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Sirpa Pahkuri

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University of Turku

Faculty of Medicine
Institute of Biomedicine
Medical Microbiology and Immunology
Turku Doctoral Programme of Molecular Medicine (TuDMM)

Supervised by

Docent Johanna Lempainen
Institute of Biomedicine
University of Turku
Department of Pediatrics and
Adolescent Medicine
Turku University Hospital
Turku, Finland

Professor Emeritus Jorma Ilonen
Institute of Biomedicine
University of Turku
Turku, Finland

Reviewed by

Associate Professor Eliisa Kekäläinen
Department of Bacteriology and Immunology
HiLIFE Helsinki Institute of Life Science
University of Helsinki
Helsinki, Finland

Docent Markku Varjosalo
Institute of Biotechnology
University of Helsinki
Helsinki, Finland

Opponent

Docent Mikko Seppänen
Rare Diseases and Pediatric Research Centers
HUS Helsinki University Hospital
Clinicum & Translational Immunology Research Program
University of Helsinki
Helsinki, Finland

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To my mom and dad

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ABSTRACT

Type 1 diabetes (T1D) is a chronic autoimmune disease which often develops during childhood in genetically susceptible individuals and is most likely triggered by some environmental factors. Around 40–50% of the genetic risk is conferred by HLA class II genes *HLA-DRB1*, *-DQA1* and *-DQB1*. In addition, single nucleotide polymorphisms (SNPs) in genes, such as *INS*, *PTPN22* and *IL2RA*, contribute by smaller effects. However, many T1D-associated SNPs are located in non-coding region and their effects are still unclear. The aim of this thesis was to examine the effect of T1D-associated genetic variation and early antibiotic exposure on DNA methylation in lymphocytes, in order to clarify their mechanisms and examine whether DNA methylation could mediate their effects in T1D and autoimmunity.

T1D-associated SNPs rs689 in *INS* and rs12722495 in *IL2RA* were found to be associated with DNA methylation in *INS* and *IL2RA* promoters, suggesting that DNA methylation may mediate the effect of some SNPs on T1D risk. However, only one CpG site had T1D-associated methylation changes in *INS* when analyzing methylation in *INS*, *IL2RA* and *PTPN22*, suggesting that T1D may have some effects on methylation but they may be cell specific with small effects. The effect of T1D susceptibility-associated HLA class II haplotypes, *DRB1*03-DQA1*05-DQB1*02* (DR3-DQ2) and *DRB1*04:01-DQA1*03-DQB1*03:02* (DR4-DQ8), and protection associated haplotype, *DRB1*15-DQA1*01-DQB1*06:02* (DR2-DQ6), on epigenome-wide methylation was localized to HLA region, especially *HLA-DRB1* locus, where the protective haplotype DR2-DQ6 was associated with hypomethylation. Antibiotic exposure during the first week of life was not associated with changes in immune cell frequencies in PBMC at the age of 3 months but it had effects on CD4+ and CD8+ T cell epigenome.

These results suggest that both T1D-associated genetic variation and early antibiotic exposure can affect DNA methylation in lymphocytes and that methylation could mediate at least part of their effects in T1D and autoimmunity.

KEYWORDS: Type 1 diabetes, autoimmunity, genetics, environmental factors, epigenetics, DNA methylation, lymphocytes

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TIIVISTELMÄ

Tyypin 1 diabetes (T1D) on krooninen autoimmuunitauti, joka kehittyy usein jo lapsuudessa geneettisesti alttiissa henkilöissä ja todennäköisesti jonkin tai joidenkin ympäristötekijöiden laukaisemana. HLA luokka II geenit *HLA-DRB1*, *-DQA1* ja *-DQB1* selittävät geneettisestä alttiudesta noin 40–50 % ja lisäksi monilla yhden nukleotidin mutaatioilla (engl. single nucleotide polymorphism, SNP) geeneissä, kuten *INS*, *PTPN22* ja *IL2RA*, on vaikutusta. Monet näistä mutaatioista sijaitsevat DNA:n koodaamattomalla alueella eikä niiden vaikutuksia vielä täysin tunneta. Väitöstutkimuksen tavoitteena oli tarkastella tyypin 1 diabetekseen liitetyn geneettisen variaation ja varhaisen antibioottialtistuksen vaikutusta DNA-metylaatioon immuunisoluissa ja selvittää voisiko DNA-metylaatio toimia niiden vaikutuksen välittäjänä T1D:n ja autoimmuunireaktioiden kehityksessä.

T1D-alttiuteen liitettyjen SNP-mutaatioiden rs689 (*INS*) ja rs12722495 (*IL2RA*) havaittiin olevan yhteydessä *INS*- ja *IL2RA*-geenien metylaatioon. Tutkittaessa koko epigenomia, T1D-alttiuteen liitettyjen HLA luokka II haplotyyppien *DRB1*03-DQA1*05-DQB1*02* (DR3-DQ2) ja *DRB1*04:01-DQA1*03-DQB1*03:02* (DR4-DQ8), sekä suojaavan haplotyypin, *DRB1*15-DQA1*01-DQB1*06:02* (DR2-DQ6), vaikutus keskittyi HLA-alueelle, erityisesti *HLA-DRB1* geeniin, jonka hypometylaatio oli yhteydessä suojaavaan DR2-DQ6-haplotyyppiin. Ensimmäisen elinviikon aikana saatu antibioottikuuri ei ollut yhteydessä immuunisolujen frekvenssien muutoksiin 3 kk:n iässä otetuissa PBMC-näytteissä, mutta se oli yhteydessä CD4+ ja CD8+ T-solujen epigenomin muutoksiin.

Nämä tulokset viittaavat siihen, että tyypin 1 diabetekseen liitetty geneettinen variaatio sekä varhainen antibioottialtistus voivat vaikuttaa immuunisolujen DNA-metylaatioon, mikä voisi toimia niiden vaikutuksen välittäjänä T1D:n ja autoimmuniteetin kehityksessä.

AVAINSANAT: Tyypin 1 diabetes, autoimmuniteetti, genetiikka, ympäristötekijät, epigenetiikka, DNA-metylaatio, lymfosyytit,

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Abbreviations

AATK	apoptosis associated tyrosine kinase
ADA	American Diabetes Association
AOAH	acyloxyacyl hydrolase
APC	antigen presenting cell
BCR	B cell receptor
CD	celiac disease
CGI	CpG island
CVB4	coxsackievirus B4
DC	dendritic cell
DIPP	Finnish Type 1 Diabetes Prediction and Prevention project
DMR	differentially methylated region
DMS	differentially methylated CpG site
DMSO	dimethyl sulfoxide
DNMT	DNA methyltransferase
EM	effector memory cell
ENDIA	Environmental Determinants of Islet Autoimmunity study
EWAS	epigenome-wide association study
FBS	fetal bovine serum
FFGC	Finnish Functional Genomics Centre
FPDR	Finnish Pediatric Diabetes Register
GADA	glutamic acid decarboxylase autoantibodies
GEE	generalized estimating equations
GR	glucocorticoid receptor
GWAS	genome-wide association study
HDAC	histone deacetylase
HLA	human leukocyte antigen
HT	Hashimoto thyroiditis
IA-2A	islet antigen-2 autoantibodies
IAA	insulin autoantibodies
ICA	islet cell autoantibodies
IFN	interferon
IGF2	insulin-like growth factor 2
INS	insulin

IL	interleukin
IL2RA	interleukin 2 receptor subunit alpha
LCT	lactase
LD	linkage disequilibrium
LINE1	long interspersed nuclear element-1
LP	lamina propria
LPS	lipopolysaccharide
MAF	Mutation Analysis Facility
MBD	methyl-binding-domain
MHC	major histocompatibility complex
mQTL	methylation quantitative trait loci
MS	multiple sclerosis
NK	natural killer cell
PAH	polycyclic aromatic hydrocarbons
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
POP	persistent organic pollutants
PTPN22	protein tyrosine phosphatase non-receptor type 22
QC	quality control analysis
RPMI	Roswell Park Memorial Institute 1640 medium
RRBS	reduced representation bisulphite sequencing
RT	room temperature
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
T1D	type 1 diabetes
TCR	T cell receptor
TEDDY	The Environmental Determinants of Diabetes in the Young study
TET	ten-eleven translocation methylcytosine dioxygenase
TF	transcription factor
Tfh	follicular helper T cell
TGF	transforming growth factor
TOP1MT	DNA topoisomerase I mitochondrial
TP53I13	tumor protein P53 inducible protein 13
Treg	regulatory T cell
TSS	transcription start site
VNTR	variable number of tandem repeats
WGBS	whole genome bisulphite sequencing
ZnT8A	zinc transporter 8 autoantibodies

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Pahkuri S, Ekman I, Vandamme C, Näntö-Salonen K, Toppari J, Veijola R, Knip M, Kinnunen T, Ilonen J, Lempainen J. DNA methylation differences within INS, PTPN22 and IL2RA promoters in lymphocyte subsets in children with type 1 diabetes and controls. Autoimmunity, 2023;56(1):2259118. https://doi:10.1080/08916934.2023.2259118

II Pahkuri S, Katayama S, Valta M, Nygård, L, Knip M, Kere J, Ilonen J, Lempainen J, Finnish Pediatric Diabetes Register. The effect of type 1 diabetes protection and susceptibility associated HLA class II genotypes on DNA methylation in immune cells. HLA, 2024;103(6):e15548. https://doi:10.1111/tan.15548

III Pahkuri S*, Valta M*, Junttila S, Schroderus AM, Kettunen J, Ollila H, Laine AP, Hyöty H, Knip M, Veijola R, Toppari J, Ilonen J, Rautava S, Kinnunen T*, Elo LL*, Lempainen J*. The impact of early life antibiotic exposure on the immune system. Manuscript. *Equal contribution.

1 Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease that develops when insulin producing beta cells in the pancreas are destroyed by the immune system, leading to life-long dependence of exogenous insulin injections (Katsarou et al., 2017). T1D can develop in any age but there is a peak in incidence in childhood before puberty (Gregory et al., 2022). The diagnosis is usually preceded by a non-symptomatic period of beta cell specific autoimmunity, which can be detected by the appearance of autoantibodies in blood, often years before diagnosis (Katsarou et al., 2017). According to current understanding, T1D develops in genetically susceptible people due to one or several environmental triggers.

About 50% of the disease risk is explained by genetics (Onengut-Gumuscu et al., 2025) and to this day more than 150 genes have been associated with T1D (Barrett et al., 2009; Onengut-Gumuscu et al., 2025; Robertson et al., 2021). HLA region has major role for the disease risk: 40–50% of the genetic risk is explained by polymorphisms of HLA class II genes in short arm of chromosome six (Noble et al., 1996). In addition, numerous other genes in multiple chromosomes have been found to have smaller impact (Onengut-Gumuscu et al., 2025). *INS*, *PTPN22* and *IL2RA* are the most important of these. However, T1D incidence has increased worldwide during the last decades (Onkamo et al., 1999), which is believed to be caused by changes in the environment. Viral infections, nutritional factors, changes in microbiome and antibiotics have been suspected to have a role (Rewers & Ludvigsson, 2016).

Epigenetics has been suggested to explain the interaction between genetics and environmental factors in the disease process. Epigenetics refers to heritable changes in gene expression that are not due to changes in DNA sequence (Cavalli & Heard, 2019). Epigenetic mechanisms include DNA methylation, post-translational modifications of histone proteins and RNA-mediated mechanisms. DNA methylation is perhaps the most studied of the mechanisms and means adding methyl groups to cytosines in the DNA strand (Jones, 2012). These methyl groups can then prevent transcription factors from binding to their target sequences, or attract methyl-binding domain proteins that recruit silencing complexes, thereby regulating gene

expression (Yin et al., 2017). DNA methylation can be affected both by genetic variation and by environmental stimuli (Cavalli & Heard, 2019).

The aim of this study was to clarify how genetic variation, associated with T1D, affects DNA methylation in immune cells like B cells and CD4+ and CD8+ T cells believed to have a significant role in the beta cell destruction leading to T1D development. We also examined how early antibiotic exposure affects long-term methylation in these cells. The aim was to better understand the mechanisms of genetic and environmental factors in T1D development. As the early antibiotic exposure and studied HLA haplotypes both have an effect on other autoimmune diseases as well, this study could also help clarify the mechanisms behind other common, polygenic immune-mediated diseases.

2 Review of the Literature

2.1 The epidemiology of type 1 diabetes

Type 1 diabetes incidence started to increase worldwide from the 1960s forward both in high and low incidence countries (Onkamo et al., 1999) although in some countries this increase may have already begun decades earlier (Gale, 2002). In Finland, the incidence more than doubled during 1980–2005 (Harjutsalo et al., 2008) and currently Finland has the highest T1D incidence in the world (Ogle et al., 2022). T1D incidence varies significantly between countries from 52.2 and 44.1 cases per 100 000 people in Finland and Sweden to 0.1–0.3 in low incidence countries such as Papua New Guinea, Venezuela and Ethiopia (Ogle et al., 2022).

Type 1 diabetes can develop in any age but there is a peak in incidence at 10–14-years old (Gregory et al., 2022). The biggest increase in incidence has been seen in the youngest children possibly due to an earlier age at diagnosis (Dahlquist et al., 2000; EURODIAB ACE Study Group, 2000; Harjutsalo et al., 2008; Karvonen et al., 1999; The DIAMOND Project Group, 2006).Therefore, it has been suggested that the rise in incidence is due to earlier clinical manifestation of T1D (Gale, 2005). Unlike most autoimmune diseases, T1D incidence is higher in men than in women although this seems to depend on the incidence level and population (Karvonen et al., 1997; Kyvik et al., 2004; Östman et al., 2008). However, no large differences seem to exist between girls and boys, although T1D incidence peaks at a younger age in girls compared to boys (Harjutsalo et al., 2008; Patterson et al., 2019; The DIAMOND Project Group, 2006).

Lately, some countries have reported a plateau or a decrease in the incidence, (Berhan et al., 2011; Cinek et al., 2012; Harjutsalo et al., 2013; McKenna et al., 2021; Parviainen et al., 2020; Skrivarhaug et al., 2014) but in general the incidence worldwide is still increasing about 3% annually (Patterson et al., 2019; The DIAMOND Project Group, 2006). In 2021 there were 8.4 million people diagnosed with T1D (Gregory et al., 2022). Especially in low-income and lower-middle-income countries the prevalence is increasing and by 2040 it is estimated that there will be 13.5–17.4 million people with T1D worldwide (Gregory et al., 2022).

The quick rise in T1D incidence worldwide is believed to be caused by changes in the environment. This is indicated by the speed of the change, the existence of

large differences in incidence between neighbouring countries and regions with similar genetic background (Kondrashova et al., 2005) and the fact that more individuals with lower genetic risk seem to be developing T1D (Gillespie et al., 2004; Hermann et al., 2003). Several different environmental exposures have been suspected to have a role (Rewers & Ludvigsson, 2016) but the results have often been contradictory or inconclusive.

2.2 The immunology of type 1 diabetes

Type 1 diabetes develops when insulin producing beta cells in pancreas are destroyed due to an autoimmune attack. This islet specific autoimmune response can last years before the actual clinical diagnosis and it can be detected by measuring islet specific autoantibodies in the blood (Katsarou et al., 2017). These are the non-specific islet cell autoantibodies (ICA) (Bottazzo et al., 1974) and antigen-specific insulin autoantibodies (IAA) (Atkinson et al., 1986), glutamic acid decarboxylase autoantibodies (GADA) (Baekkeskov et al., 1990), islet antigen-2 autoantibodies (IA-2A) (Bonifacio et al., 1995) and zinc transporter 8 autoantibodies (ZnT8A) (Wenzlau et al., 2007). Based on the presence of these autoantibodies and blood glucose levels, T1D development can be divided into three stages (Insel et al., 2015). In stage 1, two or more islet cell specific autoantibodies can be detected in blood but blood sugar is still normal. In stage 2, there are two or more autoantibodies and abnormal blood sugar. In stage 3, T1D is diagnosed. According to the American Diabetes Association, the criteria for type 1 diabetes diagnosis are hemoglobin-A1C ≥48 mmol/mol or fasting plasma glucose ≥7.0 mmol/l or 2-hour plasma glucose ≥11.1 mmol/l during an oral glucose tolerance test or plasma glucose level ≥11.1 mmol/l at any time of the day if the person also has symptoms (American Diabetes Association Professional Practice Committee, 2024). At this stage, critical amount of beta cells, although not all, have either been destroyed or they are not functional anymore, leading to insufficient amount of insulin production, hyperglycaemia and requirement of exogenous insulin (Atkinson & Mirmira, 2023).

The autoimmune attack in pancreatic islets that leads to beta cell destruction is called insulitis. Insulitis was first described in detail by Gepts et al. who observed significant beta cell destruction in individuals recently diagnosed with type 1 diabetes and also observed immune cell infiltration in the islets (Gepts, 1965). Later Willcox et al. observed that CD8+ T cells seem to be the most abundant immune cells in insulitic islets (Willcox et al., 2009). CD4+ T cells were also found but in fewer numbers. Other cells observed in islets were CD20+ B cells, which seem to be recruited more in later stages of insulitis, and macrophages. Natural killer (NK) cells and plasma cells were also found although more rarely. Based on their observations, insulitis affects insulin positive islets and the immune cells appear in a certain order as insulitis progresses. This is in line with

current understanding that both the innate and adaptive immune system take part in the autoimmune attack that finally leads to beta cell destruction and type 1 diabetes.

2.2.1 Innate immunity

The term innate immunity refers to the parts of our immune system and bodies, that function as a first line of defence against pathogens (Pradeu et al., 2024). This includes physical and chemical barriers that ward off pathogens, such as intact skin, stomach acid and enzymes in tears and saliva, but also immune cells, such as macrophages and dendritic cells, capable of phagocytosis, and natural killer (NK) cells, which can kill virus-infected cells. Additionally, innate cells like macrophages and dendritic cells have an important role as antigen presenting cells (APCs), meaning they can activate the adaptive immune system by engulfing pathogens and presenting their peptides to lymphocytes. Innate immunity uses receptors which recognize certain structural patterns often associated with pathogens. Innate immunity is faster than adaptive immunity, but it does not develop immunological memory but rather responds similarly to pathogens every time.

The role of innate immunity in T1D has been investigated, especially the role of professional APCs, like macrophages and dendritic cells, since in autoimmune reactions they could potentially present antigens to autoreactive T cells and activate them. Macrophages are phagocytes which, in addition to defence against pathogens, are also important for tissue homeostasis and clearing dying or damaged cells (Park et al., 2022). They originate from their embryonic progenitors during early development or from circulating monocytes, which differentiate into macrophages when they migrate from blood to tissues (Guan et al., 2025). Macrophages are therefore mainly found in tissues where they have varying roles (microglia in the brain, osteoclast in the bone tissue, Kupffer cells in the liver etc). Their scarcity in peripheral blood has made them challenging to study in humans. In animal models, macrophages seem to have a dramatic effect on T1D development (Hutchings et al., 1990; Parsa et al., 2012), but in humans their role is still unclear. However, they have been found from pancreatic islets in individuals with recent onset T1D, where a portion of them secreted pro-inflammatory cytokines (Uno et al., 2007). In one study, they were found to be more sensitive to LPS treatment than macrophages extracted from healthy controls (Plesner et al., 2002).

