

Duplicate events were removed using scatterplot of FS Area and FS Height. Isolated cell populations were identified in an initiall gate using forward scatter (FSC) and side scatter (SSC). Up to 10 000 events were recorded. The cell population was plotted in a histogram using the appropriate cell specific antibodies. Fluorescence intensity was identified in a histogram using an appropriate cell specific or HLA-DQ antibody. Unstained and not sorted PBMC or erythrocyte pellet were used as negative controls. The image was created using BioRender.com.

HLA high-resolution sequencing

In papers I, II, and IV, HLA high-resolution sequencing, a type of next-generation sequencing (NGS), was used to determine Class II *(-DRB3, -DRB4, -DRB5, -DRB1, -DQA1, -DQB1, -DPA1, and -DPB1)* haplotypes. The principle of HLA NGS can be divided into target generation, library preparation, clonal amplification, sequencing, and data analysis. **Target generation** includes PCR amplification of specific HLA loci to generate and obtain a larger amount of target DNA. Longer amplicons are obtained, and DNA concentration is quantified. **Library preparation** includes three vital steps: DNA fragmentation, adapter ligation, and barcoding. DNA fragmentation is a random process which guarantees that the complete gene is encompassed by shorter overlapping sequencing reads. Enzymatic cleavage is followed by DNA repair and A-tailing in a joint reaction. The goal of adapter ligation is to add primers to the end of the DNA fragments with A-overhang; adapters come with T-overhang to enable ligation. Illumina sequencing primers later use the sequences of adapters for NGS. Following the adapter, ligation is a step that involves DNA cleaning and size selection. This step removes components which could eventually interfere with sequencing.

Selection with SPR1 (Solid Phase Reversible Immobilization) beads is aimed at sorting larger fragments, which is preferable in the later process of phasing. Barcoding enables samples from different individuals to be pooled and run in a single sequencing analysis. Indexing PCR is performed as each fragment is elongated to contain individual and flow cell attachment cites. The resulting library-pooled sample includes DNA from several individuals and loci. **Clonal amplification** is where DNA is amplified to increase the amount of DNA available for sequencing. Clonal amplification is performed within the sequencer through bridge amplification resulting in millions of clusters, each including copies of the same DNA fragment. **Sequencing** of DNA molecules generates reads. A principle of sequencing by synthesis is conducted. Fluorescently labelled nucleotide bases are incorporated for detection. A large number of short reads are obtained. The short reads overlap so that each nucleotide is covered multiple times. **Data analysis** is performed by software phasing short reads and obtaining longer sequences. Statistical computations are used to phase the complete sequences together. An advantage of using this technology for short reads is that it has a lower error rate than methods using long reads. A library of previously known sequences is used as a reference to yield HLA typing results for the newly included samples.

Dried blood spots (6-mm punch-outs) were sent blinded to Scisco Genetics Systems and analysed with a ScisGo HLA v4 typing kit (Scisco Genetics Inc., Seattle, WA, US) using MiSeq v2 PE500 (Illumina, San Diego, CA, US)[83,130,131]. DNA was extracted from the 6-mm dried blood spots using a scalable protocol[132]. NGS was performed using DNA sequencing (Sequence-Based Typing). HLA-*DRB3, -DRB4, -DRB5,-DRB1, -DQA1, -DQB1, -DPA1,* and *-DPB1* haplotypes were determined from the allelic information, an online database (Allele Frequencies in WorldwidePopulation, http://www.allelefrequencies.net)[133].

Radio-binding assay

In papers I, II, and IV, IAA, GADA, IA-2A, and the three variants ZnT8A were analysed by radio-binding assay (RBA). RBA is a method of detecting and quantifying antibodies targeted toward a specific antigen. A thorough description of the autoantibody analysis by RBA can be found in paper I, II, and IV; a brief description will follow.

Coupled in vitro transcription and translation replicate the process of protein synthesis from a template DNA to synthesise a radiolabelled protein (antigen) (Figure 16). Radiolabelled ^{35}S-methionine is incorporated during protein synthesis resulting in radiolabelled antigen for the detection of GADA, IA-2A or the three variants of ZnT8A, depending on the plasmid template. The specific antigen is later used in RBA for the detection of autoantibodies against the specific antigen in serum and plasma (Figure 17). In this thesis, we used ready-made ^{125}I-insulin antigen in the detection of IAA developed by PerkinElmer (Massachusetts, USA).

In RBA, duplicate samples of serum or plasma are incubated at 4°C overnight with a radiolabelled antigen. Free labelled antigen is separated from antibody bound by Protein A-Sepharose (Invitrogen, Carlsbad, California, USA). Bound radioactivity was determined in a β-counter (2450 Microplate Counter, PerkinElmer, Waltham, Massachusetts, USA) and levels expressed in U/mL using in-house standards. Levels (Units/mL) of GADA, IAA, IA-2A, and the three variants of ZnT8A were determined, using in-house verified threshold values, in serum or plasma as previously described[65,134-136].

Our laboratory participates in the Islet Autoantibody Standardization Program (IASP), which aims to improve the performance of immunoassays measuring type 1 diabetes-associated autoantibodies and the concordance among laboratories. IASP organizes international interlaboratory assay comparison studies in which blinded serum samples are distributed to participating laboratories, followed by centralized collection and analysis of results, providing participants with an unbiased comparative assessment. IASP is performed in 18-month intervals. The 2018 analysis was close to the autoantibody analyses performed in the papers included in this thesis.

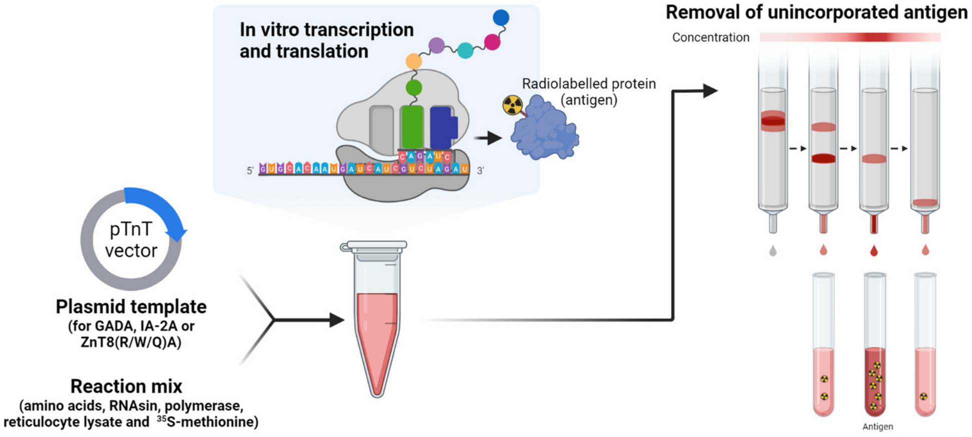

In vitro transcription and translation
5'
3'
Radiolabelled protein (antigen)
Removal of unincorporated antigen
Concentration
pTnT vector
Plasmid template
(for GADA, IA-2A or ZnT8(R/W/Q)A)
Reaction mix
(amino acids, RNAsin, polymerase, reticulocyte lysate and ³⁵S-methionine)
Antigen

Figure 16. Coupled in vitro transcription and translation.
In vitro transcription and translation replicate the process of protein synthesis from a template DNA to synthesise radiolabelled protein (antigen). The process can be summarized in two steps: 1) transcription of cDNA template into mRNA and 2) translation of mRNA into ^{35}S-labelled protein. Unincorporated antigen is removed using a purification size exclusion column. The purified antigen is later used in radio-binding assays for the detection of autoantibodies against the specific antigen in serum and plasma. The image was created using BioRender.com.

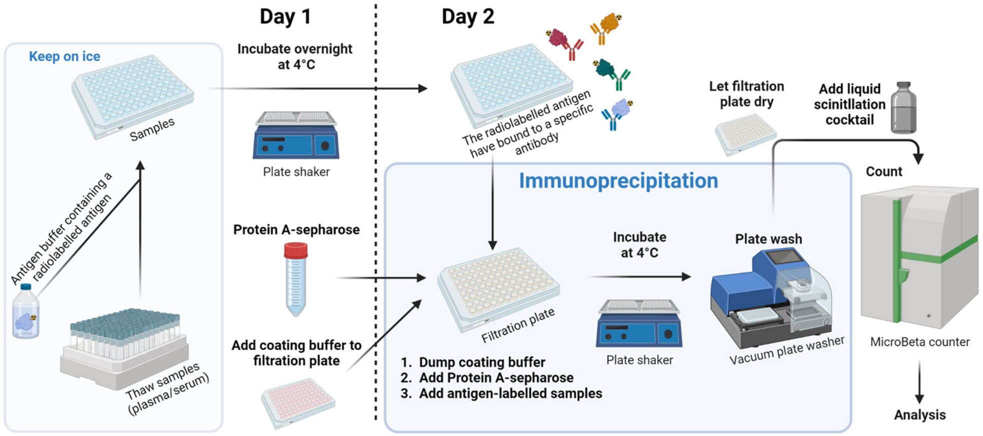

Day 1
Day 2
Keep on ice
Samples
Antigen buffer containing a radiolabelled antigen
Thaw samples (plasma/serum)
Incubate overnight at 4°C
Plate shaker
Protein A-sepharose
Add coating buffer to filtration plate
The radiolabelled antigen have bound to a specific antibody
Let filtration plate dry
Add liquid scinitllation cocktail
Count
Immunoprecipitation
Filtration plate
1. Dump coating buffer
2. Add Protein A-sepharose
3. Add antigen-labelled samples
Incubate at 4°C
Plate shaker
Plate wash
Vacuum plate washer
MicroBeta counter
Analysis

Figure 17. Radio-binding assay (RBA) is performed to detect autoantibodies against a specific antigen.
Serum or plasma samples are incubated with radiolabelled antigen overnight in a 96-well plate format, with each serum tested in duplicate. Antibody-bound antigen is precipitated with Protein A Sepharose. Unbound antigen is removed by washing. The radioactivity of antibody bound antigen is counted in a ß-counter. The image was created using BioRender.com.

Lipidomics

In Paper II, a lipidomics approach was used to analyse lipid profiles in plasma originating from whole blood samples. A thorough description of the lipidomics analysis can be found in Paper II; a brief description is given below.

Lipidomics is the study of the structure and function of the complete lipids (the lipidome) produced in a cell or organism and their interactions with other lipids, proteins, and metabolites. Lipidomic profiles were determined using ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS). The two techniques, UHPLC and Q-TOF-MS, have been combined to employ fast, high-resolution separations with required sensitivity (UHPLC) and structure elucidation and identification of fragmentation patterns of the compounds (Q-TOF-MS) (Figure 18).

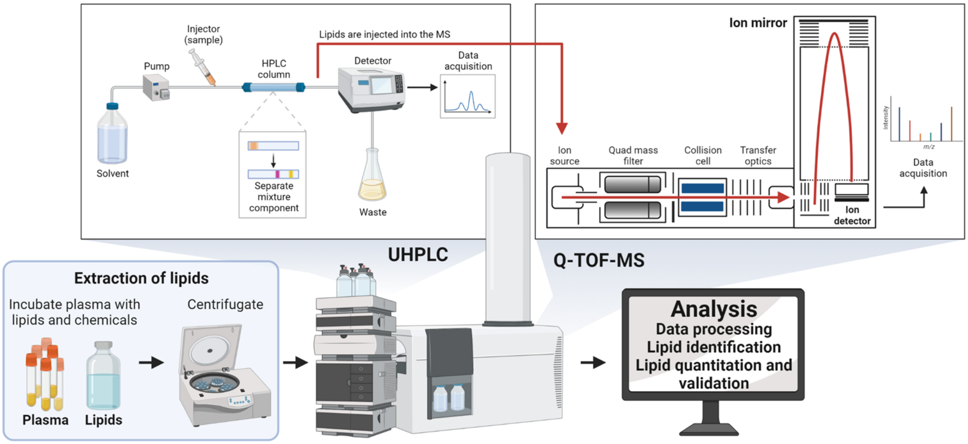

Injector (sample)
Pump
HPLC column
Lipids are injected into the MS
Detector
Data acquisition
Solvent
Separate mixture component
Waste
Ion mirror
Ion source
Quad mass filter
Collision cell
Transfer optics
Intensity
m/z
Data acquisition
Ion detector
UHPLC
Q-TOF-MS
Extraction of lipids
Incubate plasma with lipids and chemicals
Centrifugate
Plasma
Lipids
Analysis
Data processing
Lipid identification
Lipid quantitation and validation

Figure 18. Schematic of ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometer (UHPLC-Q-TOF-MS).
UHPLC contribute fast separation, high resolution, and high sensitivity while Q-TOF-MS contribute accurate mass, reliable fragmentation, structure elucidation. After HPLC separation, the lipids are injected into the Q-TOF-MS where they are further separated by size and charge that leads to the development of the chromatogram. The image was created using BioRender.com.

UHPLC is a commonly used separation method using liquid chromatography (LC). LC is a technique in analytical chemistry that separates components in a mixture. The separation occurs due to the selective distribution of analytes between a mobile and a stationary phase. In LC, the mobile phase is the liquid, and the stationary phase is the separation column with a granular material with very small porous particles. The mobile phase is a solvent or solvent mixture forced at high pressure through the separation column and the rest of the system. The separation rate depends on the affinity of the analytes to the stationary phase. A sample injected into a column with the mobile phase is dissolved. Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase. After leaving the column, the individual substances are detected by a detector and passed on as a signal to the software on the computer. A chromatogram is created in the software, allowing the identification and quantification of the different substances.

Q-TOF mass spectrometers combine quadrupole technologies with a TOF mass analyser, resulting in high mass accuracy for precursor and product ions, strong quantitation capability, and fragmentation experiment applicability. The Q-TOF MS uses a quadrupole (four parallel rods arranged in a square formation), a collision cell, and a time-of-flight unit to produce spectra. Lighter ions accelerate faster down the flight tube to the detector, thus determining the ions' mass-to-charge ratios.

Plasma samples originating from whole blood samples diluted 1:2 in RPMI1640 media before isolation of plasma were sent blinded to Steno Diabetes Centre Copenhagen in Gentofte, Denmark. Lipidomic profiling Lipid extracts were analysed using an ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometer (UHPLC-Q-TOF-MS). The column was an Acquity UPLC™ BEH C_{18} 2.1 × 100 mm with 1.7 µm particles from Waters (Milford, CT, USA). The mass spectrometer was a 6550 iFunnel quadrupole time of flight from Agilent Technologies (Agilent) interfaced with a dual jet stream electrospray ion source. Data were acquired using the MassHunters B.06.01 (Agilent). The open-source software processing tool MZmine 2.21 was used to process the data obtained from the lipidomic analysis. Features in the spectra were annotated based on the internal spectral library and the LipidMaps online database.

Molecular Inversion Probe Sequencing

Molecular inversion probe (MIP) sequencing is a method for assessing genetic information in many loci. It is cost-effective, scalable, and an efficient technology for large-scale SNP analysis. MIPs are single-stranded DNA molecules containing two regions complementary to regions in the target DNA that flank the SNP in question. Each probe contains universal primers sequences separated by an endoribonuclease recognition site and a 20-nt tag sequence. During the assay, the probes undergo a unimolecular rearrangement: they are (1) circularized by filling gaps with nucleotides corresponding to the SNP in four separate allele-specific polymerizations (A, C, G, and T) and ligation reactions; and (2) linearized in the enzymatic reaction. As a result, they become "inverted"(Figure 19). This step is followed by PCR amplification. Further processing of the probes depends on specific assay[137,138]. MIP permits identifying and quantifying SNPs in all genes and loci targeted for analysis[139]. In Paper III, MIP capture was used to sequence regions of interest in all samples[140,141]. Variant frequency was enhanced by incorporating of unique molecular identifiers within each molecule captured by a MIP[142]. This paper was a collaborative effort, and the MIP sequencing and analysis was performed by Özkan Aydemir and Jeffrey A. Bailey.

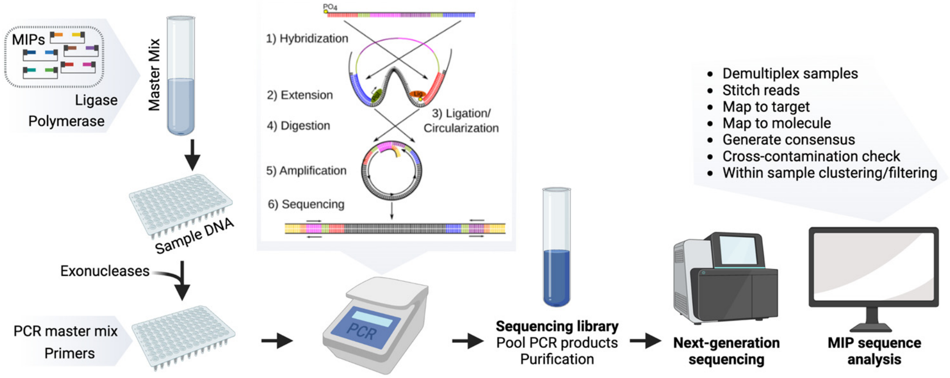

MIPs
Ligase
Polymerase
Master Mix
Sample DNA
Exonucleases
PCR master mix
Primers
PO4
1) Hybridization
2) Extension
3) Ligation/ Circularization
4) Digestion
5) Amplification
6) Sequencing
PCR
Sequencing library
Pool PCR products
Purification
Next-generation sequencing
MIP sequence analysis
Demultiplex samples
Stitch reads
Map to target
Map to molecule
Generate consensus
Cross-contamination check
Within sample clustering/filtering

Figure 19. Molecular inversion probe (MIP) library sequencing
The MIP assay include a capture carried as a single reaction per MIP panel per sample by combining sample DNA, MIP panel, polymerase and ligase. MIPs bind to their targets, followed by polymerase extension and single stranded circular formation by ligase. Exonuclease treatment remove all remaining linear DNA (unbound probes, original template DNA). Captured products are amplified using universal primers with sample barcodes. Each probe have important components such as the extension arm (blue), ligation arm (red), molecular identifiers (green), and a shared backbone complementary to the target region (pink and purple). Barcoded samples are pooled to create a sequencing library that is purified before sequencing. Important components are color coded: extension arm (blue), ligation arm (red) molecular identifiers (green), and backbone (pink or purple). The image is adapted from Aydemir et. al.[140] using BioRender.com.