Studies have also found T1D-associated gene expression changes in monocytes extracted from individuals with established T1D (Irvine et al., 2012) and from autoantibody-positive individuals, where gene expression especially in genes related to viral infections was increased (Valta et al., 2022). Monocytes circulate in peripheral blood and they can migrate to tissues to differentiate into certain macrophage types but they can also secrete pro-inflammatory cytokines, function as

phagocytes and present antigens (Ożańska et al., 2020). Dendritic cells (DCs) are also effective APCs but additionally they can direct T cells by secreting cytokines, such as IL-12 and IL-15, and they can promote Treg differentiation (Cabeza-Cabrerizo et al., 2021). Altered DC frequency in blood has been reported in recent onset T1D (J. S. Allen et al., 2009; Nieminen et al., 2012; Vuckovic et al., 2007) and functional changes have also been reported (Nieminen et al., 2012; Rodriguez-Fernandez et al., 2019; Skarsvik et al., 2004).

2.2.2 Adaptive immunity

Adaptive immunity is much slower to initiate than innate immunity and first it has to be activated by innate immune cells. However, when activated, it creates a strong pathogen-specific immune response (Chi et al., 2024). It also generates immunological memory by producing memory cells capable of faster reaction if the same pathogen is encountered again. Adaptive immunity includes B cells and T cells which both originate from hematopoietic stem cells in the bone marrow. Thymus-seeding progenitors then migrate from the bone marrow to the thymus where T cell maturation takes place (Liang et al., 2025), whereas B cells continue their development in the bone marrow and almost completely reach their maturation and finally migrate to spleen to become fully functional (Gómez-Manríquez et al., 2025). T cells and B cells can then be activated when their target antigen peptide is presented to them along with other necessary signals. B cells recognize their target antigen using a B cell receptor (BCR) and T cells use a T cell receptor (TCR). Both are produced by random recombination of the immunoglobulin or T cell receptor gene segments in order to produce a completely unique receptor. This process produces countless variety of B and T cells with different receptor specificities and ensures that when a pathogen is encountered, there are lymphocytes capable of recognising it.

2.2.2.1 B cells and autoantibodies

The main function of B cells is to produce antigen-specific antibodies to fight pathogens. Antibodies are a soluble version of a B cell receptor. When B cells are activated, they can differentiate into antibody-producing plasma cells and the secreted antibodies can then coat pathogens in order to prevent them from infecting cells. Antibodies can also enhance phagocytosis of pathogens and even lead to the destruction of bacteria by activating the complement system. Some activated B cells form memory cells. In addition to antibody production, B cells are capable of presenting antigens to T cells (Rastogi et al., 2022) and there are several different B cell subsets such as follicular B cells, marginal zone B cells, B1 cells and regulatory B cells with different functions (Vale et al., 2015).

In T1D, B cells produce islet-specific autoantibodies IAA, GADA, IA-2A and ZnT8A that are used to detect beta cell-specific autoimmunity. These autoantibodies can appear very early in life and they predict T1D risk quite well: in individuals with a genetic susceptibility, the risk to develop T1D before the age of 15 years increases from 14.5% to 69.7% depending on whether they are positive for one or multiple autoantibodies (Ziegler et al., 2013). According to the first appearing autoantibody, T1D can also be divided into different endotypes (Battaglia et al., 2020). IAA is the most common first-appearing autoantibody (43.7% of the antibody-positive individuals) and GADA is the second most common (37.7%) (Krischer et al., 2015). IAA usually appears quite early: IAA incidence peaks within the first or second year of life and then declines whereas GADA incidence peaks 1–3 years later (Ilonen et al., 2013; Krischer et al., 2015). Individuals with IAA as the first-appearing autoantibody also seem to be diagnosed earlier than individuals with GADA (Ilonen et al., 2022), and they seem to have different genetic associations as well (Ilonen et al., 2013, 2022; Krischer et al., 2015).

Despite the importance of the autoantibodies in predicting T1D development, according to current hypothesis the autoantibodies are not involved in the actual beta cell destruction (Ziegler et al., 2013). However, it does still indicate that B cells have an important role in T1D development. Another strong indicator of this is that depletion of B cells in animal models prevents the development of T1D (Akashi, 1997; Xiu et al., 2008). Similar results have been seen in humans in the rituximab trial where anti-CD20 monoclonal antibody treatment was given to individuals with recent onset T1D (Pescovitz et al., 2009). Four injections during 22 days were given which depleted B cells and resulted in higher C-peptide levels and lower insulin requirement for the patients even a year later. B cells have also been found in pancreatic islets in individuals recently diagnosed with T1D (Leete et al., 2016; Willcox et al., 2009) It was observed that there were only few B cells in the beginning stage of insulitis but their numbers increased as insulitis progressed (Willcox et al., 2009) Additionally, people with higher numbers of B cells in the islets seem to develop T1D at a younger age (Leete et al., 2016).

Studies have reported T1D-associated changes in B cell frequency or function (De Filippo et al., 1997; El-Mokhtar et al., 2020; Ling et al., 2022; Nicholas et al., 2025; Smith et al., 2015). For example, Smith et al. showed that there were insulin-specific B cells among the anergic B cell compartment in healthy individuals, but these were absent in individuals with autoantibodies or recent onset T1D, and they suggested that this is possibly due to their activation by infection (Smith et al., 2015). They also showed that this loss of anergy was associated with genetic risk for T1D both in HLA and non-HLA risk genes (Smith et al., 2018). It is still unclear how B cells affect the autoimmune process but in animal studies there are observations that

it might be by B cells promoting the survival of cytotoxic CD8+ T cells (Brodie et al., 2008) or by presenting autoantigens to CD8+ T cells (Mariño et al., 2012).

2.2.2.2 CD4+ and CD8+ T cells

T cells can be divided into two major subsets: CD4+ and CD8+ T cells. CD4+ T cells are also known as helper T cells which, as the name suggests, coordinate immune responses and assist other immune cells. After being activated by APCs, they can differentiate into several different subsets depending on the signals they acquire (Table 1). Among the most well-known are Th1, Th2, Th17, follicular helper T (Tfh) cells, and regulatory T (Treg) cells (Saravia et al., 2019). Th1 cells are important for defence against both intra- and extracellular pathogens. They help macrophages kill bacteria that persist within their vesicles and also assist B cells in antibody production. Th2 cells are especially important against extracellular pathogens like parasites and they also assist B cells in antibody production and class switching, whereas Th17 cells are important against extracellular pathogens like fungi and they among other things enhance neutrophil functions. Tfh cells are specialized to provide help to B cells in antibody production. Treg cells on the other hand help maintain tolerance and suppress autoimmune reactions.

Table 1. Properties of major CD4+ helper T cell subsets: cytokines that induce their differentiation, major transcription factors, cytokines produced by the subsets and their main functions (Zhu & Zhu, 2020).

CD4+ T cell subset	Inducing cytokines	Transcription factors	Cytokines produced	Functions
Th1	IFN-γ, IL-12	STAT1, STAT4, T-bet	IFN-γ	Defense against intra- and extracellular pathogens
Th2	IL-2, IL-4	GATA3, STAT5, STAT6	IL-4, IL-5, IL-13	Defense against extracellular parasites
Th17	IL-1β, IL-6, IL-23, TGF-β	STAT3, RORγt	IL-17A, IL-17F, IL-22	Defense against extracellular pathogens
Tfh	IL-6, IL-21, ICOS	STAT3, Bcl6	IL-21	Supporting B cell maturation and antibody production
Treg	IL-2, TGF-β	FOXP3	IL-10, TGF-β	Immune regulation, suppressing autoimmunity

CD8+ T cells are also known as cytotoxic T cells. Although there are several CD8+ T cell subsets with different functions, their main task is to recognize and kill infected or malignant cells (Koh et al., 2023). After activation, they differentiate into effector cells which can kill infected or mutated cells using cytotoxic granules or by inducing apoptosis. They recognize their target antigen presented by HLA class I molecules, which all nucleated cells express on their surface to display their intracellular peptides.

Both CD4+ and CD8+ T cells are believed to have an important role in beta cell destruction and T1D development. One indication is the strong association between HLA class I and II genes and T1D (Noble et al., 1996). HLA molecules present antigen peptides to CD4+ and CD8+ T cells and are required for their activation and effector functions. Another indicator is the success of anti-CD3 immunotherapy, which specifically targets T cells: anti-CD3 antibody called Teplizumab significantly delayed T1D onset in high-risk subjects although it did not prevent it (Herold et al., 2019). Both CD4+ and CD8+ T cells have also been discovered from islets in samples from subjects with recent onset T1D (Willcox et al., 2009).

CD8+ T cells are believed to be responsible for the actual destruction of beta cells (Pinkse et al., 2005). They are the most abundant immune cell population in islets in insulitis (Willcox et al., 2009), and both islet- and insulin specific CD8+ T cells have been found from islets of subjects recently diagnosed with T1D (Anderson et al., 2021; Coppieters et al., 2012). Circulating CD8+ T cells that recognize insulin peptides have also been found in blood from people with T1D (Skowera et al., 2008). Coppieters et al. observed HLA class I hyperexpression in the islets, also reported in other studies (Foulis et al., 1987; Richardson et al., 2016), which is typical during an infection. When Krogvold et al. examined samples of pancreatic islets harvested from subjects with recent onset T1D, they found hyperexpression of HLA class I in all case samples but only one of the nine control samples (Krogvold et al., 2015). All samples from case subjects were positive for enterovirus capsid protein 1 (VP1) and three out of the six samples were also positive for enteroviral RNA indicating an infection of the islets during the T1D onset. HLA class I molecules are required for cytotoxic CD8+ T cells to target and destroy cells and in NOD mice both HLA class I expression and CD8+ T cells have been shown to be a requirement for insulitis and T1D development (Wicker et al., 1994).

Although CD8+ T cells seem to be the final effectors causing the beta cell death, they seem to require help from CD4+ T cells. CD4+ T cells have been found from the islets during insulitis (Willcox et al., 2009) and some of them have also been shown to be insulin-specific (Kent et al., 2005; Pathiraja et al., 2014). Th1 cells were long considered the most relevant CD4+ T cells in T1D, for example in NOD mouse it was shown that Th1 cells promoted T1D whereas Th2 cells had no effect (Katz et al., 1995). However, it seems that other CD4+ T cell subsets also have varying roles

in T1D. Increased frequency of Tfh cells have been reported in peripheral blood from individuals with T1D (Ferreira et al., 2015) and their memory T cells have been reported to express higher levels of Tfh markers (Kenefeck et al., 2015). Th17 cells have also been investigated: Marhawa et al. reported that subjects with T1D had a higher portion of IL-17 secreting CD4+ and CD8+ T cells in the peripheral blood than their controls (Marwaha et al., 2010) and Bradshaw et al. showed that monocytes extracted from diabetics more efficiently induced memory T cells to differentiate into IL-17 secreting cells (Bradshaw et al., 2009). There is also evidence that Treg cell frequency might be altered or their function impaired in people with T1D (Hull et al., 2017), perhaps due to genetics (Valta et al., 2020), which could then lead to activation of autoreactive T cells.

2.3 Genetics of type 1 diabetes

Genetics has a major impact on T1D development: T1D seems to develop mainly in people with genetic susceptibility, although only a small portion of the susceptible people develop the disease. 90% of new T1D cases do not have a close a relative with T1D (Turtinen et al., 2019) but having a sibling or parent with T1D increases the risk (Harjutsalo et al., 2006; Mrena et al., 2006; Turtinen et al., 2019). In monozygotic twins the concordance rate is more than 50% (Redondo et al., 1999, 2008) whereas in dizygotic twins the risk seems to be fairly similar to non-twin siblings at 6–13% (Mrena et al., 2006; Redondo et al., 1999; Triolo et al., 2018).

It has been estimated that genetics account approximately 50% of the T1D risk (Onengut-Gumuscu et al., 2025). Genes in the human leukocyte antigen (HLA) region have the biggest impact, especially alleles in HLA class II genes which account for 40–50% of the genetic risk (Lambert et al., 2004; Noble et al., 1996). The association between HLA region and T1D was first reported already in 1970s (Nerup et al., 1974; Singal & Blajchman, 1973). After that many new gene associations have been found, although with much smaller effects, and to this day over 150 gene regions outside the HLA have been associated with T1D risk (Barrett et al., 2009; Onengut-Gumuscu et al., 2025; Robertson et al., 2021).

2.3.1 Human leukocyte antigens (HLA)

Human leukocyte antigen (HLA) region is located in the short arm of the chromosome six and it contains over 200 genes, many of which have functions in the immune system (Figure 1) (Cruz-Tapias et al., 2013). It is the human equivalent of the major histocompatibility complex (MHC) in animals. HLA class I and II genes encode molecules that are expressed on the cell surface to present internal (HLA class I molecules) or external (HLA class II molecules) peptide antigens to the

immune system (Cruz-Tapias et al., 2013). There is also a variable group of genes in the region including e.g., complement components and cytokines often called as group III genes.

HLA class I genes HLA-A, -B and -C are expressed in almost all cells of the human body and, together with the invariable beta-2-microglobulin, they create HLA class I molecules (Figure 1) which present peptide antigens from inside the cell to cytotoxic CD8+ T cells (René et al., 2016). HLA class II genes, *HLA-DP, -DQ* and *-DR*, are expressed only in antigen presenting cells (APCs) (Jurewicz & Stern, 2019). Professional APCs include dendritic cells, macrophages and B cells. APCs use HLA class II molecules to present external peptides, that the cells have picked up, to CD4+ T cells, and they are required for activating immune responses. HLA class II molecules have an alpha and a beta chain: alpha chains are encoded by *HLA-DPA*, *-DRA*, and *-DQA* genes and beta chains are encoded by *HLA-DPB*, *-DRB* and *-DQB* genes (Figure 1).

HLA class I genes *HLA-A*, *-B* and *-C* and class II genes *HLA-DPB1*, *-DRB1* and *-DQB1* are the most variable genes in the human genome with thousands of known alleles, and because the HLA class II molecules are created from two chains, the number of possible combinations is even higher (Dendrou et al., 2018). This variability is centred in the DNA sequence coding the peptide binding groove, which determines the kind of peptides immune system can recognize and create an immune response to.

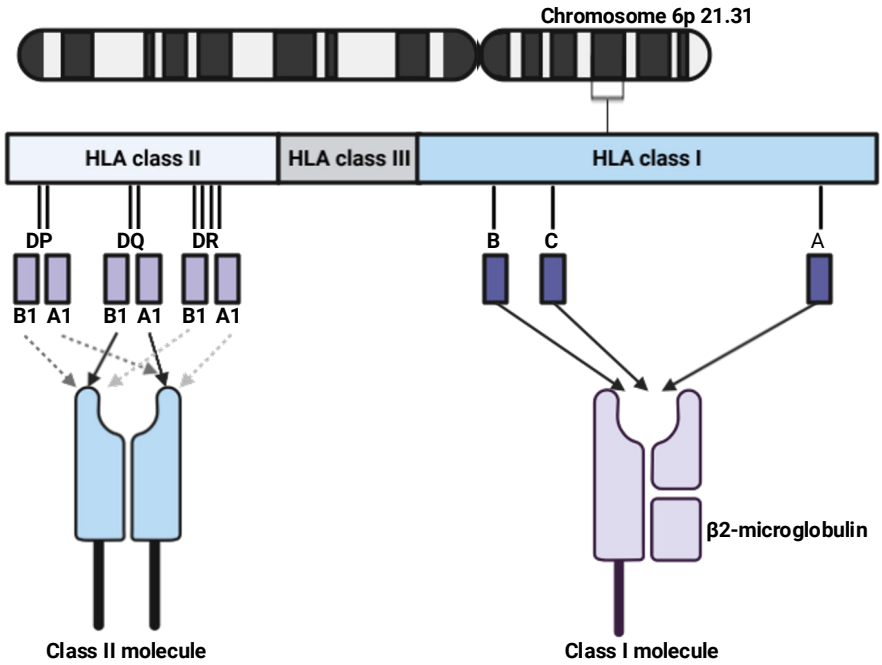

Figure 1. Schematic picture of the HLA region, located in the short arm of the chromosome six, and HLA class I and class II molecules. Created in BioRender. Lähteenmäki, S. (2026)

The most important T1D risk genes are located within the HLA region. HLA class II genes alone contribute about 40–50% of the genetic risk to T1D (Lambert et al., 2004; Noble et al., 1996). Due to the strong linkage disequilibrium, *DRB1*, *DQA1* and *DQB1* alleles are usually inherited together creating haplotypes with specific risk for T1D. Based on these haplotypes, people can be divided into multiple risk groups from strongly decreased risk to high risk (Ilonen et al., 2016). Two haplotypes, the *DRB1*03-DQA1*05-DQB1*02* (shortened as DR3-DQ2) and the *DRB1*04:01/2/4/5-DQA1*03-DQB1*03:02* (DR4-DQ8) are especially significant predisposing haplotypes: 90% of people with diabetes have either DR3-DQ2 or DR4-DQ8 or both (Redondo et al., 2018) and the highest risk is conferred by the DR3-DQ2/DR4-DQ8 heterozygote (Noble & Valdes, 2011). Interestingly the subtype of DRB1*04 allele in DR4-DQ8 haplotypes is also important. *DRB1*04:01, 04:02* and *04:05* are associated with high risk, whereas *DRB1*04:04* is associated with a moderate risk and further, *DRB1*04:03* and *DRB1*04:06* with protection (Thomson et al., 2007). On the other hand, the haplotype *DRB1*15-DQA1*01-DQB1*06:02* (DR2-DQ6) is strongly protective from T1D even when it is combined with a high-risk haplotype (Noble et al., 1996; Thomson et al., 2007). HLA class I genes HLA-A, -B and C have been associated with T1D development as well, independent of the HLA class II effects, although their contribution is smaller (Howson et al., 2009; Nejentsev et al., 2007; Noble et al., 2010; Valdes et al., 2005). Especially the HLA-A*24 (Noble et al., 2002, 2010) and HLA-B*39 (Howson et al., 2009; Valdes et al., 2005) alleles have been reported to predispose to T1D.

The same HLA haplotypes affecting T1D risk are also significant in some other autoimmune diseases as well. The DR3-DQ2 and DR4-DQ8 haplotypes, which are both significant predisposing haplotypes for T1D, also confer risk for celiac disease, although in celiac disease the DR3-DQ2 is the major risk haplotype (Liu et al., 2014). On the other hand, the *DRB1*15-DQB1*06:02* (DR2-DQ6), protective for T1D, confers risk to narcolepsy with cataplexy (Capittini et al., 2018) and multiple sclerosis (MS) (De Silvestri et al., 2019).

HLA haplotypes conferring high or moderate risk, compared to lower risk haplotypes, are associated with earlier disease onset and more aggressive symptoms at the time of diagnosis (Kieleväinen et al., 2023; Taka et al., 2022). There are differences in the disease process between the high risk haplotypes as well: the DR4-DQ8 has been associated with IAA as first appearing autoantibody with earlier disease onset, whereas the DR3-DQ2 has been associated with GADA as first appearing autoantibody with later disease onset (Krischer et al., 2015, 2017). Therefore, it has been hypothesized that these two haplotypes associate with different disease endotypes that have different disease etiologies (Battaglia et al., 2020). Whereas the HLA class II haplotypes seem to be important for the initiation of beta cell autoimmunity, HLA class I genes on the other hand have been associated

with progression from autoimmunity to T1D (Balke et al., 2018; Lipponen et al., 2010). This is not surprising considering that the HLA class II genes encode molecules used by APCs for peptide presentation, important for the initiation of immune responses, whereas HLA class I molecules present peptides to already activated cytotoxic CD8+ T cells.

2.3.2 Type 1 diabetes susceptibility genes outside the HLA region

After the initial discovery of the association between HLA region and T1D, many other risk loci have been discovered: first *INS* in 1984 (G. I. Bell et al., 1984), then *CTLA4* (Nisticò et al., 1996), *PTPN22* (Bottini et al., 2004), *IL2RA* (Vella et al., 2005) and *IFIH1* (D. J. Smyth et al., 2006). Similarly to HLA region, most of these are associated with immune system functions either in T cells (*CTLA4, PTPN22* and *IL2RA*) or in innate immunity (*IFIH1*). In later genome-wide analyses, more associations have been found: currently over 70 genes and over 100 gene regions have been associated with T1D risk (Barrett et al., 2009; Onengut-Gumuscu et al., 2015; Robertson et al., 2021; The Wellcome Trust Case Control Consortium et al., 2007). Many of the new discovered genes are involved with immune system as well (Pociot & Lernmark, 2016) and they have been reported to be enriched especially in enhancer regions which are active in CD4+ T cells (Onengut-Gumuscu et al., 2015; Robertson et al., 2021) but also in other immune cells such as CD8+ T cells and B cells (Onengut-Gumuscu et al., 2015).

Some risk SNPs are associated with beta cell autoimmunity and some with progression to clinical T1D and some of these associations may be endotype specific (Laine et al., 2022; Lempainen et al., 2015; Sharma et al., 2018). Despite the new discoveries, most of these regions have very small effects and outside the HLA region, strongest effects are still for *INS, PTPN22* and *IL2RA* (Laine et al., 2013).

2.3.2.1 Insulin (*INS*)

Insulin gene (*INS*) is located in the short arm of chromosome 11 and it encodes the peptide hormone insulin. It was first associated with T1D in 1984 in Caucasians (G. I. Bell et al., 1984) and later more specifically mapped to a variable number of tandem repeats (VNTR) locus in the region upstream from *INS* (Bennett et al., 1995). The *INS-VNTR* alleles can be split into three groups based on their size: class I (small), class II (intermediate) and class III (large), where the class I is associated with increased T1D risk and class III is protective of T1D (G. I. Bell et al., 1984; Bennett et al., 1996).

Allelic variation in *INS-VNTR* correlates with *INS* transcription and, compared to the class I, the protective class III is associated with lower expression of *INS* in the pancreas (Bennett et al., 1995, 1996; Vafiadis et al., 1996) but higher expression of *INS* in the thymus (Pugliese et al., 1997; Vafiadis et al., 1997). Because of this it has been suggested that protective class III allele leads to higher presentation of insulin peptides in the thymus leading to more effective pruning of autoreactive T cells during T cell selection and therefore lower risk of beta cell specific autoimmunity (Pugliese et al., 1997).

INS-VNTR is in near complete linkage disequilibrium (LD) with two SNPs in the region: -23HphI (rs689) and -1140A/C (Barratt et al., 2004) which is why rs689 has been used as a marker for *INS-VNTR* in genetic studies. The association between rs689 and T1D has later been replicated in Finnish population (Laine et al., 2007, 2013). It has also been associated with seroconversion to first beta cell specific autoantibody (Hermann et al., 2005; Laine et al., 2022). In addition to rs689, there is also other variation in the *INS* locus that have been associated with T1D such as rs7111341 (Barrett et al., 2009).

2.3.2.2 Protein tyrosine phosphatase non-receptor type 22 *(PTPN22)*

Protein tyrosine phosphatase non-receptor type 22 *(PTPN22)* gene is expressed in lymphocytes and some other immune cells such as neutrophils (Armitage et al., 2021). In T cells, it is a part of the TCR signaling, and functions as a negative regulator of T cell activation (Cloutier & Veillette, 1999; Vang et al., 2012). It is also involved in BCR signaling in B cells (Arechiga et al., 2009). A mutation 1858 C/T in *PTPN22* was first associated with T1D risk in 2004 (Bottini et al., 2004) and this association has later been replicated in other studies and populations (Hermann et al., 2006; Laine et al., 2013; Onengut-Gumuscu et al., 2004; D. Smyth et al., 2004). The mutation, also known as rs2476601, causes an arginine-to-tryptophan substitution at position 620 (R620W) (Bottini et al., 2004). It has been associated with seroconversion to the first beta cell specific autoantibody and also progression to clinical diagnosis after seroconversion (Hermann et al., 2006; Krischer et al., 2019; Laine et al., 2022; Lempainen et al., 2012; Steck et al., 2014).

Despite these associations, it is still unclear what the impact of the SNP is on *PTPN22* function and whether it is a gain-of-function or loss-of-function mutation. In the case of gain-of-function, the molecule encoded by the risk allele would be more active and dephosphorylate signaling molecules more efficiently leading to decreased signaling from the TCR and decreased T cell activation. In the case of loss-of-function, there would be less efficient dephosphorylation, stronger signal from TCR and more T cell activation. Most research supports the gain-of-function

hypothesis (Aarnisalo et al., 2008; Rieck et al., 2007; Vang et al., 2005), although there are also some contradictory results (J. Zhang et al., 2011).

The risk allele has been associated with alterations in T cell and B cell compartments (Chemin et al., 2018; Rieck et al., 2007) and reduced response to antigen stimulation by CD4+ T cells (Aarnisalo et al., 2008; Rieck et al., 2007). Increased frequency of total and naïve Treg cells in peripheral blood has also been reported (Valta et al., 2020). In addition to rs2476601, other SNPs in *PTPN22* region have also been examined but only rs56048322 has been associated with T1D independent of rs2476601 (Ge et al., 2015; D. J. Smyth, Cooper, et al., 2008).

2.3.2.3 Interleukin 2 receptor subunit alpha *(IL2RA)*

Interleukin 2 receptor subunit alpha *(IL2RA*) gene encodes the high affinity alpha chain of IL-2 receptor, called CD25. IL-2 signaling is important for T cell activation and proliferation but especially for regulatory T cell development, maintenance and homeostasis in the periphery (Toomer et al., 2019; J. Wang et al., 2009). The high affinity alpha chain encoded by *IL2RA* is expressed continuously in Tregs, but only when induced in other T cells where it is considered a marker for activation (J. Wang et al., 2009). *IL2RA* gene region was first associated with T1D in 2005 (Vella et al., 2005). The association was soon confirmed in other studies, which identified several T1D-associated SNPs in the region (Lowe et al., 2007; Maier et al., 2009; Qu et al., 2007). Due to their linkage disequilibrium, it has been difficult to determine the causal SNPs but there seems to be several SNPs with an independent effect, among others rs12722495 (Laine et al., 2013; D. J. Smyth, Plagnol, et al., 2008) and rs2104286 (Laine et al., 2013; Maier et al., 2009; The Wellcome Trust Case Control Consortium et al., 2007). Several studies have reported that T1D associated *IL2RA* genotypes correlate with lower level of soluble sIL-2RA (Keindl et al., 2020; Lowe et al., 2007; Maier et al., 2009) but the effects of this are still unclear. In addition, they have been reported to affect surface expression of CD25 in CD4+ memory T cells (Dendrou et al., 2009).

2.4 Epigenetics of type 1 diabetes

2.4.1 Epigenetics

The term epigenetics refers to heritable changes in DNA expression that are not caused by changes in DNA sequence. It includes several different mechanisms that regulate DNA expression in long term fashion and which can be inherited in mitosis to daughter cells or even in meiosis to offspring, called transgenerational epigenetic inheritance (Cavalli & Heard, 2019). These mechanisms include DNA methylation,

histone modifications and RNA-mediated mechanisms (Figure 2). DNA methylation is a mechanism where gene expression is regulated by adding a methyl group to cytosines in the DNA strand (Jones, 2012). These methyl groups can then work as attachment sites for transcription factors or other molecules and their effect on gene expression depends on their location in the strand (promoter, coding sequence, insulator etc). For example, active genes are often unmethylated in their promoter regions and methylated in gene body (Ball et al., 2009; H. Han et al., 2011). Gene expression can also be regulated by attaching molecules, such as methyl, acetyl and phosphate groups, to histones – either histone tails or their globular domains – called post-translational histone modifications (Millán-Zambrano et al., 2022). These groups often then recruit other proteins able to bind to them or they can directly control gene expression by affecting the charge of the histone proteins: this affects how tightly the histones are bound to the DNA strand and therefore the structure of the chromatin and whether the DNA strand is accessible to transcription factors or not (Millán-Zambrano et al., 2022). RNA-mediated mechanisms often function together with other epigenetic modifications: for example, non-coding RNAs can bind to their complementary sites and then create scaffold structures that function as binding sites for histone and DNA methyltransferases (Holoch & Moazed, 2015). All these mechanisms interact with each other to jointly regulate gene expression often in long term but reversible manner and together they form the epigenome of the cell.

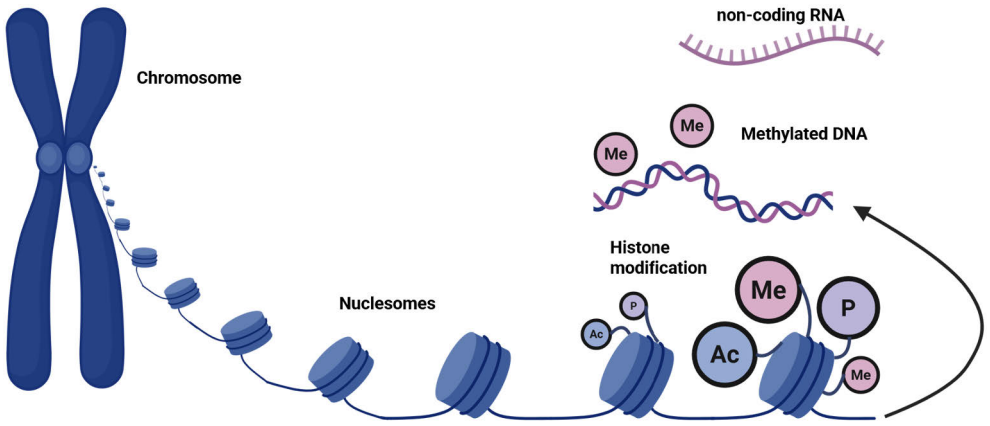

Figure 2. Depiction of the most important epigenetic mechanisms, which include DNA methylation, histone modifications and non-coding RNA-mediated mechanisms. Ac, acetylation; Me, methylation; P, phosphorylation. Created in BioRender. Lähteenmäki, S. (2026)

2.4.2 DNA methylation

DNA methylation is an epigenetic mechanism where gene expression is regulated by attaching methyl groups to cytosines in the DNA strand, specifically to cytosines that are next to a guanine, in cytosine-guanine-dinucleotides known as CpG sites (Jones, 2012). CpG sites are distributed unevenly in the genome: most of the genome has less CpG sites than expected, but there are also CpG dense regions called CpG islands (CGI) (Figure 3) (R. S. Illingworth & Bird, 2009). This is most likely because most CpG sites outside CGIs are methylated (Lister et al., 2009), and methylated cytosines are vulnerable to mutations (Duncan & Miller, 1980), whereas over 90% of CpG islands are unmethylated both in somatic tissues and in germ line cells (R. Illingworth et al., 2008; R. S. Illingworth & Bird, 2009) which protects them from mutations and keeps them CpG dense. Therefore, the genome can be divided into CpG enriched CGIs, the edges of CGIs knowns as shores, shelve regions outside the shores, and finally to CpG-poor open sea (Figure 3). About 70% of human genes have a CGI in their promoter region, many of them housekeeping genes (Saxonov et al., 2006).

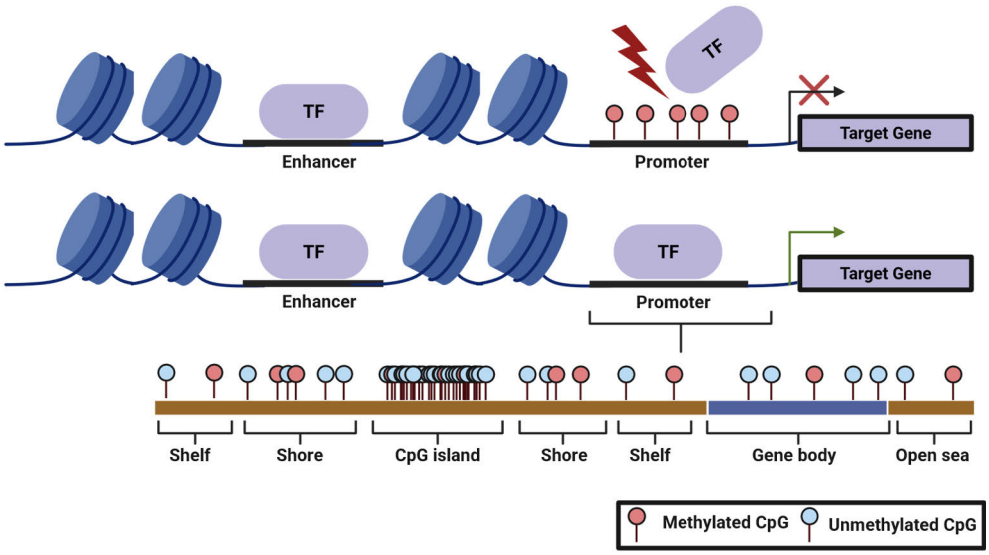

Figure 3. Simplified depiction of the effect of DNA methylation in promoter regions on gene expression: methylated CpG sites can prevent transcription factors from binding to the promoter leading to gene silencing. CpG islands are often located in gene promoters and are usually unmethylated. The regions surrounding them are knowns as shores and shelves. TF, transcription factor. Created in BioRender. Lähteenmäki, S. (2026)

CpG sites can be methylated by enzymes called DNA methyltranferases (DNMTs) (Lyko, 2018). There are three DNMTs with an enzymatic activity:

DNMT1, DNMT3a and DNMT3b. DNMT3a and DNTM3b attach methyl groups to novel sites (de novo methylation) and they are important for establishing original methylation patterns in early development. DNTM1 takes care of maintaining the methylation patterns after mitosis by targeting hemimethylated sites where only one strand is methylated. Demethylation can happen passively during mitosis: when DNA is replicated, the new strand has no methyl groups unless the DNMT1 adds them. Demethylation can also happen actively by enzymes called ten-eleven translocation methylcytosine dioxygenases (TETs) (X. Zhang et al., 2023).

When DNA methylation was first discovered, it was associated with gene silencing, but later it has been shown that the effect depends on the gene region. In actively transcribed genes, promoter regions or regions close to the transcription start site (TSS) are usually unmethylated, whereas methylation of these regions usually associate with gene silencing (Anastasiadi et al., 2018; Brenet et al., 2011; H. Han et al., 2011) Promoters can be divided into two groups: promoters with a CGI and promoters lacking a CGI, i. e. CpG-poor promoters (Saxonov et al., 2006). Many genes with CGI promoters are housekeeping genes whereas genes with CpG-poor promoters seem to be more often tissue-specifically expressed (Saxonov et al., 2006). It should be noted that some CGIs also exist in gene bodies where some of them have been shown to be methylated in tissue-specific manner and it has been hypothesized that they may be alternative promoters (R. Illingworth et al., 2008; Maunakea et al., 2010; Sarda et al., 2017).

Unlike promoters, gene bodies in actively transcribed genes are usually methylated (Ball et al., 2009), possibly to prevent improper transcription initiation (Neri et al., 2017) or to assist in splicing (Maunakea et al., 2013). Therefore, it seems that methylation suppresses transcription initiation but not elongation. Methylation also seems to be important for regulation of enhancer activity: enhancers are usually CpG-poor and variable methylated regions (Lister et al., 2009; Stadler et al., 2011). Stadler et al. distinguished them from other regions based on their low methylation level (~ 30%). Enhancers have cell subset specific methylation (Schmidl et al., 2009; Stadler et al., 2011) and their methylation status inversely correlates with their activity (Wiench et al., 2011).

Methyl-groups in CpG sites can prevent gene expression by preventing transcription factors (TF) from binding to the promoter, although sometimes they can also enable the attachment of certain TFs (Yin et al., 2017). Methyl-groups can also function as attachment sites for proteins with a methyl-binding-domain (MBD) which then usually recruit histone modifying enzymes such as histone deacetylases (HDAC) leading to repression of gene expression (Du et al., 2015). Histone modifications are also important for directing de novo methylation (Ooi et al., 2007; Weinberg et al., 2019).

During early development, cells undergo methylation and demethylation in large waves, and proper function of DNMTs and TETs is essential (Messerschmidt et al., 2014). Therefore, it is not surprising that DNA methylation has been shown to have important role in processes such as X-chromosome inactivation (Sado et al., 2000), genomic imprinting (Pervjakova et al., 2016) and cellular differentiation (Reik, 2007).

2.4.3 Genetic variation and DNA methylation

Genetic variation has an impact on DNA methylation. Loci that impact DNA methylation are called methylation quantitative trait loci (mQTL or meQTL): if the locus or SNP is close to the CpG site, it is called cis-mQTL, and if it is farther away, sometimes even on a different chromosome, it is called trans-mQTL (Villicaña & Bell, 2021). The association between mutation and methylation could be due to a SNP directly at the CpG site: if the cytosine or guanine changes into another nucleotide, the site can no longer be methylated. SNPs can also affect attachment sites for methyl-CpG-binding proteins or transcription factors (Banovich et al., 2014; M. Wang et al., 2019), which could then directly recruit DNMTs or TETs to the region. SNPs in coding region of enzymes involved in methylation such as DNMTs or TETs could lead to wider methylation changes (Potter et al., 2013).

mQTLs have been discovered in many studies and using different tissues (J. T. Bell et al., 2011; Gaunt et al., 2016; Hannon et al., 2016; Min et al., 2021). The associations seem to be stable as shown when comparing fetal and adult brains (Hannon et al., 2016) or blood samples from different stages of life (Gaunt et al., 2016) although there appears to be some mQTLs specific for a developmental stage (Hannon et al., 2016). It has been estimated that genetic variation explains about 20% of the variation in CpG methylation (Gaunt et al., 2016; McRae et al., 2014; van Dongen et al., 2016). This number seems to decrease slightly over time from childhood to middle-age possibly due stochastic changes or changes caused by the environment (Gaunt et al., 2016).

Studies have shown that 45–48.5% of all CpG sites are associated with a mQTL (McRae et al., 2014; Min et al., 2021). Majority of mQTLs are cis-associations and only about 5–8% trans-mQTLs (Gaunt et al., 2016; Hannon et al., 2016; Min et al., 2021; Volkov et al., 2016). Although there are less trans-mQTLs than cis-mQTLs, they also seem to be important and it seems that in general cis- and trans-mQTLs are located in different genomic regions with possibly different roles (Lemire et al., 2015; McRae et al., 2018; Shi et al., 2014).

2.4.4 Environmental factors and DNA methylation

In addition to genetic variation, also environmental exposures can affect the epigenome (E. M. Martin & Fry, 2018) and some of these changes have been shown to persist even years after the environmental exposure is gone (Kodal et al., 2018).

One example of this was seen in animals when Weaver et al. observed that maternal behaviour in rats had an effect on DNA methylation in offspring (Weaver et al., 2004). They divided rat mothers with their pups into two groups based on whether the mothers showed high or low levels of maternal behaviours including pup licking, grooming and nursing. Compared to the low-level group, offspring of mothers with high levels of these maternal behaviours had significantly lower methylation in the promoter region of glucocorticoid receptor (*GR*) gene. This methylation difference appeared during the first week of life and persisted into adulthood.

In humans a famous example comes from the Dutch famine in 1944–1945 when Germans imposed a food embargo on the region: using samples from individuals, who were conceived during the famine, and their siblings, who were conceived before or after the famine, Heijmans et al. examined the effects of maternal malnutrition during early pregnancy on the offspring (Heijmans et al., 2008). They found that offspring, who were exposed to famine during pregnancy, had lower DNA methylation in five CpG sites in the insulin-like growth factor 2 (*IGF2*) gene than their control siblings and that this difference persisted over six decades.