SNP Genotyping

SNP genotyping measures genetic variations of single nucleotide polymorphisms (SNPs) between species members. It is a form of genotyping that measures more general genetic variation. SNPs are one of the most common types of genetic variation. An SNP is a single base pair mutation at a specific locus. SNP genotyping was performed in papers III and IV.

In Paper IV, SNP genotyping was used to investigate the genetic variation of SNPs in the cohort of 67 DiPiS subjects. A thorough description of the cell isolation can be found in Paper III; a brief description is given below.

Genotyping of HLA-DRA1 SNPs was performed using DNA isolated from previously obtained PBMC[143]. Polymorphisms of three SNPs (tri-SNP) in intron 1 of the HLA-DRA1 gene were investigated with predesigned TaqMan® SNP Genotyping Assays using the dried-down DNA delivery method, as described in the TaqMan® SNP Genotyping Assays User Guide (Thermo Fisher Scientific) (Figure 20). The dried-down DNA delivery method can be used with low DNA concentration, a limited number of DNA templates repeatedly tested on different SNP targets or when many DNA samples are prepared in plates, dried down, and stored before use. The workflow of SNP genotyping includes the preparation of a reaction mixture, performing the PCR, setting up plate documentation, performing a post-PCR plate read, analysing the plate, reading the document, and calling allele types.

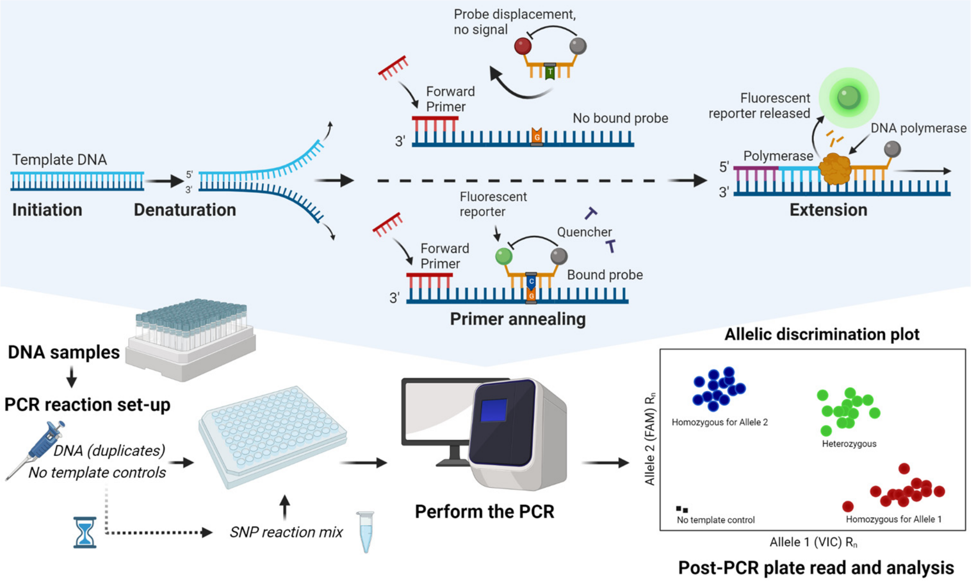

Probe displacement, no signal
T
Forward Primer
3'
G
No bound probe
Template DNA
5'
3'
Initiation
Denaturation
Fluorescent reporter
Quencher
Forward Primer
C
G
Bound probe
3'
Primer annealing
Fluorescent reporter released
DNA polymerase
Polymerase
5'
3'
Extension
DNA samples
PCR reaction set-up
DNA (duplicates)
No template controls
SNP reaction mix
Perform the PCR
Allelic discrimination plot
Allele 2 (FAM) Rn
Homozygous for Allele 2
Heterozygous
No template control
Homozygous for Allele 1
Allele 1 (VIC) Rn
Post-PCR plate read and analysis

Figure 20. SNP genotyping.
Extract and purify genomic DNA, Quantitate gDNA (Concentration, Equalize sample concentration), set up PCR reactions (Add gDNA to the bottom surface of an optical reaction plate duplicate samples no template controls). Add gDNA to the bottom surface of an optical reaction plate duplicate samples no template controls. DNA sample is dried down completely by evaporation dark room temperature amplicon-free location. DNA dry-down method is appropriate when• low DNA concentration results in large sample volumes (2 to 5 µL) to run the assay. limited number of DNA templates tested repeatedly on different SNP targets. large number of DNA samples prepared in plates, dried down, and stored before use. The image was created using BioRender.com.

The reaction mixture containing the assay was added to dried-down DNA samples in a 96-well plate. In the polymerase chain reaction (PCR), the target DNA region determined by the assays is amplified and copied. During the post-PCR plate read, fluorescence measurements collected during the PCR are used to plot the reporter signal, and this data is used to determine the genotypes present in the DNA samples. Each of the three genotyping assays consists of two sequence-specific primers and two TaqMan® minor groove binder (MGB) probes with non-fluorescent quenchers (NFQ). Allele 1 and 2 sequences are detected by one probe. each labelled with VIC and FAM dye, respectively. The software automatically assigned the SNP transition substitutions for each of the assays were automatically assigned by the software as Homozygous Allele 1, Homozygous Allele 2, or Heterozygous Allele 1/Allele 1 2.

The context sequence [VIC/FAM], provided by the manufacturer, identified the transition substitution for rs3135394, rs9268645, and rs3129877 as [A/G], [C/G], and [A/G] respectively. Hence, the SNP typing results were decoded to the corresponding polymorphism. Haplotypes of the SNPs, a tri-SNP (rs3135394, rs9268645 and rs3129877), were assembled in association with HLA-DRB345-DRB1-DQA1-DQB1 haplotypes.

In Silico Class II HLA Gene Expression

In silico gene expression analysis is an experiment performed on a computer or via computer simulation. With this technique, it is possible to profile gene expression for studying biological processes at the molecular level as most biological distinctions are now observed at a genomic level, and a large amount of expression information is now openly available via public databases[144,145]. In Paper III, the expression of Class II HLA genes in defined tissues and cell types was extracted from published databases. Effects of each high-risk SNP allele on gene expression were identified using the database at https://www.gtexportal.org/. The 1000 Genomes Project[124] database was also interrogated for individuals expressing the three-SNP risk haplotype we discovered (see below) using four populations (CEU, FIN, GBR, and TSI) that reflect the ancestral origins of our type 1 diabetes cohort. In addition, Class II gene expression data were also collected from experiments using Epstein-Barr virus (EBV)-transformed lymphocytes available for 483 individuals in the 1000 Genomes Project. Class II gene expression was then stratified according to tri-SNPs of interest. This paper was a collaborative effort, and the *in silico* analyses were performed by Elizabeth P. Blankenhorn and John P. Mordes.

Data management

R is a free software environment for statistical computing and graphics[146]. Creating a database in R is essentially writing a script specifying what will happen to the information loaded into the software. R never automatically overwrites or changes the original data. Each time R is opened, the data is loaded anew. In the script, you can change the data without altering the raw data file, create tables and plots, and model data. The most important aspect is the traceability of the analyses. With the script, it is possible to know exactly what has been done regarding data handling and analyses. Thus, it is easier to backtrack and troubleshoot than with other software types. Data can be saved in many different formats enabling you to send and receive data to and from collaborators using different software.

R statistics have been used to create, update, and maintain “the 67 DiPiS subject cohort” database used in papers I, II, and IV. Descriptive data along with DiPiS follow-up data was obtained from the DiPiS study database. Data generated with each method (complete blood count, magnetic-activated cell separation, flow cytometry, HLA high-resolution sequencing, lipidomics and SNP genotyping) was added to the database. The software was also used to create plots and tables and perform statistical computations in Paper I and IV.

Generating and managing lipidomics data (Paper II), MIP data (Paper III) as well as obtaining *In silico* gene expression data (Paper III) were managed by co-authors in the respective published papers.