Some other environmental exposures, that have been associated with methylation changes in humans, are air pollution, smoking, nutritional factors, some chemicals such as aflatoxin B_1, arsenic, bisphenol A, persistent organic pollutants (POPs) like polycyclic aromatic hydrocarbons (PAHs) and also some nonchemical exposures such as maternal anxiety and depression (E. M. Martin & Fry, 2018). Based on the observations, the effects may depend on the timing of the exposure: for example, air pollution was reported to have opposite effects on long interspersed nuclear element-1 (LINE1) methylation during first and third trimester of pregnancy (Breton et al., 2016). LINE1 is a group of transposons in human genome and it has recently been associated with long-range transcription changes (Li et al., 2024). The importance of timing is not surprising considering that somatic cells and germ cells undergo large methylation changes during early development (Messerschmidt et al., 2014) and it is possible that certain developmental phases are more sensitive than others for disruption. There is little information yet about the molecular mechanisms behind these associations or their possible consequences for health. However, one possible route is that malnutrition, lack of specific nutrients or certain chemicals could affect the availability of methyl group donors and therefore lead to methylation changes (Agodi et al., 2015).

2.4.5 Type 1 diabetes and DNA methylation

Since both genetic variation and environment have an impact on DNA methylation and type 1 diabetes develops in genetically susceptible individuals due to environmental triggers, it has been proposed that DNA methylation could explain how these factors interact in the disease pathogenesis. Many T1D-risk alleles especially outside the HLA region are located in non-coding regions and have no clear functional effects on the genes, but they could have an effect on the methylome either close-by (cis-mQTLs) or farther in the genome (trans-mQTLs). On the other hand, environmental triggers could cause long-lasting changes in the epigenome leading to gene expression differences, even without genetic differences.

T1D-associated methylation differences have been discovered from T1D risk genes, such as *INS* (Fradin et al., 2012), *IL2RA* (Belot et al., 2013) and *TNF* (Arroyo-Jousse et al., 2016). Fradin et al. reported T1D-associated methylation in four CpG sites in *INS* promoter (Fradin et al., 2012). Others have later confirmed this result (Carry et al., 2020) and it has also been shown that methylation in this region correlates with *INS* expression (Kuroda et al., 2009). Belot et al. reported similar results in *IL2RA* promoter (Belot et al., 2013) and Arroyo-Jousse et al. in *TNF* promoter (Arroyo-Jousse et al., 2016).

Methylation changes have also been found in epigenome-wide analyses (Belot et al., 2017; Johnson et al., 2020; Rakyan et al., 2011; Stefan et al., 2014). Rakyan et al. examined monocytes extracted from T1D-discordant monozygotic twins and discovered 132 differentially methylated sites: 58 hyper and 74 hypomethylated sites with small or moderate methylation changes (0.13–6.6 %) (Rakyan et al., 2011). These included immune system related genes *HLA-DQB1*, *RFXAP*, *NFKB1A* and *TNF* (Rakyan et al., 2011). Stefan et al. examined an immortalized B cell line, also extracted from T1D-discordant twins, and found 88 differentially methylated sites (55 hyper and 33 hypomethylated) including some in known T1D risk genes such as *HLA-DQA2*, *INS*, *IL2RB* and *CD226* (Stefan et al., 2014). In another array study, Belot et al. analyzed over 900 000 CpG sites in whole blood but discovered only one significant DMR: the region contained gene called *RTL1*, expressed only in placenta, and two miRNAs called miR136 and miR432 (Belot et al., 2017).

Most of these analyses have been done with samples from people with an established disease. This raises the question, whether the methylation differences are a result of the disease process rather than causal in T1D pathogenesis. However, Rakyan et al. observed some methylation differences already in stage 1 (2≤ autoantibodies, normoglycemia) in individuals who later progressed to T1D (Rakyan et al., 2011). This observation is also supported by results from Starskaia et al. who examined epigenome-wide methylation in longitudinal samples from autoantibody-positive children and their healthy controls and found methylation changes associated with beta cell autoimmunity, some of which existed already before

seroconversion (Starskaia et al., 2022). In another longitudinal study, Johnson et al. analyzed samples collected before T1D onset, both from before and after seroconversion (Johnson et al., 2020). They found 28 longitudinally differentially methylated regions between cases and controls. They also analyzed cord blood samples and some of these changes existed already at the time of birth. However, Laajala et al. also analyzed cord blood samples and did not see changes in children who later developed T1D (Laajala et al., 2022). Therefore, it seems that some differences may exist already at birth but most of them probably appear later in the disease process.

In an interesting study, Carry et al. compared methylation in reverters (people who were autoantibody positive but then lost the autoantibodies), maintainers (people who stayed autoantibody-positive) and progressors (people who progressed from autoantibody-positivity to T1D), and also compared the methylation changes between samples collected before and after seroconversion (Carry et al., 2024). They found one CpG site (cg16066195), located near a gene called *CLIP2* relevant for beta cell function, and 11 gene regions, where DNA methylation changes were different between the groups. They also found regions where methylation levels both before and after seroconversion were different between the groups (*NRIP2*, *ITFG2*).

In addition to T1D-associated methylation changes, T1D-associated genetic variation also seems to have an independent effect (Carry et al., 2020; Kindt et al., 2018; Ye et al., 2018). In their 2012 study, Fradin et al. observed that rs689, a known T1D risk SNP, was associated with methylation in four CpG sites in *INS* promoter (Fradin et al., 2012), result later confirmed by others (Carry et al., 2020; Ye et al., 2018). Similarly, Belot et al. discovered an association between 28 SNPs located in the *IL2RA* region and methylation at one CpG site in *IL2RA* promoter (Belot et al., 2013). Later, in wider analyses many T1D-associated SNPs have been associated with methylation epigenome-wide (Carry et al., 2020; Ye et al., 2018). Ye et al. examined the effect of 64 top GWAS results on methylation in 55 non-HLA loci and found 95 cis-mQTLs and 1 trans-mQTL (Ye et al., 2018). They concluded that especially in five genes, *ITGB3BP*, *AFF3*, *PTPN2*, *CTSH* and *CTLA4*, DNA methylation might mediate the effect of the SNP on the gene expression. Carry et al. examined 13 T1D-associated SNPs on epigenome-wide methylation using Illumina 450K chip and found 13 SNP-CpG pairs: 4 SNPs, located in *GSDMB*, *C1QTNF6*, *IL27* and *INS*, had significant effect on methylation in 13 CpG sites. One study also examined the effect on T1D-associated HLA haplotypes: Kindt et al. compared epigenome-wide methylation between DR3-DQ2 homozygotes, DR3-DQ2/DR4-DQ8 heterozygotes and DR4-DQ8 homozygotes and found 196 differentially methylated sites, many of which were located in chromosome six and HLA region (Kindt et al., 2018). These results indicate that at least part of the effect of T1D-associated genetic variation could be mediated through methylation.

Some studies have also found interesting interactions between genetic variation and environmental factors. Ye et al. showed that methylation in a known T1D-risk gene *CTSH* correlated with gene expression and that the methylation was affected by both genetic variation and cytokines (Ye et al., 2021). Interestingly, they also showed that people with the protective variant seemed to have more variation in the methylation, indicating that they might be more sensitive to the effect of cytokines. Vigers et al. on the other hand, examined associations between the methylome, certain metabolites and islet autoimmunity and found seven CpG-metabolite pairs (Vigers et al., 2021). The metabolites were positively associated with islet autoimmunity whereas the methylation of the CpG sites was negatively associated with both the metabolite and islet autoimmunity. Vigers et al. concluded that the protective effect of the CpG methylation might be due to their effect on the metabolite levels. These studies highlight the complex interactions between the epigenome and environment and their possible effects on T1D development.

2.5 Environmental factors and type 1 diabetes

Although genetics has a major impact on T1D development, the increase in T1D incidence during the last century (Onkamo et al., 1999) is believed to be caused by changes in the environment. In addition to the quick change in incidence, this is indicated by the fact that more people with lower genetic risk are developing the disease (Fourlanos et al., 2008; Gillespie et al., 2004; Hermann et al., 2003) and there are large differences in incidence between regions close to each other and with similar HLA genotype frequencies (Kondrashova et al., 2005). There is also evidence that T1D incidence increases for children of migrants from low incidence countries if they are born in a high incidence country (Oilinki et al., 2012; Söderström et al., 2012). Due to the early development of beta cell autoimmunity and T1D, environmental exposures during pregnancy or early childhood most likely have the biggest impact. There has been some large study projects designed to investigate the effect of environmental factors in the development of T1D, such as the BABYDIAB (Hummel & Ziegler, 2011), the Diabetes Autoimmunity Study in the Young (DAISY) (Rewers et al., 1996), the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) (Nejentsev et al., 1999), The Environmental Determinants of Diabetes in the Young (TEDDY) (Lernmark et al., 2025; The TEDDY Study Group, 2007) and the Environmental Determinants of Islet Autoimmunity (ENDIA) (Oakey et al., 2023). Many environmental factors have been investigated over the years including infections (Sioofy-Khojine et al., 2018; Vehik et al., 2019), vaccinations (Elding Larsson et al., 2018; Morgan et al., 2016), nutrition (Hummel et al., 2017; Virtanen, 2016), birth mode (Clausen et al., 2016), microbiome (Vatanen et al.,

2018), antibiotics (Kilkkinen et al., 2006) and psychological and psychosocial factors (Nygren et al., 2013, 2015) but many of these results have been contradictory or inconclusive.

2.5.1 Infections

Infections have long been suspected of having a role in T1D development. There is seasonal variation in T1D incidence that could reflect seasonal variation in some infections (Gamble & Taylor, 1969; Green et al., 2001; Turtinen et al., 2022) and ketoacidosis and beta cell destruction have also been observed after acute infections (Yoon et al., 1979). Direct infection of pancreas or beta cells could cause beta cell destruction and trigger beta cell specific autoimmunity. Infection could also accelerate an on-going autoimmunity. It is also possible that some viral peptides resemble autoantigens in beta cells and infection leads to a cross reaction, phenomenon known as molecular mimicry.

Already in 1969 Gamble et al. reported an association between recent onset T1D and antibodies against Coxsackievirus B4 (CVB4) (Gamble et al., 1969). Subsequently, it has been shown that infections in early life are associated with autoimmunity and T1D diagnosis (Hyöty et al., 1995; Kordonouri et al., 2022; Lönnrot et al., 2017; Salminen et al., 2003) and that also maternal infections during pregnancy might have an effect on T1D risk in offspring (D. W. Allen et al., 2018; Bélteky et al., 2020; Viskari et al., 2012).

In addition to evidence from association studies, enterovirus RNA and viral protein VP1 have been discovered in pancreas of people who have recently been diagnosed with T1D (Krogvold et al., 2015; Oikarinen et al., 2021; Ylipaasto et al., 2004). Krogvold et al. even demonstrated that the samples collected from patients with recent onset T1D contained infectious viruses, as they were able to infect new cell cultures (Krogvold et al., 2022). They did not find signs of other viruses (Krogvold et al., 2022). Some enteroviruses are able to cause persistent infection of human islets (Chehadeh et al., 2000) and the results from Krogvold et al. also point towards a long-term mild infection due to the observed low RNA and VP1 levels (Krogvold et al., 2015). Recently they also showed that using antivirals pleconaril and ribavirin preserved beta cell function in people with recent onset T1D (Krogvold et al., 2023).

The effect of SARS-CoV-2 infection on T1D incidence has also been investigated but the results have been contradictory (Kamrath et al., 2022; Knip et al., 2023; Reschke et al., 2022; van den Boom et al., 2022; Zareini et al., 2023). However, most studies seem to indicate that the possible increase in T1D incidence is not caused directly by the SARS-CoV-2 infection but rather other effects of the pandemic such as lockdowns (Kamrath et al., 2022; Zareini et al., 2023).

2.5.2 Nutrition

Several nutritional factors have been investigated in relation to T1D, but most studies have had contradictory results. Breastfeeding and infant formula are perhaps the most studied nutritional factors. Some studies have found breastfeeding duration to be associated with islet autoimmunity or T1D (Holmberg et al., 2007; Lund-Blix et al., 2014, 2017) but other studies have failed to find an association (Frederiksen et al., 2013; Virtanen et al., 2006; Ziegler et al., 2003). However, in a meta-analysis by Cardwell et al. an exclusive breastfeeding for more than two weeks protected from T1D (Cardwell et al., 2012). This is in line with results from Lund-Blix et al. who reported that breastfeeding duration did not have an effect but, if there were no breastfeeding at all, the risk was increased (Lund-Blix et al., 2017).

Studies have also examined the effect of formula and in some studies early introduction of formula has been associated with increased risk of islet autoimmunity (Holmberg et al., 2007; Hummel et al., 2017; Wahlberg et al., 2005). Similarly, there are also some reports that early introduction of solid food or certain foods like cereal, gluten containing foods, fruits, berries or roots can increase risk for islet autoimmunity or T1D (Norris et al., 2003; Virtanen et al., 2006; Ziegler et al., 2003). It is of note that both breastfeeding and introduction of formula and solid food have an effect on gut microbiome, which then has significant impact on the early development of the immune system (Donald & Finlay, 2023).

Of other childhood exposures, cow's milk consumption and vitamin D are perhaps the most studied. It has been suggested that bovine insulin in cow's milk could stimulate the immune system to produce insulin-binding antibodies (Vaarala et al., 1999). Higher cow's milk consumption has been associated with increased risk of islet autoimmunity and T1D (Koivusaari et al., 2020; Lamb et al., 2015; Virtanen et al., 2000). Cow's milk in infant formula has also been investigated with varied results (Hummel et al., 2017; Knip et al., 2010; Knip & Group, 2018). Vitamin D has been considered due to the seasonal variation in T1D incidence and the incidence peak during winter months and some studies have reported that vitamin D supplementation protects from T1D (Hyppönen et al., 2001; The EURODIAB Substudy 2 Study Group, 1999). There also seems to be temporal association between vitamin D supplementation and the plateau in T1D incidence in Finland (Mäkinen et al., 2014). However, later research has been contradictory to this (Mäkinen et al., 2016; Reinert-Hartwall et al., 2014; Simpson et al., 2011) and the role of vitamin D in T1D development is still unclear.

2.5.3 Microbiome

During the first years of life, microbiome has a significant impact on immune system development (Donald & Finlay, 2023). Microbiome in the gut also has an impact on

the intestinal mucosal barrier (Kinashi & Hase, 2021). This epithelial barrier separates the contents of the intestinal lumen, such as food molecules, commensal gut microbes and pathogens, from the internal parts of the body. Gut epithelial cells are connected to each other with tight junctions to prevent molecules or bacteria leaking through between the cells. Epithelial cells and commensal microbes in the gut lumen also produce molecules such as mucin and antimicrobial peptides that prevent pathogens from attaching to the epithelium. Dysfunctional mucosal barrier can lead to commensal gut bacteria, pathogens or other molecules gaining access into the body. This increased intestinal permeability is called "leaky gut" and it can cause inflammation and it has been associated with autoimmune diseases (Kinashi & Hase, 2021). It has therefore been hypothesized that changes in the microbiome can have an impact on T1D development. This has been shown in animal studies, where differences in microbiota have been observed in animals that later developed T1D (Brugman et al., 2006) and where T1D development has been shown to be affected by antibiotics (Brugman et al., 2006; X.-S. Zhang et al., 2018) or transfer of individual microbes (Hänninen et al., 2018).

T1D-associated differences in gut microbiota have also been reported in humans including a lower diversity of the gut microbiota (de Goffau et al., 2014; Leiva-Gea et al., 2018), increased abundance of Bacteroidetes (de Goffau et al., 2014; Leiva-Gea et al., 2018; Murri et al., 2013) and decreased abundance of Bifidobacterium (Leiva-Gea et al., 2018; Soyucen et al., 2014). When considering functionality, studies have observed a decreased abundance of butyrate producing species (de Goffau et al., 2014; Groot et al., 2017). Butyrate is a short chain fatty acid, which among other things increases mucin production, and it is believed to be important for gut mucosal barrier. In addition to changes in microbiota, studies have found T1D-associated inflammation in the gut mucosal tissue (Leiva-Gea et al., 2018; Pellegrini et al., 2017) and changes in gut permeability (Leiva-Gea et al., 2018). However, these changes were observed in people with established T1D and could be caused by the disease process itself, especially when the plasma glucose levels have been shown to correlate with some microbiota changes (Murri et al., 2013). This raises the question, whether there are differences before seroconversion or already in early childhood.

This has been examined in autoantibody-positive children and their healthy controls with observation that autoimmunity was associated with scarcity of lactate and butyrate producing microbes (de Goffau et al., 2013) in-line with studies from established T1D, although another study in autoantibody-positive children did not find any changes in microbiota (Endesfelder et al., 2014). Giongo et al. examined this using longitudinal samples from children who later developed autoimmunity and their healthy controls. The first of the three samples was taken before autoimmunity. They found that bacterial diversity diminished in case children over time when

compared to controls, and they also saw reverse trends in the abundance of Bacteroidetes and Firmicutes (Giongo et al., 2011). These differences were significant in all three time points. In another longitudinal study, Vatanen et al. examined the development of microbiota in childhood using samples from the TEDDY study (Vatanen et al., 2018). The control children had more microbes that were related to fermentation and biosynthesis of short chain fatty acids, once again indicating an important role for short chain fatty acids and their producers in protection from T1D development.

2.5.4 Antibiotics

Use of antibiotics in early childhood has been associated with increased risk of later life health problems, such as asthma, allergies and other atopic and autoimmune disorders, obesity and psychiatric disorders (Aversa et al., 2020; Fishman et al., 2019; Räty et al., 2024). This association is believed to be mediated by the gut microbiota, which antibiotic exposure has modified (Coker et al., 2020; Reyman et al., 2022; Uzan-Yulzari et al., 2021), and the effect of this microbiota on the developing immune system (Donald & Finlay, 2023). Because of widespread use of antibiotics during childhood (Aversa et al., 2020; Giannoni et al., 2022; Slykerman et al., 2023) and the possible connection between early antibiotics exposure and later autoimmune diseases, antibiotics use has also been examined in relation to type 1 diabetes.

Most studies have not found an association between antibiotics use in early childhood and later islet autoimmunity (Kemppainen et al., 2017; Virtanen et al., 2014), nor between antibiotics use during pregnancy (Haupt-Jørgensen et al., 2018; Kilkkinen et al., 2006; Tapia et al., 2018) or during childhood (Antvorskov et al., 2020; Clausen et al., 2016; Hviid & Svanström, 2009; Kilkkinen et al., 2006; Mikkelsen et al., 2017; Tapia et al., 2018) and later T1D. However, there are some contradictory results where for example an association with later T1D was found in children born with caesarean section (Clausen et al., 2016; Wernroth et al., 2020) or in children who had multiple prescriptions at an early age (Boursi et al., 2015; Mikkelsen et al., 2017). The effect might depend on the type of antibiotics used (Boursi et al., 2015; Clausen et al., 2016; Kilkkinen et al., 2006; Ternák et al., 2021; Wernroth et al., 2020) although the results are unclear concerning this as well since many studies did not see this kind of effect (Antvorskov et al., 2020; Hviid & Svanström, 2009; Mikkelsen et al., 2017). It is possible that the effect seen in some studies is actually caused by the underlying infection being treated. In addition, since the association is seen in children born with caesarean section, which has a significant impact on gut microbiome, it is possible that there are other confounding factors such as gut microbiota or genetics, which make certain individuals more susceptible to the effect of antibiotics.

3 Aims

The aim of the study was to assess the effect of T1D-associated genetic variation and environmental factors to immune cell DNA methylation in cells associated with T1D development, in order to clarify their mechanisms and whether DNA methylation mediates their effects in T1D pathogenesis. The specific aims were:

1. To examine the effect of four T1D susceptibility associated SNPs (rs12722495 and rs2104286 in *IL2RA*, rs689 in *INS* and rs2476601 in *PTPN22*) on DNA methylation in their proximal gene promoters in immune cells (study I).

2. To compare DNA methylation in *IL2RA*, *INS* and *PTPN22* promoters in immune cells between children with newly diagnosed T1D and healthy controls (study I).

3. To examine the effect of two T1D susceptibility associated HLA class II haplotypes: *DRB1*03-DQA1*05-DQB1*02* (DR3-DQ2) and *DRB1*04:01-DQA1*03-DQB1*03:02 (*DR4-DQ8*)* and one protection associated haplotype, *DRB1*15-DQA1*01-DQB1*06:02* (DR2-DQ6), on epigenome-wide DNA methylation in immune cells of healthy autoantibody-negative individuals (study II).

4. To assess whether antibiotic exposure during the first week of life has long-term effects on immune system by examining immune cell frequencies in PBMC and DNA methylation in immune cells in samples collected at the age of 3 months (study III).

4 Materials and Methods

4.1 Subjects

4.1.1 Diabetes Prediction and Prevention (DIPP) project (studies I and III)

Since 1994, as a part of the Diabetes Prediction and Prevention project, new-borns born in University Hospital in Turku, and later also in Oulu (from 1995) and Tampere (from 1997), have been screened for T1D-susceptibility associated HLA genotypes after written informed consent is provided by the parents or legal guardians. Children with increased genetic risk for T1D have then been recruited for follow-up visits and to give blood samples every three months and then later every 6 months/yearly/every three years depending on the child's age, until they are diagnosed with T1D or until they are 15 years old. T1D is diagnosed based on criteria by the American Diabetes Association (ADA) (American Diabetes Association, 2020).

In study I and III we had samples collected as a part of the DIPP study. In study I, we had samples from 25 children recently diagnosed with T1D (mean age 7.5 ± 3.7; 1.6–14.5 years) and their healthy autoantibody-negative control subjects (mean age 7.3 ± 3.9; 1.1–14.4 years). The case subjects and their controls were matched based on HLA class II genotype, sex, age and date of sampling. Samples were collected in Turku University Hospital within 6 days after the diagnosis.

In study III, we had samples from 58 autoantibody-negative healthy children who had not been diagnosed with T1D. This included 29 case subjects who had been exposed to antibiotics during the first week of life and their matched controls who had not been exposed to antibiotics and who were matched based on mode of birth, gestational age, time of sampling and sex. For case subjects, antibiotics were started during the first 48 hours and they were all prescribed Benzylpenicillin and gentamicin. Thirteen case subjects had a confirmed infection and received antibiotics for seven days, and 14 subjects had a suspected infection and received antibiotics for two days. Two case subjects and their controls were excluded from the analyses (antibiotic course three and five days). Samples for case subjects and controls were collected at the age of three months.

4.1.2 Finnish Pediatric Diabetes Register (FPDR) (study II)

In study II, our samples came from the Finnish Pediatric Diabetes Register (FPDR). Since 2002, FPDR has collected samples and data from children who are diagnosed with diabetes before the age of 16 years. Samples are collected within a few days after the diagnosis when written informed consent is provided by the parents or legal guardians. Samples are also collected from family members. The participants are genotyped for major T1D-associated HLA class II alleles and screened for T1D-associated autoantibodies (IAA, GADA, IA-2A, ZnT8A).

Our study samples were from healthy autoantibody-negative children who had a sibling diagnosed with T1D and who were homozygous either for *DRB1*15-DQA1*01-DQB1*06:02* (DR2-DQ6) (n = 14), *DRB1*03-DQA1*05-DQB1*02* (DR3-DQ2) (n = 19) *or DRB1*04:01-DQA1*03-DQB1*03:02* (DR4-DQ8) (n = 17).

DR3-DQ2 and DR4-DQ8 are associated with increased risk for T1D whereas DR2-DQ6 haplotype is protective against T1D and rather rare in FPDR. All suitable DR2-DQ6 homozygotes were chosen for the study and then individuals for DR3-DR2 and DR4-DQ8 groups were chosen based on the age and sex distribution in DR2-DQ6 group. The mean ages were 15.0 (± 8.3), 11.1 (± 5.6), and 11.8 (± 7.9) years for DR2-DQ6, DR3-DQ2, and DR4-DQ8, and their sex ratios (male/female) were 8/6, 9/10, and 11/6.

4.2 Methods

4.2.1 Sample material

Whole blood samples were collected from the Finnish Pediatric Diabetes Register (FPDR) and the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) project. Peripheral blood mononuclear cells (PBMCs) were then isolated from the samples using Ficoll Paque Plus density gradient centrifugation and resuspended in RPMI 1640 medium. The PBMC samples were then stored in a viable state at -150°C in 10% DMSO. Depending on the project, immune cell subsets were later isolated from the cryopreserved PBMC samples using either flow cytometric sorting or magnetic bead separation.

4.2.2 Genotyping

4.2.2.1 HLA genotyping

Study subjects were genotyped for major T1D-associated HLA class II alleles as has been described previously using an in-house test with sequence specific

oligonucleotides for DQB1 alleles (Kiviniemi et al., 2007) and using the DELFIA® assay for DQA1 and DRB1 alleles (Nejentsev et al., 1999).

Briefly, DQB1 alleles relevant for T1D risk were first determined, including probes for DQB1*02, *03:02, *06:02/3 and *06:03/4. Then DQA1 alleles were determined including probes for DQA1*02:01, *03 and *05. Depending on the results, DRB1 alleles were also analyzed for certain relevant DRB1*04 alleles when necessary.

4.2.2.2 SNP genotyping

SNP genotyping in study I was done using TaqMan SNP Genotyping Assays (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific) according to the manufacturer's instructions. Based on the results, genotypes were determined using QuantStudio™ Design & Analysis Software. The study subjects were genotyped for four T1D-risk associated SNPs: rs12722495 and rs2104286 in *IL2RA*, rs689 in *INS* and rs2476601 in *PTPN22*.

4.2.3 Autoantibody analyses

Both in DIPP and FPDR, the participants were screened for T1D-associated autoantibodies to insulin (IAA), glutamic acid decarboxylase 65 (GADA), and islet antigen-2 (IA–2A) using specific radio-binding assays as has been previously described (Siljander et al., 2009) and later also autoantibodies to zinc transporter 8 (ZnT8A) (Salonen et al., 2013).

4.2.4 Isolation of immune cell subsets from PBMC samples

4.2.4.1 Isolation of immune cell subsets using flow cytometric sorting (study I)

In study I, (CD20+) B cells, (CD3+CD4+CD25lowCD127+) CD4+ T cells and (CD3+CD8+) CD8+ T cells were isolated from the cryopreserved PBMC samples using flow cytometric sorting. For the sorting, the cells were stained with antibodies to CD3 (APC-F750, Biolegend), CD4 (PE-Cy7, Biolegend), CD8 (FITC, Biolegend), CD20 (PE-CF594, BD), CD127 (APC, Biolegend), CD25 (PE, Miltenyi) and CD45RO (AF700, Biolegend). Additionally, 7-AAD (Biolegend) was used for excluding the dead cells. Sorting was conducted using FACSAria III flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA). The cell pellets were then stored at -80 °C until further analyses.

4.2.4.2 Isolation of immune cell subsets using magnetic bead separation (study II and III)

In studies II and III, immune cell subsets were extracted separately from the cryopreserved PBMC samples using consecutive magnetic bead separations. In study II, CD4+ T cells and CD19+ B cells were isolated using Dynabeads™ CD4 Positive Isolation Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), Dynabeads™ CD19 Pan B (Thermo Fisher Scientific) and DETACHaBEAD™ CD19 Kit (Thermo Fisher Scientific), and in study III, CD4+ and CD8 T cells were isolated using Dynabeads CD4 Positive Isolation Kit (Thermo Fisher Scientific) and Dynabeads CD8 Positive Isolation Kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

Briefly, the PBMC samples were first thawed and washed with RPMI 1640 medium (Thermo Fisher Scientific) which was supplemented with CTL Anti-Aggregate Wash™ 20x Solution (ImmunoSpot, Cleveland, Ohio, USA) in order to perform DNase treatment. Then the samples were centrifuged (1500 rpm, 10 min) and resuspended with phosphate buffered saline (PBS) supplemented with 2% Fetal bovine serum (FBS) (Biowest, Florida, USA). Then the individual subsets were isolated with two consecutive magnetic bead separations so that the remaining PBMC fraction from the first isolation was used as a starting material for the second isolation. Finally, the isolated cells were lysed and homogenized using a syringe and needle and Buffer RLT Plus lysing buffer (Qiagen, Hilden, Germany) supplemented with 1% 2-Mercaptoethanol (Thermo Fisher Scientific) by passing the sample through the needle several times. The homogenized samples were stored at -80 °C.

4.2.5 Isolation of genomic DNA and bisulphite conversion

In study I, DNA was extracted from the frozen cell pellets using NucleoSpin Tissue Kit (Macherey-Nagel, Germany) and bisulphite conversion was done using EZ DNA Methylation-Gold Kit (Zymo Research Corporation, California, USA), according to the manufacturer's protocols. In studies II and III, sample material was frozen cell lysates from magnetic bead separation and DNA was extracted from the samples using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany). In study II, bisulphite conversion was done using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, California, USA) and in study III using the Nugen Ovation RRBS Methyl-Seq with TrueMethyl BS kit. In all studies, DNA concentrations were measured using Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific) and the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

4.2.6 Methylation analyses

4.2.6.1 Pyrosequencing (study I)

Pyrosequencing is a targeted method for DNA methylation analysis and can be used for determining methylation for a specific region and for all individual CpG sites in the region instead of a mean for several sites. In pyrosequencing the region of interest is first amplified with a PCR reaction and a biotinylated primer is used to mark which resulting strand will be used as a template for pyrosequencing. Then in pyrosequencing reaction, a separate sequencing primer is hybridized to the template to guide the sequencing reaction. Nucleotides are then added to the mixture one nucleotide at a time. Due to enzymes such as luciferase in the mixture, each time a nucleotide is incorporated to the growing strand, a light is generated which the pyrosequencing machine can detect. Based on a pyrogram® generated from these signals, the nucleotide sequence can then be determined.

In study I, pyrosequencing was used for measuring DNA methylation in the proximal promoters of three T1D-susceptibility genes: *IL2RA* (-459, -456, -373, -356, -272, -241 and -134), *INS* (-234, -206, -180, -135, -102 and -69) and *PTPN22* (-558, -546, -288, -164, -96 and -68). First, the PyroMark Assay Design software 2.0 (Qiagen, Hilden, Germany) was used to design primers for PCR and pyrosequencing assays for the regions. Bisulphite converted sample DNA was then amplified using these primers and PyroMark PCR kit (Qiagen), according to the kit protocols. After PCR reaction, PyroMark Q24 Vacuum Workstation (Qiagen) was used to purify the biotinylated strand. This strand was used as a template for the pyrosequencing which was performed using PyroMark Q24 pyrosequencing instrument (Qiagen) and PyroMark Q24 Advanced Reagents (Qiagen). These were conducted according to the manufacturer's instructions. PyroMark Q24 Advanced software (Qiagen) was used to analyze the results and calculate the methylation percentage for each CpG site.

4.2.6.2 Infinium Methylation EPIC microarray (study II)

In study II, we examined epigenome-wide DNA methylation using Infinium MethylationEPIC microarray (Illumina, San Diego, California, USA), which includes over 850000 CpG sites and covers about 3% of all CpG sites in the genome. The analysis included DNA samples extracted from CD4+ T cells and CD19+ B cells from individuals homozygous for DR2-DQ6 (n = 14), DR3-DQ2 (n = 19) and DR4-DQ8 (n = 17).

For the methylation analysis, DNA samples within the HLA-groups were first pooled to create 11 pooled samples for both CD4+ T cells and B cells (4–5 samples per

pooled sample) which were then bisulphite converted using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, California, USA). Then DNA methylation was examined with the Infinium MethylationEPIC BeadChip (Illumina) according to the manufacturer´s protocol. Briefly, the bisulphite converted DNA samples were first whole genome amplified and then enzymatically fragmented. The fragmented DNA samples were then hybridized onto the BeadChips. After hybridization, the BeadChips were stained using the Tecan Freedom Evo robot (Tecan Group Ltd., Switzerland) and finally imaged using the iScan System (Illumina). Genome Studio v2011.1 software (Illumina) was used for the quality control (QC) analysis.

Mutation Analysis Facility (MAF) in Karolinska University Hospital (Stockholm, Sweden) conducted the bisulphite conversion of pooled samples, DNA methylation analysis with Infinium MethylationEPIC BeadChip and preliminary QC analysis with Genome Studio v2011.1.

4.2.6.3 Reduced representation bisulphite sequencing (study III)

In study III, epigenome-wide DNA methylation was examined using reduced representation bisulphite sequencing (RRBS). RRBS was developed by Meissner et al. (Meissner et al., 2005) and it is described well by Gu et al. (Gu et al., 2011). Briefly, in RRBS, DNA is first fragmented using methylation-insensitive restriction enzymes such as MspI, which recognizes sequence 3´-CCGG-5´. This means that each produced fragment has at least one CpG site. After fragmentation, end repair is done to prevent the fragments from sticking to each other. Then A-tailing is done in order to enable attaching adapters to the fragments. These adapters are used later for PCR amplification. After adapter ligation, the fragments are bisulphite converted and then fragments of certain size (160–410) are selected and PCR amplified. Finally, these libraries are sequenced. This method and using restriction enzymes like MspI lead to libraries that are biased towards CpG rich regions. Although it means little coverage for CpG poor regions, it also means that much higher portion of CpG sites in genome can be analyzed compared to for example Illumina microarrays such as Infinium MethylationEPIC microarray and it is much more affordable than whole genome bisulphite sequencing (WGBS).

In our study, DNA methylation was analyzed in CD4+ T cells (56 samples) and CD8+ T cells (42 samples). Libraries for RRBS were prepared using the Nugen Ovation RRBS Methyl-Seq with TrueMethyl BS kit (Tecan Group Ltd.) according to the M01394 protocol. Advanced Analytical Fragment Analyzer (Agilent Technologies, Santa Clara, California, United States) was used to examine the quality of the samples both before and after the library preparation. Sample concentration was also measured both times using Qubit (Thermo Fisher Scientific). Sequencing of the libraries was done using NovaSeq 6000 S2 v1.5 (Illumina). Library preparation and

sequencing was conducted by the Finnish Functional Genomics Centre (FFGC) (University of Turku, Åbo Akademi and Biocenter Finland).

4.2.7 Detection of immune cell subsets using flow cytometry (study III)

In study III, after thawing and washing the PBMC samples, but before the magnetic bead separations, 1.5–3 million cells per sample were set aside for examining immune cell subsets using flow cytometry. For the analysis, the cells were stained using fluorochrome-labelled antibodies listed in Table 2. There were three different panels focusing on different subsets: panel 1 for monocytes, DCs, invariant T cells and NK cells, panel 2 for B cells and Treg cells, and panel 3 T cell subsets. 0.5–1 million cells of each sample were used per panel. Briefly, first Zombie Aqua dye (Biolegend, San Diego, California, USA) was used for excluding dead cells and then immunostaining for other markers were done in dark and in room temperature (RT) with 20 min incubation time. Brilliant Stain Buffer Plus (BD Biosciences, Franklin Lakes, New Jersey, USA) was used for the mixes in immunostaining. For panel 1, Fc receptor blocking for the samples was done with TruStain FcX (Biolegend) before immunostaining. The flow cytometry analysis was performed using Novocyte Quanteon flow cytometer (Agilent Technologies, USA) and results were analyzed with FlowJo Software v10.7.1 (BD Biosciences). All analyzed immune cell subsets and their phenotypes are listed in Table 3.

Table 2. List of markers used in the immunostainings. From unpublished manuscript (Pahkuri S, Valta M, Junttila S et al.) with permission from all authors.

Staining panel	Fluorochrome	Marker	Clone	Manufacturer
Purity stainings				
	APC-F750	CD3	SK3	Biolegend
	PE-Cy7	CD4	RPA-T4	Biolegend
	BV510	CD19	HIB19	Biolegend
	PE	CD8	RPA-T8	Biolegend
	FITC	CD14	HDC14	Biolegend
	7-AAD			Biolegend
Panel 1: Monocyte, DC, Invariant T, NK panel				
	BV421	TCR γδ	B1	BioLegend
	BV570	CD19	HIB19	BioLegend
	BV605	CD56	HCD56	BioLegend
	BV650	CD123	6H6	BioLegend
	BV711	CD14	MφP9	BD Biosciences
	BV786	TCR Vα7.2	3C10	BioLegend
	BB515	CD11c	B-ly6	BD Biosciences

Staining panel	Fluorochrome	Marker	Clone	Manufacturer
	BB700	CD27	M-T271	BD Biosciences
	PE	TCR Vα24-J18	6B11	Biolegend
	PE/Dazzle 594	CD16	3G8	Biolegend
	PE-Cy5	HLA-DR	G46-6	BD Biosciences
	PE-Cy7	CD8	RPA-T8	BD Biosciences
	APC	CD161	HP-3G10	Biolegend
	R718	CD3	UCTH1	BD Biosciences
	APC-F750	CD45	HI30	Biolegend
Panel 2: B cell/Treg panel				
	BB515	CD25	MA251	BD Biosciences
	BB700	CD38	HIT2	BD Biosciences
	BV421	CD127	A019D5	Biolegend
	BV510	CD14	MφP9	BD Biosciences
	BV510	CD16	3G8	Biolegend
	BV510	CD56	HDC56	Biolegend
	BV570	IgM	MHM-88	Biolegend
	BV605	IgG	G18-145	BD Biosciences
	BV650	CD3	UCTH1	BD Biosciences
	BV711	CD21	B-ly4	BD Biosciences
	BV786	IgD	IA6-2	Biolegend
	APC	CD4	RPA-T4	Biolegend
	R718	CD20	2H7	BD Biosciences
	APC-F750	CD19	HIB19	Biolegend
	PE	IgA	IS11-8E10	Miltenyi
	PE-CF594	CD27	M-T271	Biolegend
	PE-Cy5	CD10	HI10a	Biolegend
	PE-Cy7	CD45RA	HI100	Biolegend
Panel 3: T cell panel				
	BV421	ICOS	C398.4A	Biolegend
	BV510	CD19	HIB19	Biolegend
	BV510	CD14	MφP9	BD Biosciences
	BV510	CD16	3G8	Biolegend
	BV510	CD56	HDC56	Biolegend
	BV570	CD8	RPA-T8	BioLegend
	BV605	CXCR3	G025H7	Biolegend
	BV650	CCR7	G034H7	BioLegend
	BV786	CD45RA	HI100	BD Biosciences
	BB515	CXCR5	RF8B2	BD Biosciences
	PE	PD-1	EH12.2H7	Biolegend
	PE/Dazzle 594	CrTh2	BM16	Biolegend
	PE-Cy5	CD38	HIT2	Biolegend
	PE-Cy7	CD27	M-T271	Biolegend
	APC	CCR10	REA326	Miltenyi
	R718	CD3	UCTH1	BD Biosciences
	APC-F750	CD4	RPA-T4	BioLegend
	BB700	CCR4	1G1	BD Biosciences
	BV711	CCR6	G034E3	Biolegend

Table 3. List of all analyzed immune cell subsets and their phenotypes. Modified from unpublished manuscript (Pahkuri S, Valta M, Junttila S et al.) with permission from all authors.