Statistical methods

Autoimmunity burden at sampling (sAB) and cumulative autoimmunity burden (cAB) were used to group subjects in paper I, II, and IV. sAB is defined as the number of autoantibodies at the time of sampling, and cAB is defined as the number of autoantibodies over a period of time and is calculated as the sum of the products of the number of autoantibodies by the period of time (in years) the subject had that number of autoantibodies. In the DiPiS study sAB was the number of autoantibodies at the time of sampling of the 67 DiPiS subjects, and cAB was the sum of the products of the number of autoantibodies and time over the entire DiPiS follow-up from 2 years of age until the time of sampling.

Boxplots and likelihood ratio tests were used to examine and test, respectively, the associations between a parameter of interest and autoimmunity burden level or group the associations between a parameter in groups or with varying autoimmunity burden (Paper I and IV). Histograms were used to assess the distribution and identify potential outliers in different parameters (Paper I and IV).

In papers I and IV, linear mixed-effects models (LMEM) were used to examine the association between HLA-DQ cell surface MFI and autoimmunity burden. LMEMs are used when independence between data points cannot be assumed. One example of when this occurs is when more than one data point was collected from the same individual, as was the case in our study. We fit linear models with HLA-DQ as the outcome, autoimmunity burden (or tri-SNP haplotypes), as the main predictor, with a random intercept. The models were adjusted for age at sampling, sex, HLA-DQ2/8 (Paper I and IV) as well as CBC (Paper I). To determine whether autoimmunity was a mediator of the association between the outcome and predictor, additional models were fitted adjusting for autoimmunity burden (sAB and cAB). Due to the small sample size, the standard errors were estimated using robust methods, as well as model based as a sensitivity analysis. Robust standard errors adjust the model-based standard errors using the empirical variability of the model residuals that are the difference between observed outcome and the outcome predicted by the statistical model[147]. For example, in estimating the mean difference between two groups, the residuals are the difference between the observed outcome and the mean in each group. A sensitivity analysis is an approach used to determine how sensitive the results are to the modelling assumptions.

Lipidomics data were analysed according to genotype. Hierarchical clustering was used to cluster subjects based on their HLA genotype. First, a dissimilarity matrix was calculated by computing all pairwise dissimilarities (distances) between the individual data points using “Gower’s distance” as the distance metric. The subjects were clustered using agglomerative hierarchical clustering using the complete linkage method. Agglomerative clustering initially starts with n clusters, where n is the number of observations, assuming each is its own separate cluster. Then, the algorithm identifies the most similar clusters using the complete linkage method and groups them into larger clusters. This process was repeated until four clusters were identified, and cluster statistics were assessed.

In papers I, III and IV, statistical analyses were performed using R[146] (versions 3.6.1 and 4.2.1). Statistical analyses and hierarchical cluster analyses were performed using SPSS from IBM and R, respectively. Differences between groups were assessed using one-way ANOVA), Chi-squared test, Student’s t-test, Mann-Whitney, Kruskal-Wallis, or likelihood ratio tests in paper I, II, and IV.

P-values reported in paper I, II, and IV were corrected for multiple comparisons using the Benjamini-Hochberg method. A p-value of <0.05 was considered statistically significant. In Paper III, odds ratios (ORs) were calculated from 2 × 2 tables. Two-sided p-values were calculated using Fisher’s exact tests to analyse 2 × 2 tables. The Chi-square statistic was used to analyse higher-order tables with Bonferroni adjustment for multiple comparisons. For evaluating published Class II RNA expression data from EBV transformed lymphoblasts, RPKM (reads per kilobase of transcript per million mapped reads) values were stratified by haplotype and tested for significance by two-way ANOVA (GraphPad Prism), corrected for multiple comparisons by the Holm-Sídák method, with a = 0.05.

Results

The results of each paper are briefly summarized below.

Paper I

A differential expression of HLA-DQ on isolated peripheral blood cells, relative autoimmunity burden, and Class II HLA were investigated. At the time of sampling into the present study, 13 children were autoantibody negative at any measured, 23 had a single autoantibody and 18 had multiple autoantibodies (Stage I diabetes). Detailed information was obtained on HLA-DR, -DQ, and -DP genes.

Decreased HLA-DQ cell surface MFI was observed with an increasing number of autoantibodies on $CD16^+$, $CD14^+CD16^-$, $CD4^+$, and $CD8^+$. HLA-DQ cell surface MFI was associated with HLA-DQ2/8 in $CD4^+$ T lymphocytes, marginally in $CD14^+CD16^-$ monocytes and $CD8^+$ T lymphocytes. These associations appeared to be related to autoimmunity burden. The results suggest that HLA-DQ cell surface expression was related to HLA and autoimmunity burden. Additionally, differential HLA-DQ cell surface MFI was observed with age and HLA-DQ2/8 genotype in the two models not adjusted for autoimmunity burden.

Paper II

The lipidomic profiling in this study provides insight into the lipid composition in children with an increased genetic risk for type 1 diabetes and at different stages of autoimmunity. Analysis of lipids revealed four major clusters depending on HLA haplotypes. Additionally, HLA seems to influence levels of long chain TG. Variations in type and levels of several specific lipid species were related to the number of beta cell autoantibodies.

At cross-sectional sampling, 13 of the subjects had never had detectable autoantibodies at any time measured. Five subjects had progressed to type 1 diabetes after the cross-sectional sampling in the present investigation. These five subjects diagnosed with type 1 diabetes 6–26 months after the cross-sectional sampling did not differ in lipid composition compared to the other subjects with multiple autoantibodies at sampling.

The lipidomic analysis detected 128 lipid features annotated based on the internal standard. The detected lipids were members of the following lipid classes: sphingomyelin (SM), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine, alkylphosphatidylcholine (PC(O)), alkenylphosphatidylcholine, alkylphosphatidylethanolamine or phosphatidylethanolamine, phosphatidylinositol, cholesterol ester, ceramide (Cer), and triacylglycerol (TG).

A hierarchical cluster analysis grouped the subjects based on their HLA profiles and haplotypes, followed by the generation of heat maps of lipid expression based on the observed clusters, was used to identify lipidomic specific phenotypes in relation to HLA haplotypes.

According to the HLA haplotypes, four major clusters, according to the HLA haplotypes were identified in the hierarchical cluster analysis. The largest cluster contained many different haplotypes, whereas more specific haplotypes defined the other three clusters. Cluster 3 was the most distinct as it was characterized by the presence of the DPB1*20:01:01, DRB4*01:03:01:02N, and DPB1*13:01:01 alleles in 50% of the participants and the DRB4*01:01:01 allele in 75% of the participants. In addition, levels of PC (O-38:6) (a) were lower and PC (36:5) higher in this cluster. Overall, the levels of TG (18:2/18:2/18:2) or TG (18:3/18:2/18:1) decreased from clusters 1–4, and several long chain TG were lower detected in cluster 4.

Variation in the lipidome due to autoimmunity was assessed by dividing the subjects into two groups: negative for any autoantibody or positive for one or more autoantibodies

Three LPCs and two PCs were detected at significantly higher levels in children positive for autoantibodies. A single SM was detected in higher levels in autoantibody-positive research subjects. One PC and one TG were detected in lower amounts in subjects positive for autoantibodies.

Glycosylated ceramides (GlcCer) were investigated in relation to specific autoantibodies. Five GlcCer differed between the positive or negative for IAA groups. Individuals who were positive for IAA, independent of other autoantibodies, had significantly higher levels of the five GlcCer. No correlations with any of the other autoantibodies were observed.

Paper III

A previously unreported and unexpected haplotype of tri-SNP, located in intron-1 of the HLA-DRA1 gene, was identified. The tri-SNP was identified because the MIPs designed to interrogate rs9268645 revealed the presence of the other SNPs (Figure 21).

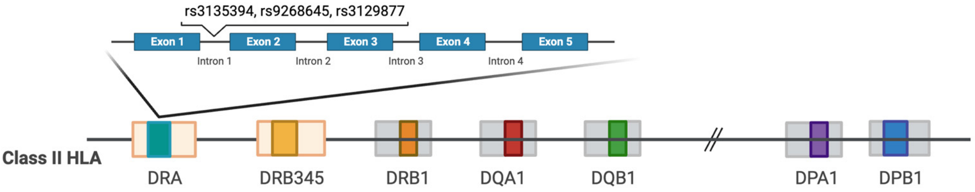


Figure 21. Schematic of the tri-SNP in intron 1 of the DRA gene and certain Class II HLA genes
The tri-SNP is a haplotype of the transition substitutions in rs3135394, rs9268645, and rs3129877 in intron-1 of the HLA-DRA1 gene on chromosome 6. Tri-SNPs were assembled in association with HLA-DRB345-DRB1-DQA1-DQB1 haplotypes. The image was created using BioRender.com.