Cell subset	Phenotype
Activated Tfh CD4+ T cells (CD45RA-CXCR5+PD-1+ICOS+)	CD3+CD4+CD45RA-CXCR5+PD1+ICOS+ of CD3+CD4+CD45RA-
CD16+CD56- NK cells	HLADR-CD3-CD123-CD16+CD56low of CD45+
CD16+CD56bright NK cells	HLADR-CD3-CD123-CD16-CD56bright of CD45+
CD27- effector memory (EM) CD4+ T cells (CD45RA-CCR7-CD27-)	CD4+CD45RA-CCR7-CD27- of CD4+
CD27- effector memory (EM) CD8+ T cells (CD45RA-CCR7-CD27-)	CD8+CD45RA-CCR7-CD27- of CD8+
CD27+ effector memory (EM) CD4+ T cells (CD45RA-CCR7-CD27+)	CD4+CD45RA-CCR7-CD27+ of CD4+
CD27+ effector memory (EM) CD8+ T cells (CD45RA-CCR7-CD27+)	CD8+CD45RA-CCR7-CD27+ of CD8p
CD27-IgD- Memory CD19+ B cells	CD19+CD27-IgD- of CD19+
Central memory (CM) CD4+ T cells (CD45RA-CCR7+CD27+)	CD4+CD45RA-CCR7+CD27+ of CD4+
Classical dendritic cells (cDC) (CD11c+CD14-CD16-)	HLADR+CD3-CD56-CD11c+CD123-CD14-CD16- of CD45+
Classical monocytes (CD14+CD16-)	HLADR+CD3-CD56-CD11c+CD14+CD16- of CD45+
CM CD8+ T cells (CD45RA-CCR7+CD27+)	CD8+CD45RA-CCR7+CD27+ of CD8+
IgA+ CD19+ memory B cells (IgA+CD27+IgD-)	CD19+CD27+IgD-IgM-IgG-IgA+ of CD19+
IgG+ CD19+ memory B cells (IgG+CD27+IgD-)	CD19+CD27+IgD-IgM-IgG+ of CD19+
IgM+ CD19+ memory B cells (IgM+CD27+IgD-)	CD19+CD27pIgDmIgMpIgGm of CD19+
Intermediate monocytes (CD14-CD16+)	HLADR+CD3-CD56-CD11c+CD14-CD16+ of CD45+
Memory Tregs	CD3+CD4+CD45RA-CD127lowCD25+ of memory T cells
Naïve CD19+ B cells (CD27-IgD+)	CD19+CD27-IgD+ of CD19+
Naïve CD4+ T cells (CD45RA+CCR7+CD27+)	CD4+CD45RA+CCR7+CD27+ of CD4+
Naïve CD8+ T cells (CD45RA+CCR7+CD27+)	CD8+CD45RA+CCR7+CD27+ of CD8+
Naive Tregs	CD3+CD4+CD45RA+CD127lowCD25+ of naïve T cells
Non-classical monocytes (CD14+CD16+)	HLADR+CD3-CD56-CD11c+CD14+CD16+ of CD45+
Plasmablasts (CD19+CD38+CD20-)	CD19+CD38+CD20- of CD19+
Plasmacytoid dendritic cells (pDCs) (CD123+CD11c-)	HLADR+CD3-CD56-CD11c-CD123+ of CD45+
Switched memory CD19+ B cells (CD27+IgD-)	CD19+CD27+IgD- of CD19+
TCRγδ T cells	HLADR-CD3+TCRγδ+ of CD3+

Cell subset	Phenotype
TEMRA CD4+ T cells (CD27-CD45RA+CCR7-)	CD4+CD45RA+CCR7-CD27- of CD4+
TEMRA CD8+ T cells (CD27-CD45RA+CCR7-)	CD8+CD45RA+CCR7-CD27- of CD8+
Tfh CD4+ T cells (CXCR5+)	CD3+CD4+CD45RA-CXCR5+ of CD3+CD4+CD45RA-
Th1 CD4+ T cells (CXCR3+CCR6-)	CD3+CD4+CD45RA-CXCR3+CCR6-CCR4- of CD3+CD4+CD45RA-
Th1/Th17 CD4+ T cells (CXCR3+CCR6+)	CD3+CD4+CD45RA-CXCR3+CCR6+CCR4- of CD3+CD4+CD45RA-
Th17 CD4+ T cells (CXCR3-CCR6+)	CD3+CD4+CD45RA-CXCR3-CCR6+CCR4- of CD3+CD4+CD45RA-
Th2 CD4+ T cells (CCR4+CrTh2+CXCR3-CCR6-)	CD3+CD4+CD45RA-CXCR3-CCR6-CCR4+CrTh2+ of CD3+CD4+CD45RA-
Total B cells (CD19+)	CD19+ of viable cells
Total CD3+ T cells	CD3+ of viable cells
Total CD4+ T cells	CD4+ of CD3+
Total CD8+ T cells	CD8+ of CD3+
Total MAIT T cells (CD3+TCRVα7.2+CD161+)	HLADR-CD3+TCRγδ-TCRVa7,2+CD161+ of CD3+
Total memory CD4+ T cells (CD45RA-CD4+)	CD3+CD4+CD45RA- of CD4+
Total naïve CD4+ T cells (CD45RA+CD4+)	CD3+CD4+CD45RA+ of CD4+
Total Tregs (CD4+CD25+CD127lo)	CD3+CD4+CD127lowCD25+ of CD3+CD4+
Tph CD4+ T cells (CD45RA-CXCR5-PD-1hi)	CD3+CD4+CD45RA-CXCR5-PD1hi of CD3+CD4+CD45RA-
Transitional CD19+ B cells (IgD+CD38+CD10+)	CD19+CD27-IgD+CD38+CD10+ of CD19+
Unswitched memory CD19+ B cells (CD27+IgD+)	CD19+CD27+IgD+ of CD19+

4.2.8 Statistical analyses

4.2.8.1 Study I

In study I, methylation at three gene promoters (*IL2RA*, *INS* and *PTPN22*) was compared between groups at variably methylated CpG sites, defined as CpG sites with at least a 5-percentage point difference between the second highest and second lowest value. Mann-Whitney U-test was used to compare methylation between T1D cases and healthy controls, and to compare methylation between different SNP genotypes. Methylation was also compared between different cell subsets using Kruskal-Wallis H test. The analyses were conducted with IBM SPSS Statistics 27.0 software (Armonk, New York, USA) and p-values were corrected for multiple comparisons using the Benjamini-Hochberg method. P values <0.05 were

considered statistically significant. When necessary, post hoc analyses were conducted.

4.2.8.2 Study II

In study II, DNA methylation was compared between three different HLA genotype carriers using the Infinium MethylationEPIC microarray (Illumina). The preliminary quality control analysis for the array data was done with Genome Studio v2011.1 at MAF. Then an open-source SeSAMe package (Zhou et al., 2018) (version 1.16.1) was used in R (version 4.2.3) for analysis of the data including additional quality control and preprocessing of the data done according to Illumina's instructions. Results with p values < 9.42×10^{-8} and more than 10% difference in the β values were considered statistically significant.

4.2.8.3 Study III

4.2.8.3.1 Flow cytometry

Immune cell frequencies between cases and controls were compared using generalized estimating equations (GEE). Each result for individual subset was analyzed separately with their own model. Models took into account the case-control pairing, HLA genotype (three groups: DR3-DQ2, DR3-DQ2/DR4-DQ8, DR4-DQ8), sex and sampling age (months). The results were corrected for multiple comparisons using FDR and p values < 0.05 were considered significant. Normal distribution assumption was checked from Pearson residuals. The analysis was done using SAS software (version 9.4 for of the SAS System for Windows) (Cary, North Carolina, USA).

4.2.8.3.2 RRBS data

The data from the sequenced libraries first needed to be prepared before methylation analysis could be conducted. For this, first the adapter sequences and low quality sequences were removed from the data using bioinformatics software tools called TrimGalore (v.0.6.10) (<https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/>) and Cutadapt (v.4.4) (M. Martin, 2011). Additionally, diversity trimming was done using a Python script from NuGen's GitHub repository (<https://github.com/nugentechnologies/NuMetRRBS>). Diversity trimming means removing certain sequences that were added earlier to the ends of the amplicons in order to improve sequencing.

After trimming, the Bismark package (v.0.24.2) (Krueger & Andrews, 2011) was used for aligning the reads to the hg38 reference genome and for the methylation calls. CpG sites with C/T SNPs were filtered out from the results as were also sites with 0.1 percentile highest coverage, in order to remove outliers and possible technical artifacts. Additionally, at least 10 reads in at least 10 case-controls pairs were required for a site to be included in the analysis. Then, individual CpG sites were combined into 100bp bins. Methylation value of a bin was determined as the median value of the sites in the region. Bins with no variation in methylation between samples were filtered out.

Finally, methylation between cases and controls was compared using R software (v.4.3.0) and a PQLseq package (v.1.2.1) (Sun et al., 2019). The comparison was done with a binomial mixed model and covariates included in analysis were the case-control pairing, HLA group, sex and sampling age. Results were corrected for multiple comparisons using the Benjamini-Hochberg method and p values <0.1 were considered statistically significant.

5 Results

5.1 Type 1 diabetes-associated single nucleotide polymorphisms in *INS* and *IL2RA* have an impact on DNA methylation in their proximal promoters in lymphocyte subsets (study I)

In study I, we examined promoter methylation in three T1D-associated risk genes, *IL2RA*, *INS* and *PTPN22*, in samples from 25 individuals with recently diagnosed T1D and their matched healthy controls. DNA methylation was examined in three different lymphocyte subsets – B cells and CD4+ and CD8+ T cells collected separately using flow cytometry – and determined at 6–7 CpG sites per promoter using pyrosequencing.

First, we compared methylation between lymphocyte subsets and found subset specific methylation patterns in all promoters (Figures 4–6), although the differences were quite small in *INS*. In *IL2RA*, methylation differences were found at six out of seven CpG sites: -459 (FDR = 2.11×10^{-15}), -456 (FDR = 2.11×10^{-15}), -373 (FDR = 1×10^{-16}), -356 (FDR = 1×10^{-16}), -272 (FDR = 4.05×10^{-4}) and -241 (FDR = 1×10^{-16}) (Figure 4). At three of these CpG sites, B cells differed considerably from other subsets: -373 (B cells 49% methylated, CD8+ T cells 30% and CD4+ T cells 31%), -356 (18%, 4% and 5%) and -241 (8%, 2% and 2%). In *INS*, methylation differed at three out of six CpG sites: -135 (FDR = 1.04×10^{-2}), -102 (FDR = 5.43×10^{-6}) and -69 (FDR = 2.04×10^{-4}) (Figure 5). At all these three sites, CD8+ T cells differed from other subsets by having lower methylation. In *PTPN22*, there were differences at two out of six CpG sites: -558 (FDR = 1×10^{-16}) and -546 (FDR = 1×10^{-16}), where all subsets differed considerably (Figure 6).

Next, we examined whether four known T1D-risk associated SNPs – rs12722495 and rs2104286 in *IL2RA*, rs689 in *INS* and rs2476601 in *PTPN22* – affect promoter methylation in these genes by comparing individuals with different genotypes. All observed significant associations are listed in Table 4. We found that rs689 was associated with methylation at four CpG sites in *INS* promoter: -234, -206, -102 and -69. In all sites with a significant difference, the T1D-risk associated AA genotype was associated with higher methylation level than other genotypes. Additionally, the rs12722495 had an association with methylation at two CpG sites, -373 and -356, in

IL2RA promoter in B cells, where the risk genotype AA was associated with lower methylation levels than other genotypes.

Additionally, in order to investigate whether there are T1D-associated methylation differences in the promoters, we compared methylation between individuals with recent onset T1D and their healthy matched controls, but we found only one CpG site with a significant difference: CpG site -135 in *INS* promoter in CD8+ T cells (FDR = 0.034), where methylation was higher in people with T1D than their controls (87.3 ± 7.2 vs. 78.8 ± 8.9). All other sites had comparable methylation between the groups.

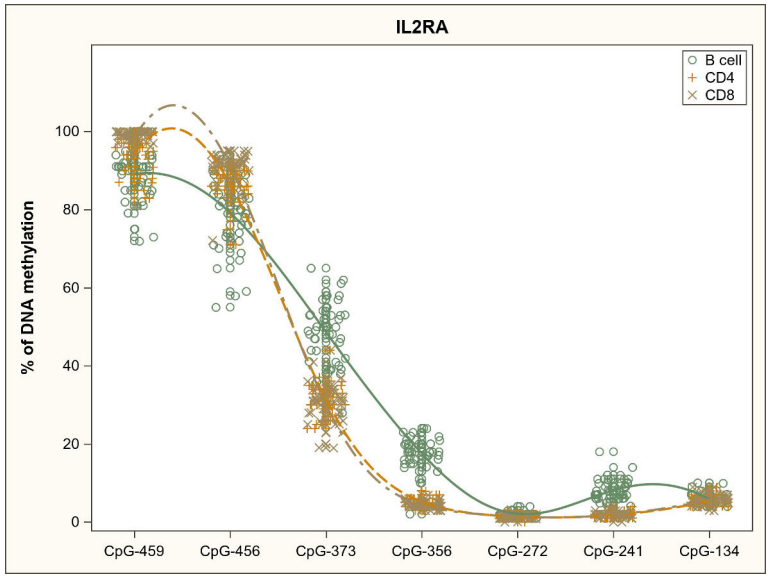

Figure 4. Levels of DNA methylation at seven CpG sites in *IL2RA* promoter in three immune cell subsets: B cells and CD4+ and CD8+ T cells. Modified from (Pahkuri et al., 2023) under the Creative Commons CC BY 4.0 license.

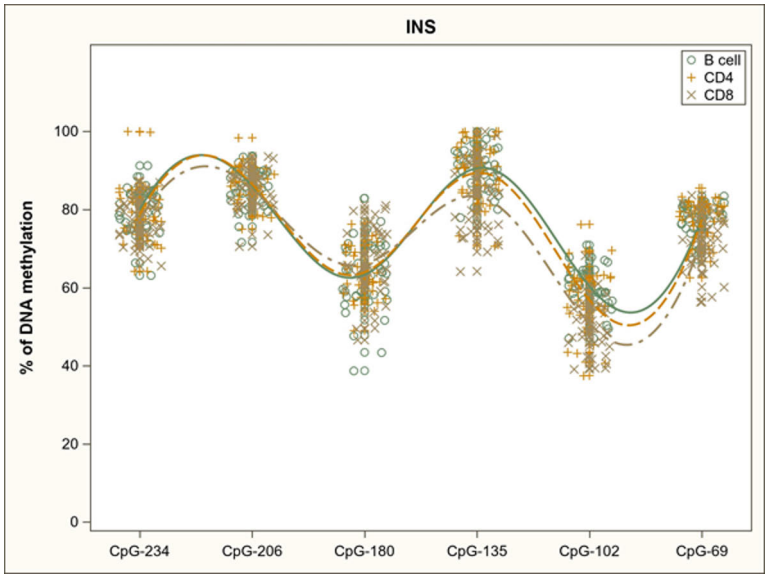

Figure 5. Levels of DNA methylation at six CpG sites in *INS* promoter in three immune cell subsets: B cells and CD4+ and CD8+ T cells. Modified from (Pahkuri et al., 2023) under the Creative Commons CC BY 4.0 license.

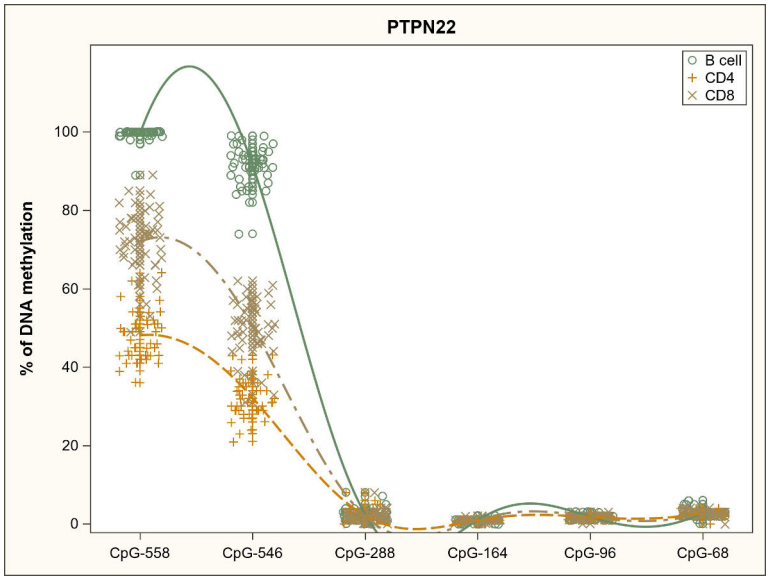

Figure 6. Levels of DNA methylation at six CpG sites in *PTPN22* promoter in three immune cell subsets: B cells and CD4+ and CD8+ T cells. Modified from (Pahkuri et al., 2023) under the Creative Commons CC BY 4.0 license.

Table 4. Results from mQTL analyses. Associations between rs689 (*INS*) and rs12722495 (*IL2RA*) SNP markers and CpG site methylation in *INS* and *IL2RA* promoters, and their FDR corrected p-values (pFDR). P values <0.05 were considered statistically significant. Modified from (Pahkuri et al., 2023) under the Creative Commons CC BY 4.0 license.

SNP	Cell subset	Gene	CpG site	pFDR
rs689	B cell	*INS*	-234	3.52×10^{-5}
	B cell	*INS*	-206	1.05×10^{-5}
	CD4+	*INS*	-234	1.05×10^{-5}
	CD4+	*INS*	-206	1.05×10^{-5}
	CD4+	*INS*	-102	0.012
	CD4+	*INS*	-69	4.89×10^{-3}
	CD8+	*INS*	-234	2.20×10^{-5}
	CD8+	*INS*	-206	7.77×10^{-4}
	CD8+	*INS*	-102	4.89×10^{-3}
rs12722495	B cell	*IL2RA*	-373	8.80×10^{-3}
	B cell	*IL2RA*	-356	4.89×10^{-3}

5.2 Type 1 diabetes protection and susceptibility associated HLA class II genotypes have an impact on DNA methylation in HLA region in lymphocyte subsets (study II)

In this study, we examined the effect of T1D-susceptibility and protection associated HLA genotypes on epigenome-wide DNA methylation in CD4+ T cells and CD19+ B cells using Infinium MethylationEPIC microarray. We had samples from healthy subjects who were homozygous either for protective DR2-DQ6 haplotype (n = 14) or T1D-risk-associated DR3-DQ2 (n = 19) or DR4-DQ8 (n = 17) haplotypes.

When DNA methylation was compared between the protective DR2-DQ6 genotype and a combined group of the risk genotypes DR3-DQ2 and DR4-DQ8, there were six differentially methylated CpG sites (DMS) (Figure 7a and Table 5). These sites were significant in both CD4+ T cells and CD19+ B cells. There were also three CD4+ T cell specific DMSs and one CD19+ B cell specific DMS.

When DR2-DQ6 was compared separately to only DR3-DQ2, eight of these same sites remained significant (Figure 7b and Table 5). There were also three novel differentially methylates sites: cg07180897 (*HLA-DQB2*), cg24668570 (*KNDC1*), and cg27576367 (*GADD45A*). When DR2-DQ6 was compared separately to only DR4-DQ8, six of the sites remained significant and no novel sites were found (Figure 7c and Table 5). Lastly, the risk genotypes DR3-DQ2 and DR4-DQ8 were compared to each other and two differentially methylated sites were discovered in CD19+ B cells (Figure 7d and Table 5). There were no DMSs between these risk-associated groups in CD4+ T cells.