The tri-SNP was strongly associated with type 1 diabetes risk in DR3/3 homozygous individuals. High-risk, intermediate-risk, and protective haplotypes were designated related to the major and minor alleles of the three SNPs as reference (0) and alternate (1), respectively. The 010 haplotypes were strongly associated with type 1 diabetes in the T1DGC and Swedish DR3/3 cohorts. The 010 haplotype appears to confer significant type 1 diabetes risk compared with the 101 haplotypes. In addition, risk association remained highly significant when data were re-analysed, restricting the Swedish cases to the Skåne region, from which all the control samples were obtained. The 101-containing haplotypes (especially 101/101) are significantly protective for type 1 diabetes compared with the 010 haplotypes. These results effectively stratify the high-risk DR3/3 individuals into two groups: those who carry the 010 haplotypes and those who do not.

The eQTL database at https://www.gtexportal.org/home/ groups gene expression in numerous tissues and cell types by the allele at any given SNP. Using this eQTL database, we found that RNA expression changes have been associated with the risk alleles of each of our three SNPs and that they are statistically significant in affecting the expression of HLA genes such as DQB1

Paper IV

Four tri-SNPs were identified in the cohort of 67 DiPiS subjects and were associated with 13 extended HLA-DR-DQ haplotypes. Among the haplotypes, AGG was the most common representing 50.0% (n = 67) of the entire cohort, followed by GCA (35.1%, n = 47), ACG (8.2%, n = 11), and ACA (6.7%, n = 9). In AGG subjects, 13 HLA-DR-DQ haplotypes were identified, distributed over three different DRB345, types where DRB4*01:03:01 (n = 57) was the most common, followed by DRB3*02:02:01 (n = 6) and DRB4*01:03:02 (n = 4). In GCA, only a single HLA-DR-DQ haplotype was identified (DRB3*01:01:02-DRB1*03:01:01-DQA1*05:01:01-DQB1*02:01:01). In ACA and ACG subjects, four and eight HLA-DR-DQ haplotypes were identified, distributed over two and six DRB345 variants, respectively. Furthermore, three prominent HLA-DRB1-DQA1-DQB1 haplotypes were identified with AGG and GCA tri-SNPs; DRB1*03:01:01-DQA1*05:01:01-DQB1*02:01:01 (AGG, n = 6; GCA, n = 47), DRB1*04:01:01-DQA1*03:01:01-DQB1*03:02:01 (AGG, n = 31), and DRB1*04:04:01-DQA1*03:01:01-DQB1*03:02:01 (AGG, n = 15). Furthermore, the AGG and GCA tri-SNPs were found in subjects with the HLA-DQ2/8 genotype (n = 32 and n = 21, respectively) and non-HLA-DQ2/8 genotype (n = 24 and n = 11, respectively). ACA and ACG tri-SNPs were only present in subjects with non-HLA-DQ2/8 genotypes (n = 9 and n = 11, respectively).

The 13 subjects that were autoantibody negative in their follow-up in DiPiS, all have the HLA-DQ2/8 genotype, the AGG (n = 14), and GCA (n = 12) tri-SNPs. We found no association between the tri-SNPs and autoimmunity burden, defined as the presence or absence of autoantibodies. However, we observed an association with autoimmunity burden defined as sAB but not cAB. We observed no indication that the HLA-DRA1 tri-SNPs were impacting the type of first appearing autoantibody, be it GADA or IAA, in subjects with HLA-DQ2/8 or non-DQ2/8 genotypes (Table VI).

A pattern of decreased HLA-DQ cell surface MFI on peripheral blood cells was observed with the AGG relative to the GCA tri-SNP. HLA-DQ cell surface MFI was lower on $CD4^+$ T lymphocytes with the AGG tri-SNP. Further stratifying HLA-DQ cell surface MFI by autoimmunity burden revealed a pattern of decreasing HLA-DQ cell surface MFI with increasing autoimmunity burden in both AGG and GCA tri-SNPs. For the AGG tri-SNP, lower HLA-DQ cell surface MFI was found on some but not all isolated peripheral blood cell types.

In this cohort, HLA-DRB3 01:01:02 and DRB3*02:02:01 is the only allele separating subjects with the GCA (n = 47) and AGG (n = 6) tri-SNPs. The HLA-DQ MFI did not differ between GCA and AGG subjects, as previously seen on $CD4^+$ T lymphocytes. However, HLA-DQ MFI still decreases with increasing cAB on some but not all isolated peripheral blood cell types. Similarly, HLA-DQ MFI decreases with increasing sAB.

Stratifying the tri-SNP genotypes by autoimmunity burden and the HLA-DQ2/8 or non-HLA-DQ2/8 genotype, we found that most subjects with HLA-DQ2/8 and AGG/AGG or GCA/AGG had no or low autoimmunity burden.

HLA-DQ MFI was lower in subjects with homozygous AGG/AGG than heterozygous GCA/AGG on some but not all isolated peripheral blood cell types. The pattern of decreasing HLA-DQ MFI with increasing cAB was identified for subjects heterozygous for GCA/AGG and homozygous for AGG/AGG.

In addition, differential HLA-DQ cell surface MFI was observed with age and HLA-DQ2/8 genotype on the isolated peripheral white blood cells without, but not when, adjusting for autoantibodies in a model based on linear regression with robust standard errors (adjusting for age at sampling, sex, and HLA-DQ). Without adjusting for autoimmunity burden, HLA-DQ cell surface MFI measured in subjects with the HLA-DQ2/8 genotype compared to non-HLA-DQ2/8 subjects were lower on $CD14^+CD16^-$, $CD4^+$, and $CD8^+$ cells, and marginally lower on $CD19^+$ and $CD16^+$ cells. Additionally adjusting for autoimmunity burden, HLA-DQ cell surface MFI measured in subjects with the HLA-DQ2/8 genotype compared to non-HLA-DQ2/8 subjects was lower on $CD8^+$ cells and marginally lower in $CD4^+$ T lymphocytes for sAB as well lower on $CD4^+$ and $CD8^+$ cells and marginally lower on $CD16^+$ and $CD14^+CD16^-$ for cAB.

Adjusting for sAB, HLA-DQ cell surface MFI was marginally lower on $CD4^+$ cells for subjects with one autoantibody compared to no autoantibodies. The association was shifted adjusted for cAB; HLA-DQ cell surface MFI was lower on $CD16^+$ cells for subjects with high compared to low autoimmunity burden.

Discussion

Type 1 diabetes is typically diagnosed based on clinical symptomatology associated with overt hyperglycaemia and metabolic imbalance. However, the disease can now be identified at earlier presymptomatic stages. Longitudinal follow-up studies have shown that patients have better metabolic control and fewer long-term complications due to less fluctuating blood glucose[8,59,60]. As only 7% of individuals with the highest HLA risk, HLA-DQ2/8, develop type 1 diabetes[47], it is important to differentiate between individuals at high-risk of developing type 1 diabetes and identify those that will develop the disease.

This study aimed to investigate the immunological process that precedes type 1 diabetes in relation to HLA and the burden of autoimmunity using flow cytometry, metabolomics, and genotyping. A subset of DiPiS subjects at risk of developing type 1 diabetes is the backbone of papers I, II, and IV. The study was designed to investigate samples from healthy children with risk for type 1 diabetes and a variable number of autoantibodies. The subjects with multiple autoantibodies were classified as stage 1 type 1 diabetes according to the current nomenclature[82]. At the time of cross-sectional sampling, none of the children with multiple autoantibodies had dysglycaemia without symptoms (Stage 2) or had developed clinical type 1 diabetes (Stage 3) at the time of sampling. Paper III was performed using cases and controls to investigate new genetic markers of type 1 diabetes.

This thesis, through four projects, investigates several factors that may shed light on the autoimmune process leading to the development of type 1 diabetes. This thesis aimed to test the hypothesis that there is an association between HLA, the capacity of the antigen-presenting cells to present antigen and chronic beta cell autoimmunity or *autoimmunity burden*. The latter is quantified by the number and type of autoantibodies at cross-sectional sampling and as well as during follow-up. The sub-aims corresponding to each of the four projects in this thesis is to test whether the expression of HLA-DQ on peripheral blood cells is associated with either the number of autoantibodies or autoimmunity burden or both (Paper I); to test the hypothesis that lipids in the blood of the children in the present cohort are associated with beta cell autoimmunity (Paper II); to investigate additional type 1 diabetes susceptibility loci in patients and controls homozygous for the HLA-DR3 high-risk haplotype (Paper III) and to explore whether the newly identified tri-SNP in intron 1 of HLA-DRA can be observed and whether it is associated with the number of autoantibodies and high-risk HLA in a cohort of a subset of DiPiS subjects (Paper IV).

Class II HLA haplotypes and risk of type 1 diabetes

Although much is known about the risk associated with certain HLA genes, haplotypes, and genotypes, much remains to be discovered and explained. It is well established that HLA-DQ2/8 contributes to the highest risk for type 1 diabetes, and the risk for progression to clinical onset increases with an increasing number of autoantibodies[56,148]. Therefore, we believe that the burden of autoantibodies over time puts a strain on the immune system and could contribute to T cell exhaustion. T cell exhaustion is thought to allow partial containment of chronic infections by the persistence of T lymphocytes, without causing immunopathy[149,150]. It has been suggested that T cell exhaustion may be important to limit immunopathology or autoreactivity[149,151], and it cannot be excluded that our observation that the decrease in HLA-DQ cell surface MFI by sAB and cAB is related to T cell exhaustion.