In summary, majority of the discovered methylation differences were found when we compared the protective DR2-DQ6 genotype to the risk genotypes DR3-DQ2 and

DR4-DQ8. Most differentially methylated sites were located in the HLA region, especially the *HLA-DRB1* gene, where the DR2-DQ6 genotype was associated with hypomethylation.

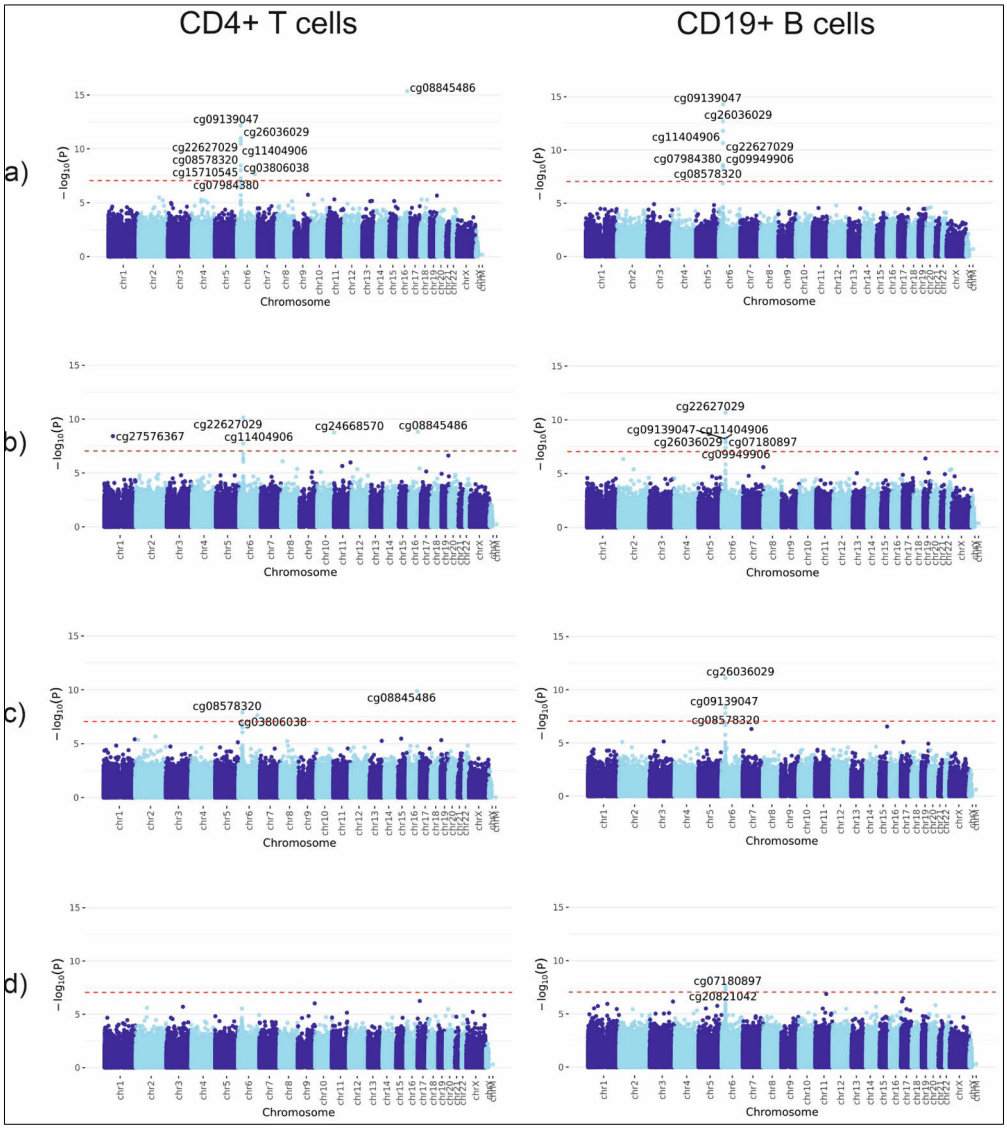

Figure 7. Manhattan plots depicting results from differential methylation analysis: differentially methylated CpG probes between a) DR2-DQ6 and the combined group of DR3-DQ2 and DR4-DQ8, b) the DR2-DQ6 and DR3-DQ2 groups, c) the DR2-DQ6 and DR4-DQ8 groups, and d) the DR3-DQ2 and DR4-DQ8 groups. The red horizontal line marks the threshold for genome-wide significance ($p < 9.42 \times 10^{-8}$). Reproduced from (Pahkuri et al., 2024) under the Creative Commons CC BY 4.0 license.

Table 5. List of all differentially methylated CpG sites in all comparisons. Reproduced from (Pahkuri et al., 2024) under the Creative Commons CC BY 4.0 license.

Comparison	Cell subset	CpG	Difference	P value	Gene	Chromosome	Location	Strand	Methylation
DR2-DQ6 vs DR3-DQ2+ DR4-DQ8	CD4	cg22627029	-0.88	1.95×10^{-11}	*RNU1-61P; HLA-DRB6*	6	32552839	-	hypermethylated
	CD19	cg22627029	-0.93	2.19×10^{-11}	*RNU1-61P; HLA-DRB6*	6	32552839	-	hypermethylated
	CD4	cg07984380	-0.27	5.44×10^{-8}	*HLA-DRB1*	6	32579243	-	hypermethylated
	CD19	cg07984380	-0.21	2.54×10^{-9}	*HLA-DRB1*	6	32579243	-	hypermethylated
	CD4	cg11404906	0.76	3.42×10^{-11}	*HLA-DRB1*	6	32583973	+	hypomethylated
	CD19	cg11404906	0.72	1.61×10^{-12}	*HLA-DRB1*	6	32583973	+	hypomethylated
	CD4	cg08578320	0.82	3.38×10^{-9}	*HLA-DRB1*	6	32584263	-	hypomethylated
	CD19	cg08578320	0.81	4.08×10^{-9}	*HLA-DRB1*	6	32584263	-	hypomethylated
	CD4	cg09139047	0.88	6.81×10^{-13}	*HLA-DRB1*	6	32584266	-	hypomethylated
	CD19	cg09139047	0.90	5.09×10^{-15}	*HLA-DRB1*	6	32584266	-	hypomethylated
	CD19	cg09949906	0.85	3.25×10^{-9}	*HLA-DRB1*	6	32584574	+	hypomethylated
	CD4	cg26036029	0.24	9.79×10^{-12}	*HLA-DRB1*	6	32584667	-	hypomethylated
	CD19	cg26036029	0.37	1.93×10^{-13}	*HLA-DRB1*	6	32584667	-	hypomethylated
	CD4	cg15710545	-0.40	9.69×10^{-9}	*HLA-DRB1; HLA-DQA1*	6	32610338	-	hypermethylated
	CD4	cg03806038	0.59	2.23×10^{-8}	*TAB2*	6	149317906	+	hypomethylated
	CD4	cg08845486	0.92	4.37×10^{-16}	*NOL3; E2F4*	16	67183822	+	hypomethylated
DR2-DQ6 vs DR3-DQ2	CD4	cg27576367	0.89	3.80×10^{-9}	*GADD45A*	1	67685889	+	hypomethylated
	CD4	cg22627029	-0.89	7.09×10^{-11}	*RNU1-61P; HLA-DRB6*	6	32552839	-	hypermethylated
	CD19	cg22627029	-0.93	2.19×10^{-11}	*RNU1-61P; HLA-DRB6*	6	32552839	-	hypermethylated
	CD4	cg11404906	0.76	1.76×10^{-8}	*HLA-DRB1*	6	32583973	+	hypomethylated
	CD19	cg11404906	0.73	5.19×10^{-9}	*HLA-DRB1*	6	32583973	+	hypomethylated
	CD19	cg09139047	0.89	5.99×10^{-9}	*HLA-DRB1*	6	32584266	-	hypomethylated

Comparison	Cell subset	CpG	Difference	P value	Gene	Chromosome	Location	Strand	Methylation
	CD19	cg09949906	0.88	3.85×10^{-8}	*HLA-DRB1*	6	32584574	+	hypomethylated
	CD19	cg26036029	0.37	1.25×10^{-8}	*HLA-DRB1*	6	32584667	-	hypomethylated
	CD19	cg07180897	-0.43	1.11×10^{-8}	*HLA-DQB2*	6	32761354	-	hypermethylated
	CD4	cg24668570	0.83	1.70×10^{-9}	*KNDC1*	10	133160275	-	hypomethylated
	CD4	cg08845486	0.92	1.48×10^{-9}	*NOL3; E2F4*	16	67183822	+	hypomethylated
DR2-DQ6 vs DR4-DQ8	CD4	cg08578320	0.83	1.26×10^{-8}	*HLA-DRB1*	6	32584263	-	hypomethylated
	CD19	cg08578320	0.85	1.64×10^{-8}	*HLA-DRB1*	6	32584263	-	hypomethylated
	CD19	cg09139047	0.90	4.81×10^{-9}	*HLA-DRB1*	6	32584266	-	hypomethylated
	CD19	cg26036029	0.38	7.78×10^{-12}	*HLA-DRB1*	6	32584667	-	hypomethylated
	CD4	cg03806038	0.62	2.28×10^{-8}	*TAB2*	6	149317906	+	hypomethylated
	CD4	cg08845486	0.92	1.29×10^{-10}	*NOL3; E2F4*	16	67183822	+	hypomethylated
DR3-DQ2 vs DR4-DQ8	CD19	cg20821042	-0.32	5.46×10^{-8}	*HLA-DQB3; MIR3135B*	6	32741382	-	hypermethylated
	CD19	cg07180897	0.41	3.13×10^{-8}	*HLA-DQB2*	6	32761354	-	hypomethylated

5.3 The effect of early antibiotic exposure on DNA methylation in lymphocyte subsets (study III)

In study III, we assessed the long-term effects of early antibiotic exposure on the immune system using PBMC samples from 3-month-old children, who had been exposed to antibiotics during the first week of life, and their healthy control children who had no antibiotic exposure.

First, flow cytometry was used for detecting different immune cell subpopulations in PBMC and for calculating their frequencies. All analyzed immune cell subsets and their phenotypes are listed in Table 3. There were no antibiotic exposure associated differences in immune cell frequencies between cases and controls after the results were corrected for multiple comparisons (FDR < 0.05). However, before correction, there was a difference in frequencies of three subsets: naïve CD4+ T cells (p value = 0.048), CD27+ effector memory (EM) CD4+ T cells (p value = 0.047) and CD27+ effector memory (EM) CD8+ T cells (p value = 0.012) (Figure 8a).

Out of the case children, 13 children had a confirmed infection and received a seven-day antibiotic course and 14 case children had a suspected infection and had antibiotics for two days. As an additional analysis, we compared cases and controls within these groups in order to see if the length of the antibiotic course would impact the results, but again we saw no significant differences after correcting for multiple comparisons (FDR < 0.05). Before correction, in the short antibiotic group (2 days, no infection) we observed a difference in one cell subset: plasmablasts (p value = 0.043) (Figure 8b). In the long antibiotic group (7 days, confirmed infection) we observed a difference in total Treg frequency (p value = 0.032) and frequency of naïve Tregs and memory Tregs (Figure 8c).

In addition to flow cytometry analyses, epigenome-wide DNA methylation in CD4+ and CD8+ T cells was also examined using RRBS and compared between cases and controls in order to investigate if early antibiotic exposure causes long-term changes in their gene expression regulation. Although initially there were 109 differentially methylated regions (DMRs) in CD4+ T cells and 182 in CD8+ T cells ($p < 0.01$; methylation difference > 10%), only four DMRs remained significant after correcting for multiple comparisons (FDR < 0.1): two DMRs in CD4+ T cells in Lactase (*LCT*) (pFDR = 0.07) and Apoptosis associated tyrosine kinase (*AATK*) (pFDR = 0.08) genes, and two in CD8+ T cells in DNA topoisomerase I mitochondrial (*TOP1MT*) (pFDR = 0.009) and Tumor protein P53 inducible protein 13 (*TP53I13*) (pFDR = 0.009) genes.

Similarly to flow cytometry analyses, DNA methylation was also analyzed separately in short and long antibiotic groups and cases and controls were compared within these groups. The results from all comparisons are summarized in Table 6 and all DMRs significant after FDR correction (FDR < 0.1) are listed in Table 7.

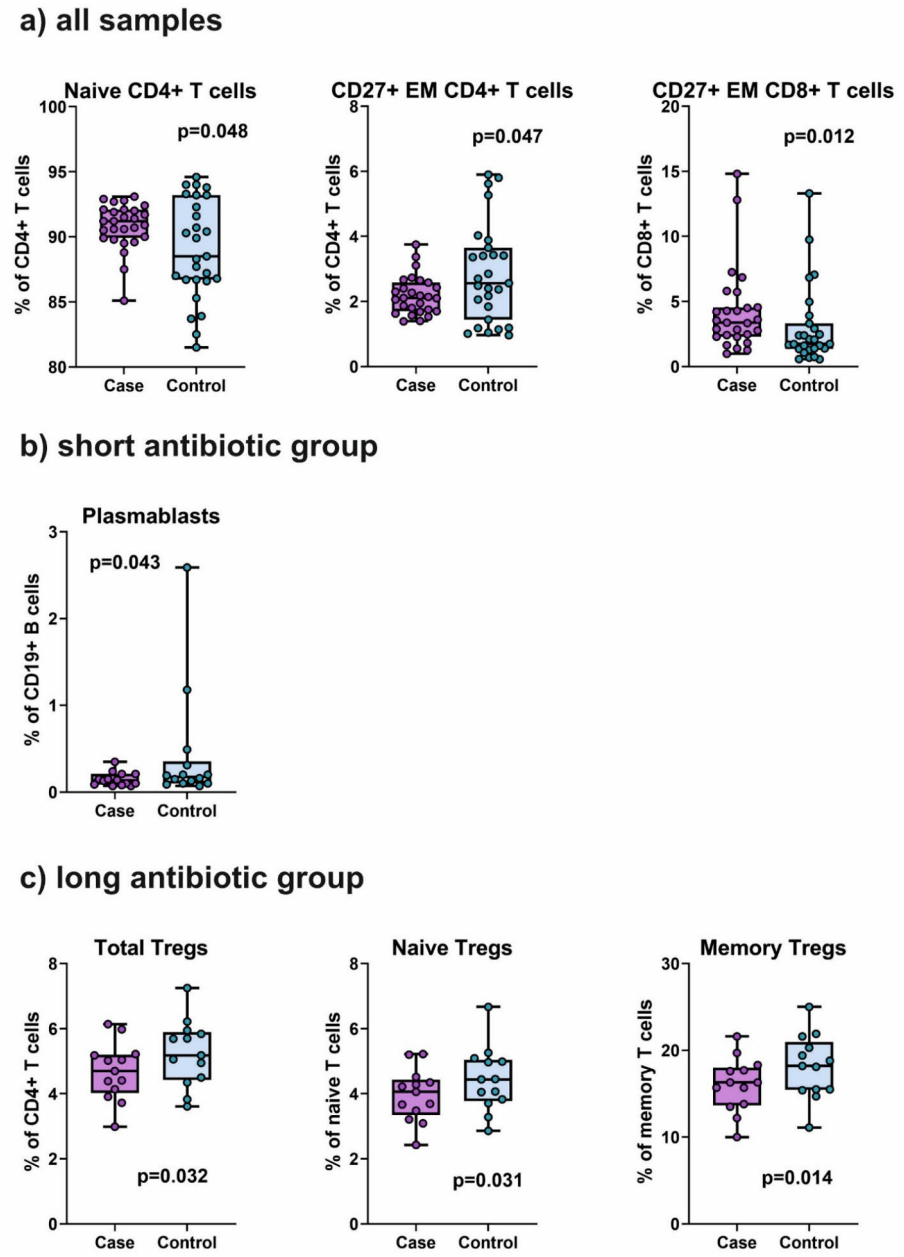

Figure 8. Box plots depicting immune cell frequencies in cases and controls in immune cell subsets where antibiotic exposure associated difference was observed before correction for multiple comparisons in a) all samples, b) short antibiotic group (2 days, no infection) and c) long antibiotic group (7 days, confirmed infection). Analysis was conducted using generalized estimating equations (GEE) and p values <0.05 were considered statistically significant. From unpublished manuscript (Pahkuri S, Valta M, Junttila S et al.) with permission from all authors.

Table 6. Results from differential methylation analysis in all comparisons summarized: the number of differentially methylated regions before and after FDR correction (pFDR). From unpublished manuscript (Pahkuri S, Valta M, Junttila S et al.) with permission from all authors. Meth diff, methylation difference.

Sample group	Cell type	Number of DMRs with p-value < 0.01 & |meth diff| > 0.1	Number of DMRs with pFDR < 0.1
All samples	CD4	109	2
All samples	CD8	182	2
Long ant	CD4	427	27
Long ant	CD8	674	44
Short ant	CD4	292	4
Short ant	CD8	246	12

Table 7. List of all differentially methylated regions (DMRs) significant after FDR correction (FDR < 0.1) in all comparisons. From unpublished manuscript (Pahkuri S, Valta M, Junttila S et al.) with permission from all authors. Meth diff, methylation difference.