In the cohort of the 67 DiPiS subjects (papers I, II, and IV), the HLA-DQ2/8 subjects are mostly autoantibody negative. This is not surprising as 3.5% of new-borns have this genotype, and only a fraction of such children will develop one or several autoantibodies, let alone autoimmune type 1 diabetes. It is also possible that some DiPiS HLA-DQ2/8 subjects have already been diagnosed with type 1 diabetes and were therefore not asked to participate in the present investigation.

Autoimmunity burden

Autoantibody data obtained from the long-term follow-up of subjects randomly selected from the DiPiS study made it possible to study the burden of autoantibodies over time during follow-up and at cross-sectional sampling (papers I, II, and IV). The number of autoantibodies defined autoimmunity burden during DiPiS follow-up or at cross-sectional sampling. For this thesis, we aimed to quantify that burden, i.e., the burden of autoantibodies over time, and estimating the area under the trajectory of the autoantibodies over time was a natural choice. The length of the prodrome period, the time from the appearance of multiple autoantibodies until the diagnosis of diabetes, is inversely proportional to the number of autoantibodies: the more autoantibodies, the faster the rate of progression to clinical onset[56,104,152].

To our knowledge, autoimmunity burden has not been considered in studies of children who have been beta cell autoantibody-positive from an early age. The subjects had been positive for autoantibodies between 3 and 13 years. Progression to clinical onset may be faster in children with seroconversion early in life[56]. Infections constantly train the immune system in young children for adaptive immune response; thus, exhaustion of immune cells could potentially speed up the progression to diabetes. Immune exhaustion refers to immune dysfunction, and poor effector function of immune cells, due to autoimmune burden (reviewed in [153,154]).

Relative levels of T-cell exhaustion are associated with clinical outcomes in chronic viral infection[151].

Interestingly, as of June 2022, there are seven subjects from the subset DiPiS cohort (papers I, II, and IV) who have subsequently developed type 1 diabetes (6.0, 8.9, 9.8, 11.3, 26.0, 49.0, and 50.6 months after sampling). Therefore, the subjects represent children at various stages of type 1 diabetes pathogenesis.

Genetic, primarily non-HLA genetic factors, in addition to the beta cell autoantibody markers and environmental factors[155,156], also seem to increase the disease's progression rate. Thus, the number of autoantibodies is a strong predictive marker of pathogenesis. However, it is still unclear when mononuclear cells begin to invade the pancreatic islet in beta cell autoantibody-positive subjects. It has been reported that pancreas organ donors with beta cells autoantibodies are negative for insulitis, which was only found in donors with multiple autoantibodies[22,157].

Identifying HLA-DQ cell surface expression in at-risk subjects

There is a lack of understanding of the triggering and progression of the autoimmune destruction of the beta cells in type 1 diabetes. An association between HLA and the risk of a first autoantibody have been suggested to be primary to an association between HLA and type 1 diabetes[21,50,64,158]. Recent data support an association between HLA-DR-DQ and the first appearing autoantibody[21,50,64], and that the first-appearing autoantibody may be associated with the age at clinical onset of type 1 diabetes. HLA-DQ cell surface expression on $CD4^+$ and $CD8^+$ T lymphocytes has previously been documented in healthy individuals[159] and in relation to autoimmune conditions (for example, type 1 diabetes, coeliac disease and vitiligo) and infectious episodes[160,161].

Variation in HLA-DQ cell surface expression was investigated on six peripheral blood cell types in children with an increased genetic risk for type 1 diabetes and at different stages of autoimmunity.

The calculated autoimmunity burden and measured HLA-DQ cell surface MFI indicated a trend of lower HLA-DQ cell surface expression in children at an increased risk for type 1 diabetes and increasing autoimmunity burden (Paper I). While we have defined autoimmunity burden as exposure to autoantibodies, it can only be speculated to what extent reduced HLA-DQ cell surface expression on T lymphocytes and monocytes reflects the type of T cell exhaustion reported in other autoimmune diseases. HLA-DQ is constitutively expressed on monocytes, and activated T lymphocytes express Class II HLA heterodimers[162]. Further studies are therefore warranted to determine whether children with multiple autoantibodies exhibit T cell exhaustion as defined by poor effector function, sustained expression of inhibitory receptors or a transcriptional pattern different from that of functional effector or memory T lymphocytes (reviewed in [153,154]).

Characterizing lipid profiles in at-risk subjects

The development and function of immune cells depend on different aspects of lipid metabolism, affecting features such as the expression of enzymes and the transport of proteins. Lipid metabolism is important for the differentiation and function of T lymphocytes, as well as maintaining immune tolerance[163]. As patients with type 1 diabetes also present with lipid disorders, lipidomic profiles have the potential to be used in assessing beta-cell function in type 1 diabetes as well as the effects of dysregulated lipid metabolism on the progression of beta-cell destruction.

Predicting disease progression and status has been the focus of establishing lipidomic profiles[164,165]. Lipidomic profiling provides a snapshot of information about lipids in a sample. Both genetic and environmental factors influence susceptibility to develop chronic diseases, such as type 1 diabetes, and reflect changes in lipidomic phenotypes as they are sensitive to subtle modifications and nutrition[166]. Distinct lipidomic signatures have been suggested to characterize children who progress to islet autoimmunity or overt type 1 diabetes, which may be helpful in the identification of at-risk children before the initiating autoimmunity. Early immune developmental processes in type 1 diabetes progressors have in previous studies been suggested to be disturbed by distinct cord blood phospholipids and TGs. A characteristic lipidomic profile of lower phospholipid levels has been found to be present already at birth in type 1 diabetes progressors[73,74].

Paper II investigated the variation in blood lipids in adolescent subjects, 10–15 years of age, with increased genetic risk for type 1 diabetes and different stages of autoimmunity. We investigated variation in the lipidome in relation to autoimmunity (positive or negative for autoantibodies) and not the number of autoantibodies, due to the cohort size and the size of our groups. Our results indicate that variation in phospholipids is related to autoimmunity but not necessarily progression to type 1 diabetes, since we did not see any differences in five subjects that developed type 1 diabetes after the follow-up. However, this may be the result of lack of power as this analysis included very few subjects.

Sphingolipids are important cellular components of membranes and regulators of immune cell activity and several cellular processes, including apoptosis, autophagy, cell cycle arrest, and cellular senescence[167-169]. Cer is a type of sphingolipid and is an important intermediate in the biosynthesis and metabolism of all sphingolipids, such as SM. Cer's have a detrimental effect on pancreatic β cells, where they activate the stress-induced apoptotic pathway. Cer also modulates many of the insulin signalling intermediates and causes insulin resistance. Cer quenches the expression of the insulin gene resulting in reduced insulin synthesis[170]. Cer's are up-regulated following seroconversion to positivity for islet autoantibodies[171]. TGs are a type of fatty acid and an important source of cellular energy but are also precursors to producing complex lipids, such as cholesterol and membrane phospholipids[172]. Reduced plasma TG levels result from lipoprotein lipase promoting the catabolism of TG-rich lipoproteins. Lipoprotein lipase is an enzyme activated by insulin[173]. TGs accumulate in intracellular droplets in macrophages as a response to inflammatory activation by pathogens. TG levels are often elevated during ketoacidosis, a state of insulin deficiency[174]. The formation of TGs is important for limiting the activation of protein kinase C[163], a family of kinases that regulate numerous cellular functions[175]. The mechanisms underlying TG accumulation and its exact role in the inflammatory response of macrophages are not fully understood[176].

In previous studies, it has been suggested that SMs and TGs are potent regulators of immunogenic processes and play a potent role in inflammatory disease[177,178]. Other studies indicate that the presence of autoantigens in the development of type 1 diabetes is preceded by modulation of multiple serum phospholipids[72,179]. Another study showed that several lipid classes were associated with a decrease in beta-cell function after diagnosis of type 1 diabetes in children and the observations support the hypothesis of lipid disturbances as explanatory factors for residual beta-cell function in children with new onset type 1 diabetes[180]. In the Finnish Type 1 Diabetes Prevention and Prediction Study (DIPP), SMs were persistently downregulated in children who progressed to type 1 diabetes compared to children with at least one autoantibody but did not progress to type 1 diabetes and control groups. TGs and PCs were mainly downregulated in children who progressed to type 1 diabetes compared to children with at least one autoantibody but did not progress to type 1 diabetes at the age of 3 months. In Paper II, we did not find any differences in lipid levels when comparing the five subjects who developed type 1 diabetes after sampling to those who did not. However, it would be necessary to investigate further as this analysis was performed with very few subjects.

Dysregulation of lipid metabolism has been suggested to precede islet autoimmunity and type 1 diabetes. Up-regulation of Cer's and down-regulation of SM and LPC have been associated with autoantibody appearance. Disruption of SM metabolism has been suggested to precede the appearance of islet autoantibodies from an early age in children progressing to type 1 diabetes[171].

Distinct lipidomic profiles associated with progression to type 1 diabetes have been most pronounced in very young children[73,181]. Children who develop type 1 diabetes later in life have distinctly different lipidomic signatures in infancy than those who do seroconvert to a single islet autoantibody but do not develop the disease[181]. Dietary patterns may mask type 1 diabetes-associated signatures, and using metabolites to predict type 1 diabetes may be most feasible early in life, before the appearance of islet autoantibodies.

Since the current stratifications in studies aimed at type 1 diabetes prevention are based on the detection of islet autoantibodies, lipidomic profiles may thus provide a valuable complementary tool for identifying children at the highest risk of progression to type 1 diabetes. In the future, we need to consider two possibilities: that increase or decrease in levels of specific lipids are associated with 1) risk of developing and maintaining autoantibodies or 2) an ongoing pathogenic process causing different lipidomic profiles in individuals without and with autoantibodies. A better understanding of lipid metabolism and lipid profiles associated with type 1 diabetes will increase our understanding of the contribution of specific lipids, such as TGs and Cer's, to the pathogenesis of diabetes and further help identify potential therapeutic targets for the management of type 1 diabetes and its complications.

The HLA-DRA1 tri-SNP

Over 60 risk regions marked by SNPs confer a genetic predisposition to type 1 diabetes have been identified in the human genome through GWAS[182]. Using gene expression as the phenotype in typical eQTL is equivalent to GWAS. A study of gene expression signatures early in life identified 67 differently expressed genes in children that developed autoantibodies relative to children who remained autoantibody negative. The genes contribute to cell-related immune responses through B and T lymphocytes and dendritic cells[183]. To better understand the impact of variants on target genes, it would be useful to determine if it is causal[52]. Analysis of peripheral blood by eQTL has suggested possible causal effects of variants associated with type 1 diabetes. Investigating specific immune cell types (such as T lymphocytes and monocytes) could improve understanding of variants' influence on target genes[182]. A gene of interest and a variant associated with disease may be in nearby. However, the variant may regulate the expression of another gene far away[182,184]. In one study, spatially regulated genes that exhibit tissue-specific effects in multiple tissues have been implicated in tissue and cell type-specific regulatory networks contributing to pancreatic beta cell inflammation and destruction, adaptive immune signalling, and immune-cell proliferation and activation[185].

In Paper III, we identified a three-SNP haplotype that appears to modify the risk of type 1 diabetes in individuals homozygous for the HLA-DR3 haplotype. This intronic SNP haplotype may function as an eQTL, affecting the expression of Class II HLA genes and perhaps other genes. The attempt to analyse genetic susceptibility to type 1 diabetes was performed by studying a population of case and control subjects homozygous for HLA-DR3. This result is consistent with discoveries reported in patients with multiple sclerosis literature[186], suggesting that HLA-specific susceptibility loci may be important. Our data support a possible explanation that most persons with homozygous HLA-DR3 (more than 90% even with the highest risk, the HLA-DR3/4 haplotype[47]) do not develop diabetes. Notably, 75% of the Swedish samples in this study are homozygous for the relatively low-risk haplotype.

The tri-SNP was an unexpected finding and made it possible to investigate if it is somehow associated with different HLA haplotypes, autoantibodies, and HLA-DQ cell surface expression on isolated peripheral white blood cells (Paper IV). In Paper III, the study cohort was limited to subjects with homozygous "HLA-DR3" haplotypes, while the subjects in Paper IV had many different HLA haplotypes. The risk and protective tri-SNPs identified in Paper III correspond to the AGG and GCA haplotypes identified in Paper IV, respectively. In Paper IV, we confirmed that the tri-SNP occurs outside of the cohort in Paper III, but whether it is diabetes predictive for other high-risk haplotypes, such as “DR4”, is unknown. The tri-SNP may add new knowledge in relation to different HLA haplotypes and the risk of developing autoantibodies. Future studies need to investigate if the tri-SNP affect the risk of developing a first autoantibody and subsequently type 1 diabetes.

Strengths and limitations

Paper I, II, and IV utilize a well-characterized prospective cohort of subjects identified by early screening as being of high risk for developing type 1 diabetes. Developing reliable genetic risk markers for type 1 diabetes could improve disease prediction and trial design for preventive therapies. We believe this is very important to investigate and are incredibly thankful for the subjects and their families participating in longitudinal follow-up.

The cohort used in papers I, II, and IV is small and only provides limited power. However, as this is a unique cohort of subjects with an incredible follow-up and monitoring of autoantibodies and well-being, we believe that the investigation and comparisons are important for the research community and to shed light on the combination of HLA cell surface expression and HLA haplotypes and genotypes combined with autoantibodies. In addition, it was possible to obtain comprehensive data on HLA by NGS, autoantibodies, CBC, and HLA-DQ cell surface MFI at the time of sampling. It is noted that HLA-DQ2/8 children in this cohort are mostly autoantibody negative. This is not surprising as 3.5% of new-borns have this genotype, and only a fraction of such children will develop one or several autoantibodies, let alone autoimmune type 1 diabetes. It is also possible that some children in DiPiS with HLA-DQ2/8 have already been diagnosed with type 1 diabetes and, therefore, not asked to participate in the present investigation. In Paper III, the cases and controls are largely derived from different cohorts – which appear to be sourced from various geographic areas. It should be noted that the Swedish cases and controls are derived from different cohorts, and there exists the possibility that the effect could be due in part to population stratification.

The HLA-DQ cell surface MFI used in Papers I and IV was obtained in two steps: manually isolating peripheral blood cells and white blood cells and applying classic flow cytometry to identify HLA-DQ cell surface MFI. Limitations of this method include large blood samples, a variety of materials and reagents that increase cost, and a time-consuming protocol. The many steps in the protocol increase risk of contamination. A possible substitute for this method is fluorescence-activated cell sorting (FACS), which automatically sorts a cell sample stained with monoclonal antibodies and simultaneously records the fluorescence emitted by all monoclonal antibodies attached to each cell. Applying FACS technology would decrease the required blood volume, cost of material and reagents, lead time, and risk of contamination as there would not be steps in the protocol.

A weakness of our study is the lack of cellular analyses during follow-up, which was impossible to accomplish for reasons of resources, place of residence, and logistics. Another potential weakness is using a pan-HLA-DQ monoclonal antibody; it would have been interesting to use allele-specific HLA-DQ antibodies. Unfortunately, such antibodies were not available. However, the pan-HLA-DQ antibody used allowed us to test the hypothesis that HLA-DQ expression was related to sAB and cAB. The follow-up in DiPiS was not critical for the lipidomics analysis in Paper II but enabled detailed interrogation of the development of autoantibodies before cross-sectional sampling in relation to HLA haplotypes.

A weakness of the study would be the limited number of participants, making it difficult to generalize since many different HLA haplotypes are represented, and cellular analyses were unavailable during follow-up. Another limitation is that the subjects were not fasting before the blood draw. The blood samples from the participants were drawn in the morning (and in the afternoon for a few), and the participants were not instructed to fast before and, therefore, must be expected to have had breakfast (and in some cases, lunch). Drawing blood from young individuals is more complicated when fasting and has to be done in the morning. The participants donated blood to the present study during their annual DiPiS visit, and on top of that, some participants had to travel a long way to take the blood sample. Ideally, we would have preferred to have used blood samples from fasting individuals; however, originally, this study cohort was obtained for other purposes and fasting before the blood draw is also not a part of the preparations before a DiPiS visit. Based on previously published data showing a small direct effect on test meal fatty acid composition and postprandial lipid composition of the blood[187] and a long-term twin study showing high heritability of particularly phospholipids, irrespective of a 5-week dietary intervention[188], we decided to conduct our study regardless.

In Paper III, the preliminary database finding and transformed cell line mRNA data are premature at this time and should be validated in primary cells from genotype-selected individuals. The analysis is limited and needs additional in vivo studies to directly address the putative enhancer's functionality. The eQTL analysis is limited due to the limited amount of data available. The analysis is limited, and there is a need for additional in vivo studies to address the functionality of the putative enhancer directly.

Future perspectives

It would be interesting for future studies to analyse autoantibodies between groups of research subjects without and with one or multiple autoantibodies. Flow cytometry could then be used to study PBMC in subjects with autoantibodies progressing from Stage 2 to Stage 3 of the current nomenclature. Planned future studies include determining whether the tri-SNP risk haplotype is present in another type 1 diabetes risk haplotype. We expect that there will be a difference in risk or protection by the tri-SNP depending on the linkage disequilibrium to either DR3-DQ2 or DR4-DQ8. We hypothesize that combining HLA, tri-SNP genotyping, and autoantibodies may improve the risk assessment of type 1 diabetes. Additionally, with variations in lipid levels, lipidomic profiling could potentially be a novel way to improve prediction and monitor disease progression. The lipidomic profiling in Paper II provides insight into the lipid composition in subjects who had developed islet autoimmunity and are at an increased risk of type 1 diabetes. Future investigations of the observed tendencies that lipidomic profiles may be associated with HLA will be necessary. However, it needs to be clarified to what extent lipids are rather associated with PBMC profiles than with autoantibodies.

We speculate that this type of analysis would increase the knowledge of lipids levels and if they tend to vary with an increasing number of autoantibodies. Future studies will have to confirm the value of combining the markers presented in this thesis and if the findings can be used as early markers for type 1 diabetes.

Conclusions

Previous studies have shown that children in close follow-up, at an increased risk of type 1 diabetes, are diagnosed earlier in the disease process and have better metabolic control, even after diagnosis, compared to children not in follow-up before diabetes diagnosis[189,190]. Suppose we can identify markers that predict type 1 diabetes with increased precision other than autoantibodies. In that case, they could be used to predict type 1 diabetes in groups that are followed with increased risk, e.g., first-degree relatives to type 1 diabetes patients and individuals with increased genetic risk, perhaps based on a genetic risk score in population screening. This could increase the knowledge behind triggers and help to discover new methods to prevent (primary prevention) or impede (secondary prevention) the autoimmune process. Identifying such triggers and, thus, preventing diabetes is the ultimate goal of diabetes research.

Summary in Swedish

Typ 1 diabetes är en autoimmun sjukdom som karaktäriseras av att kroppens förmåga att reglera nivån av socker (glukos) i blodet kraftigt försämrats eller helt slagits ut. Orsaken är en autoimmun reaktion där kroppens egna celler angriper delar av bukspottskörteln som om de vore bakterier eller virus. Insulin är ett hormon som produceras i bukspottskörteln och hjälper kroppen att ta upp glukos i olika vävnader. Kraftigt försämrad eller förlorad förmåga att producera insulin resulterar i ökande blodsockernivå. Då symptom på typ 1 diabetes uppträder, så som ökad törst, stora urinmängder, viktnedgång och trötthet, beräknas endast 10–20% av de insulinproducerande beta cellerna i bukspottskörteln fortfarande fungera. Det är då livsnödvändigt att tillföra det insulin kroppen själv inte kan producera. Perioden innan symptom framträder kan variera från månader till år. Yngre barn har ofta en kortare asymtomatisk period, månader, innan klinisk debut medan det kan ta år innan kliniska symptom framträder hos äldre personer.

Typ 1 diabetes är starkt relaterat till vissa gener i HLA-regionen på kromosom 6. HLA-gener används för att producera så kallade klass II humana leukocyt antigen (HLA). Klass II HLA är proteiner som presenteras på cellytan av immunceller. Proteinerna används av kroppens immunsystem för att signalera mellan celler och vävnader samt att upptäcka främmande antigen, bakterier och virus, för att kunna bekämpa dem. HLA-DR och HLA-DQ är två typer av gener som tillsammans bildar en haplotyp, där varianterna HLA-DR3 och HLA-DQ2 samt HLA-DR4 och HLA-DQ8 ofta ärvs tillsammans och bidrar med hög risk för sjukdomen. Varje människa har två haplotyper som tillsammans bildar en genotyp. Ungefär 90% av alla barn som utvecklar typ 1 diabetes har åtminstone en av typerna HLA-DR3-DQ2 eller HLA-DR4-DQ8, jämfört med den generella populationen. Ärvs båda haplotyperna bidrar dessa med den högsta risken att utveckla typ 1 diabetes. Barn med högst risk representerar 30% av alla barn som utvecklar typ 1 diabetes, jämfört med endast 3% i den generella populationen.

Autoantikroppar är markörer som kan mätas i blodet månader till år innan diagnos av typ 1 diabetes. Hittills har man kunnat identifiera sex olika autoantikroppar som är kopplade till typ 1 diabetes. Dessa autoantikroppar är riktade mot betacellsproteinerna GAD65, insulin, IA-2 och tre varianter av Zink Transporter 8. Förekomsten av autoantikroppar är tecken på en pågående autoimmun process och ökar risken för typ 1 diabetes.

Ett blodprov kan avgöra om en person har ärftlig risk för att utveckla typ 1 diabetes. Vissa gener brukar beskrivas som skyddande mot sjukdomen och individer med denna genuppsättning utvecklar sällan eller aldrig sjukdomen. Drygt hälften av alla barn som föds har denna typ av genuppsättning. Neutrala gener, som inte är skyddande men inte heller ger högre risk att insjukna än för hela befolkningen i övrigt, finns hos 25 procent av befolkningen. De resterande barnen, ungefär 20 procent av befolkningen, har däremot en förhöjd ärftlig risk att utveckla typ 1 diabetes. Drygt 90 procent av alla barn som insjuknar har dessa gener och har därmed ökad risk att insjukna. Slutsatsen blir att enbart ärftlig risk trots allt inte är tillräckligt för att utveckla typ 1 diabetes eftersom endast sju av hundra barn med ärftlig risk insjuknar. Därför måste det vara något annat som bidrar och triggar igång den autoimmuna processen.

Denna avhandling är uppdelad i fyra projekt som undersöker olika faktorer som skulle kunna bidra med kunskap om den autoimmuna processen som leder till typ 1 diabetes. Det övergripande syftet med avhandlingen är att testa hypotesen att det finns en association mellan HLA, förmågan att presentera proteiner på cellytan av vita blodceller och autoimmunitet. Tre projekt (delarbete I, II och IV) data från 67 friska barn, med risk för typ 1 diabetes och varierande antal autoantikroppar, som deltog i uppföljningsstudien DiabetesPrediktion i Skåne (DiPiS). Med flödescytometri kunde vi mäta typer och mängd av olika proteiner på cellytan av sex isolerade vita blodceller. Genom att analysera DNA kunde vi även ta reda på HLA-genotypen för varje individ. Typ och antal autoantikroppar kunde mätas i plasma från blodprov. Genom att individerna deltagit i DiPiS under väldigt lång tid så kunde vi beräkna ett snitt av hur lång tid varje individ hade varit utan eller haft en eller flera autoantikroppar, dvs bördan av autoantikroppar.

I delarbete I undersökte vi om utvecklingen av autoantikroppar och risk för typ 1 diabetes är associerat med olika uttryck av HLA-DQ proteiner på cellytan av vita blodceller. Vi observerade att mängden HLA-DQ proteiner på cellytan hade en nedåtgående trend med ökande börda av autoantikroppar. I delarbete II studerade vi sammansättningen av lipider vid olika stadium av autoimmunitet. Vi identifierade fyra grupper av lipider relaterade till olika HLA-typer. Typ och nivå av vissa lipider varierade med antal autoantikroppar. I delarbete III använde data från olika nationella och internationella studier och stora databaser. Vi identifierade en ny haplotyp (tri-SNP), som utgörs av tre enbaspolymorfier (substitution av en nukleotid på en specifik position i DNA), i en icke-kodande del av HLA-DRA1 genen, en gen som bidrar med risk för typ 1 diabetes. I delarbete IV undersöktes denna tri-SNP i relation till HLA, autoantikroppar och HLA-DQ uttryckt på cellytan av isolerade vita blodceller. Vi fann fyra olika tri-SNP:ar som associerades med olika HLA haplotyper, autoantikroppar och olika uttryck av HLA-DQ på cellytan av isolerade vita blodceller.

I framtida studier skulle det vara intressant att analysera autoantikroppar mellan grupper utan och med en eller flera autoantikroppar. Flödescytometri skulle sedan kunna användas för att studera vita blodceller hos individer med en eller flera autoantikroppar och som går från att inte ha några symptom till att utveckla symptom på typ 1 diabetes. Dessutom, om HLA, autoantikroppar kan kombineras med variationer i lipider så skulle lipidprofiler potentiellt kunna bidra till prediktion och övervaka utvecklingen av typ 1 diabetes. Framtida studier måste studera värdet i att kombinera markörerna presenterade i denna avhandling.

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Immunological markers of type 1 diabetes pathogenesis prior to clinical diagnosis

One in 15 (7%) of individuals with the highest genetic risk for type 1 diabetes will develop the disease over a life-time. The overall aim of this thesis was to investigate immunological markers and survey the type 1 diabetes pathogenesis. Factors that may contribute to the immunologic process preceding type 1 diabetes were investigated in four projects.

Agnes Andersson Svärd has a MSc in Biotechnology from Uppsala University. Her main interests have been immunological research and autoimmunity. In her research she focuses on markers of the type 1 diabetes pathogenesis in both national and international collaborations.

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