Comparison	Cell	DMR	FDR	Mean case	Mean control	Meth diff (%)	Strand	Gene	Genomic location
All samples	CD4	chr2:135837648-135837748	0.07	0.24	0.32	-0.08	-	LCT	promoter
All samples	CD4	chr17:81119020-81119120	0.08	0.59	0.67	-0.08	-	AATK	intron
All samples	CD8	chr17:29572420-29572520	0.01	0.66	0.77	-0.11	+	TP53I13	promoter
All samples	CD8	chr8:143360673-143360773	0.01	0.45	0.49	-0.05	-	TOP1MT	promoter
Short ant	CD4	chr20:48794673-48794773	0.00	0.67	0.87	-0.20	-	PREX1	intron
Short ant	CD4	chr20:57921973-57922073	0.01	0.70	0.79	-0.09	+	ENSG00000263453	intergenic
Short ant	CD4	chr11:73274910-73275010	0.02	0.38	0.48	-0.11	+	P2RY6	intron
Short ant	CD4	chr2:135798548-135798648	0.05	0.64	0.75	-0.12	-	Y_RNA	intron
Short ant	CD8	chr7:63541864-63541964	0.05	0.55	0.52	0.03	+	SLC29A4P2	intergenic
Short ant	CD8	chr17:7858620-7858720	0.05	0.38	0.43	-0.05	+	ENSG00000280046	promoter
Short ant	CD8	chr20:62494973-62495073	0.05	0.63	0.78	-0.15	+	ENSG00000272050	intergenic
Short ant	CD8	chr17:29572820-29572920	0.05	0.21	0.38	-0.17	+	TP53I13	promoter
Short ant	CD8	chr20:63252573-63252673	0.05	0.35	0.52	-0.18	-	NKAIN4	promoter
Short ant	CD8	chr6:77794357-77794457	0.05	0.70	0.95	-0.25	+	MEI4	intron
Short ant	CD8	chr7:2979864-2979964	0.06	0.41	0.55	-0.13	-	CARD11	promoter
Short ant	CD8	chr12:109637942-109638042	0.06	0.56	0.67	-0.12	-	RN7SKP250	intergenic
Short ant	CD8	chr21:13979745-13979845	0.07	0.42	0.35	0.07	-	ANKRD20A11P	promoter
Short ant	CD8	chr16:1942684-1942784	0.09	0.37	0.47	-0.10	-	MSRB1	promoter
Short ant	CD8	chr7:1271764-1271864	0.09	0.75	0.59	0.16	+	ENSG00000233082	intergenic
Short ant	CD8	chr14:79045132-79045232	0.09	0.28	0.45	-0.16	-	ENSG00000258829	intron
Long ant	CD4	chr15:22840024-22840124	0.01	0.24	0.47	-0.24	+	NIPA2	intron
Long ant	CD4	chr8:8845373-8845473	0.01	0.59	0.65	-0.05	-	MFHAS1	intron
Long ant	CD4	chr3:126984515-126984615	0.01	0.25	0.36	-0.11	+	PLXNA1	intron
Long ant	CD4	chr4:8114632-8114732	0.01	0.67	0.78	-0.11	-	ABLIM2	intron
Long ant	CD4	chr8:1416973-1417073	0.01	0.42	0.56	-0.14	+	ENSG00000281961	intron

Comparison	Cell	DMR	FDR	Mean case	Mean control	Meth diff (%)	Strand	Gene	Genomic location
Long ant	CD4	chr17:82298720-82298820	0.01	0.56	0.72	-0.16	+	ENSG00000260563	intergenic
Long ant	CD4	chr6:56277157-56277257	0.01	0.56	0.76	-0.20	-	DHFRP6	promoter
Long ant	CD4	chr10:27354771-27354871	0.01	0.15	0.38	-0.24	-	FAM210CP	intergenic
Long ant	CD4	chr16:55760899-55760999	0.02	0.61	0.79	-0.18	+	CES1P1	promoter
Long ant	CD4	chr4:11132-11232	0.03	0.58	0.69	-0.11	-	BNIP3P41	intergenic
Long ant	CD4	chr6:160226357-160226457	0.05	0.76	0.55	0.21	-	SLC22A2	intron
Long ant	CD4	chr1:1439697-1439797	0.07	0.66	0.79	-0.13	+	VWA1	exon
Long ant	CD4	chr1:243284597-243284697	0.07	0.33	0.47	-0.13	+	SDCCAG8	intron
Long ant	CD4	chr2:91744948-91745048	0.07	0.43	0.59	-0.16	-	KMT5AP2	exon
Long ant	CD4	chr8:2198973-2199073	0.08	0.36	0.51	-0.14	+	ENSG00000288782	intergenic
Long ant	CD4	chr17:81119020-81119120	0.08	0.55	0.71	-0.16	-	AATK	intron
Long ant	CD4	chr14:77042832-77042932	0.09	0.90	0.86	0.04	+	LINC02288	intron
Long ant	CD4	chr19:41136129-41136229	0.09	0.32	0.42	-0.11	+	CYP2T3P	exon
Long ant	CD4	chr9:136659989-136660089	0.09	0.60	0.47	0.13	-	ENSG00000228401	promoter
Long ant	CD4	chr16:49884099-49884199	0.09	0.82	0.65	0.17	+	ENSG00000279842	intergenic
Long ant	CD4	chr19:39875629-39875729	0.09	0.57	0.39	0.18	-	FCGBP	exon
Long ant	CD4	chr22:39727712-39727812	0.09	0.33	0.52	-0.20	+	MTFR2P2	intergenic
Long ant	CD4	chr8:22713573-22713673	0.10	0.55	0.64	-0.09	-	ENSG00000287812	intron
Long ant	CD4	chr2:10433048-10433148	0.10	0.73	0.61	0.12	+	HPCAL1	intergenic
Long ant	CD4	chr16:87345199-87345299	0.10	0.53	0.65	-0.12	-	ENSG00000131152	intron
Long ant	CD4	chr2:235864448-235864548	0.10	0.34	0.47	-0.13	-	TMSB10P1	intron
Long ant	CD4	chr19:22105329-22105429	0.10	0.56	0.38	0.18	-	ZNF92P2	intergenic
Long ant	CD8	chr6:170280357-170280457	0.00	0.76	0.56	0.20	+	LINC01624	intergenic
Long ant	CD8	chr10:69396971-69397071	0.00	0.27	0.66	-0.39	-	TACR2	intron
Long ant	CD8	chr4:80189332-80189432	0.00	0.52	0.68	-0.16	-	PRDM8-AS1	promoter
Long ant	CD8	chr20:29742273-29742373	0.00	0.62	0.51	0.11	-	5_8S_rRNA	promoter
Long ant	CD8	chr4:1330332-1330432	0.01	0.36	0.61	-0.24	+	MAEA	promoter

Comparison	Cell	DMR	FDR	Mean case	Mean control	Meth diff (%)	Strand	Gene	Genomic location
Long ant	CD8	chr2:26242848-26242948	0.01	0.32	0.62	-0.30	+	HADHB	promoter
Long ant	CD8	chr10:101074371-101074471	0.01	0.79	0.52	0.27	-	ENSG00000288844	intergenic
Long ant	CD8	chr6:141395157-141395257	0.01	0.60	0.77	-0.17	+	ENSG00000286452	intergenic
Long ant	CD8	chr2:10432248-10432348	0.01	0.31	0.15	0.16	+	HPCAL1	intergenic
Long ant	CD8	chr15:27828124-27828224	0.01	0.80	0.47	0.33	+	ENSG00000232394	intron
Long ant	CD8	chr16:9241884-9241984	0.02	0.51	0.74	-0.22	-	RPL21P119	intergenic
Long ant	CD8	chr6:163149257-163149357	0.03	0.56	0.34	0.22	-	ENSG00000285553	intron
Long ant	CD8	chr1:25146797-25146897	0.03	0.58	0.29	0.29	-	IFITM3P7	intergenic
Long ant	CD8	chr16:55760884-55760984	0.03	0.52	0.83	-0.32	+	CES1P1	promoter
Long ant	CD8	chr19:29183885-29183985	0.04	0.65	0.82	-0.17	-	UQCRFS1	intergenic
Long ant	CD8	chr1:62184297-62184397	0.04	0.79	0.90	-0.11	+	PIGPP2	intergenic
Long ant	CD8	chr18:48868940-48869040	0.04	0.43	0.60	-0.17	+	CTIF	intergenic
Long ant	CD8	chr7:57219664-57219764	0.05	0.77	0.87	-0.10	-	ENSG00000234089	intron
Long ant	CD8	chr8:1417273-1417373	0.05	0.52	0.74	-0.22	+	ENSG00000281961	intron
Long ant	CD8	chr2:27442548-27442648	0.05	0.70	0.47	0.23	+	KRTCAP3	promoter
Long ant	CD8	chr9:118964389-118964489	0.05	0.53	0.79	-0.27	+	TUBB4BP6	intergenic
Long ant	CD8	chr15:44426324-44426424	0.05	0.20	0.38	-0.18	-	CTDSPL2-DT	promoter
Long ant	CD8	chr17:367020-367120	0.06	0.14	0.29	-0.14	+	ENSG00000262558	intron
Long ant	CD8	chr17:38610320-38610420	0.07	0.35	0.49	-0.14	-	SRCIN1	intergenic
Long ant	CD8	chr20:19977273-19977373	0.07	0.92	0.75	0.16	+	NAA20	intron
Long ant	CD8	chr2:6772648-6772748	0.07	0.27	0.11	0.16	-	LINC00487	intergenic
Long ant	CD8	chr8:143360673-143360773	0.07	0.50	0.54	-0.03	-	TOP1MT	promoter
Long ant	CD8	chr7:76445464-76445564	0.07	0.51	0.31	0.19	+	ZP3	intergenic
Long ant	CD8	chr16:30016284-30016384	0.07	0.80	0.66	0.14	-	DOC2A	intron
Long ant	CD8	chr15:77908024-77908124	0.08	0.85	0.96	-0.11	+	ENSG00000290664	intergenic
Long ant	CD8	chr3:71754915-71755015	0.08	0.07	0.19	-0.12	-	EIF4E3	promoter
Long ant	CD8	chr17:78040920-78041020	0.08	0.71	0.54	0.17	+	TNRC6C	promoter

Comparison	Cell	DMR	FDR	Mean case	Mean control	Meth diff (%)	Strand	Gene	Genomic location
Long ant	CD8	chr6:31683057-31683157	0.08	0.76	0.59	0.17	-	LY6G5C	promoter
Long ant	CD8	chr9:130320789-130320889	0.08	0.48	0.29	0.18	+	HMCN2	exon
Long ant	CD8	chr6:163149357-163149457	0.08	0.56	0.37	0.19	-	ENSG00000285553	intron
Long ant	CD8	chr10:130301871-130301971	0.08	0.40	0.60	-0.19	-	ENSG00000276375	intron
Long ant	CD8	chr12:31119542-31119642	0.08	0.32	0.12	0.21	-	ENSG00000291250	promoter
Long ant	CD8	chr8:1417173-1417273	0.08	0.45	0.67	-0.22	+	ENSG00000281961	intron
Long ant	CD8	chr3:127227115-127227215	0.08	0.33	0.55	-0.22	-	ENSG00000290035	promoter
Long ant	CD8	chr3:492715-492815	0.08	0.66	0.38	0.27	+	LINC01266	intergenic
Long ant	CD8	chr3:126995015-126995115	0.08	0.67	0.79	-0.13	+	PLXNA1	intron
Long ant	CD8	chr19:48490785-48490885	0.08	0.49	0.64	-0.15	-	LMTK3	intron
Long ant	CD8	chr3:66798415-66798515	0.08	0.17	0.33	-0.16	+	ENSG00000285738	intron
Long ant	CD8	chr11:62464410-62464510	0.10	0.81	0.60	0.21	-	ENSG00000255446	intron

6 Discussion

In study I, we examined DNA methylation in promoters of three known T1D-risk genes: *INS*, *IL2RA* and *PTPN22*. We investigated if promoter methylation in these genes is affected by four T1D-susceptibility associated SNPs: rs689 in *INS*, rs12722495 and rs2104286 in *IL2RA* and rs2476601 in *PTPN22*, and if there are methylation differences between cases and their matched healthy controls.

We found that the rs689 SNP was associated with methylation at four CpG sites in *INS* promoter (-234, -206, -102 and -69). The association was significant in all examined cell subsets and the risk genotype AA was associated with higher methylation levels than other genotypes at all four sites. In addition, the rs12722495 SNP was associated with methylation at two CpG sites in *IL2RA* promoter (-373 and -356) in B cells where the risk genotype was associated with lower methylation levels. When we compared methylation between people recently diagnosed with T1D and their healthy controls, we saw T1D-associated methylation in only one CpG site at *INS* promoter (-135) in CD8+ T cells.

In *INS*, Fradin et al. first reported that rs689 was associated with methylation at -69, -102, -180 and -206 (Fradin et al., 2012). Others have later confirmed this and found other associated sites upstream (Carry et al., 2020; Ye et al., 2018). We now report this association in lymphocyte subsets – CD4+ and CD8+ T cells and B cells – and also first time in Finnish population where the rs689 has been shown to be significant for T1D risk (Laine et al., 2007, 2013). T1D-associated methylation changes in *INS* promoter have earlier been reported by Fradin et al. and Carry et al. at four of these studied CpG sites (Carry et al., 2020; Fradin et al., 2012). We observed only one differentially methylated site in CD8+ T cells but this discrepancy could be due to different study material (whole blood vs lymphocytes) or our smaller study size. As CD8+ T cells do not express insulin, the functional significance of this association between *INS* methylation and T1D is uncertain. It may, however, reflect some environmental influence on *INS* methylation more widely in different tissues, for example in the pancreas or the thymus, where the methylation change may have an effect on *INS* expression.

It has been shown that methylation of this region inversely correlates with *INS* expression (Kuroda et al., 2009; Yang et al., 2011) and that *INS* expression can be

repressed in vitro by methylation of the region (Kuroda et al., 2009). In vivo, *INS* is expressed by beta cells in pancreas but also by thymic epithelial cells, which function as antigen presenting cells during T cell selection, in order to prune out autoreactive T cells specific for *INS* epitopes. The rs689 marker is in linkage disequilibrium with 5′ variable number tandem repeat (VNTR) polymorphism (Barratt et al., 2004; Vafiadis et al., 1997), which has been associated with *INS* expression both in the pancreas and in the thymus: specifically, the protective allele, compared to the risk allele, has been shown to be associated with lower *INS* expression in the pancreas (Bennett et al., 1996; Vafiadis et al., 1996) but higher *INS* expression in the thymus (Pugliese et al., 1997; Vafiadis et al., 1997). Due to this, it has been hypothesized that the risk associated allele of rs689 may lead to a decreased expression of *INS* by thymic epithelial cells during T cell selection. This could lead to an insufficient presentation of INS peptides to autoreactive T cells and some INS specific T cells could evade negative selection. The association between rs689 and *INS* methylation indicates that methylation may mediate this effect on *INS* expression and T1D risk.

In *IL2RA*, we saw an association between rs12722495 and methylation at CpG sites -373 and -356 in B cells. Earlier Belot et al. has reported association between 28 SNPs in *IL2RA* region and methylation at CpG -373 in whole blood (Belot et al., 2013). Sixteen of these 28 SNPs have been associated with T1D, including rs2104286 which we also analysed. However, we did not see any association between rs2104286 and *IL2RA* methylation, and neither did Buhelt et al. who also analyzed *IL2RA* methylation in CD8+ T cells (Buhelt et al., 2021). Belot et al. also found T1D-associated methylation changes at -456 and -373, but we did not observe any T1D-associated methylation in *IL2RA*. The *IL2RA* promoter region has been shown to be methylated in cell specific manner both in the proximal promoter adjacent to TSS (Belot et al., 2013) and higher upstream (Belot et al., 2018). It may be that the methylation changes, associated either with T1D or SNPs, are cell specific which would explain the discrepancy between results. However, our results along with earlier reports from Belot et al. indicate that SNPs in the region may have an effect on *IL2RA* promoter methylation and especially at CpG site -373.

IL2RA is expressed in T cells when they are activated but continuously in Treg cells (J. Wang et al., 2009). The proximal promoter is differentially methylated between these cells and more highly methylated in tissues that do not express *IL2RA* at all (Belot et al., 2013). However, it is not clear whether methylation in the proximal promoter directly correlates with *IL2RA* expression or what the effects of methylation changes we observed at CpG sites -373 and -356 are. Belot et al. showed that *IL2RA* promoter methylation was associated with *IL2RA* expression on the surface of CD4+ T cells but the associated CpG sites were higher upstream than the sites we analyzed (Belot et al., 2018). Genetic variation in *IL2RA* has been shown to affect *IL2RA* expression (Lowe et al., 2007; Maier et al., 2009) and for example the

protective allele of rs12722495 has been associated with increased *IL2RA* expression in CD4+ memory T cells (Dendrou et al., 2009). This effect could be mediated by methylation changes in the promoter and it could lead to alterations in T cell or B cell activation or function.

In *PTPN22* we did not see any SNP or T1D-associated methylation changes. It is possible that rs2476601 conveys its effects on T1D for example by directly altering the structure of the molecule, instead of affecting methylation. Little has been published about methylation in *PTPN22*, although methylation changes in this region have been associated with Hashimoto thyroiditis (HT) (Kyrgios et al., 2021) and psoriasis (Chandra et al., 2018). Kyrgios et al. also analyzed *PTPN22* methylation in whole blood from patients with both HT and T1D and compared it with healthy controls, but did not find any HT+T1D associated methylation changes. Methylation in the promoter at four of the CpG sites we analyzed (-288, -164, -96 and -68) has been shown to inversely correlate with *PTPN22* expression (Chandra et al., 2018), but based on our findings, methylation at CpG sites -558 and -546 differentiate these examined cell subsets from each other and may be important for *PTPN22* expression regulation as well.

In conclusion, we observed that T1D-associated SNPs affect methylation in *INS* and *IL2RA*. There have also been other wider analyses that have examined the effect of T1D-susceptibility SNPs on epigenome-wide methylation and found both cis- and trans-mQTLs (Carry et al., 2020; Ye et al., 2018). These results suggest that some T1D-associated SNPs could mediate their effects by affecting methylation. It remains to be shown whether these methylation changes have downstream effects, but in their study Ye et al. came to the conclusion that at least in five genes, *ITGB3BP*, *AFF3*, *PTPN2*, *CTSH* and *CTLA4,* the observed methylation changes also affected gene expression (Ye et al., 2018). When we compared methylation between cases and controls, we saw T1D-associated methylation difference in only one CpG site in *INS* promoter. Other studies have reported T1D-associated methylation changes both in *INS* (Fradin et al., 2012) and *IL2RA* (Belot et al., 2013) and in epigenome-wide association studies also in genes such as *HLA-DQB1*, *RFXAP*, *NFKB1A* and *TNF* (Rakyan et al., 2011) and *RTL1* (Belot et al., 2017). However, the reported methylation changes have been quite small and may depend on the examined tissue or cell subset. Most of the research has been done with heterogeneous samples such as whole blood or PBMC which can lead to discrepancy in the results. Future studies concerning T1D-associated methylation should also consider the effect of susceptibility SNPs, their interaction with the environment and the analyzed sample material.

In study II, we expanded our analysis of genetic effects on DNA methylation to HLA variants. Although genes such as *INS*, *PTPN22* and *IL2RA* outside the HLA region have an impact, the majority of the genetic risk for T1D is determined by

HLA class II genes *HLA-DQB1*, *-DQA1* and *-DRB1* (Noble et al., 1996). T1D-associated alleles in these genes have an effect on structure of the peptide binding groove in HLA class II molecules but we wanted to examine if part of their effect could also be conveyed by DNA methylation and if their effect is restricted to the HLA genes or if they have wider effects on the epigenome.

For the analysis, we had samples from healthy subjects who were homozygous either for *DRB1*15-DQA1*01-DQB1*06:02* (DR2-DQ6), which is associated with a strongly decreased T1D risk, or *DRB1*03-DQA1*05-DQB1*02* (DR3-DQ2) or *DRB1*04:01-DQA1*03-DQB1*03:02* (DR4-DQ8), which are associated with a moderately increased T1D risk. We compared epigenome-wide methylation in CD4+ T cells and CD19+ B cells between these genotypes and discovered 14 differentially methylated CpG sites. 10 of these CpG sites were located in the HLA region and six were in or near the *HLA-DRB1* locus. Therefore, based on our analysis the effect of HLA variants localizes strongly to HLA region with few systemic effects.

In earlier studies, different *HLA-DQA1*, *-DQB1* and *-DRB1* alleles have been shown to be differentially expressed (Britten et al., 2009; D'Antonio et al., 2019; Donner et al., 2002; Yamamoto et al., 2020; Zajacova et al., 2015, 2018) but Houtman et al. also examined their effect on other loci: they examined the effect of *HLA-DRB1* alleles on the whole transcriptome in CD4+ and CD8+ T cells and CD14+ monocytes and found that *HLA-DRB* and *-DQ* molecules were differentially expressed in different *HLA-DRB1* allele carries but, in line with our results, they did not see any effects outside the HLA region (Houtman et al., 2021). However, some distal associations have been reported, such as an association between *HLA-DRB1* alleles and expression of acyloxyacyl hydrolase (*AOAH*) gene in chromosome 7 (Fairfax et al., 2012). DNA methylation studies in this context are still quite scarce, but at least in *HLA-DQA1* (Zajacova et al., 2015) and *-DRB1* (Kular et al., 2018) different alleles also seem to be differentially methylated. In a wider analysis, Kindt et al. examined DNA methylation in cord blood samples from DR3-DQ2 and DR4-DQ8 carriers using Infinium HumanMethylation450 BeadChip microarray (Illumina, San Diego, California, USA) which determines methylation in 450 000 CpG sites epigenome-wide (Kindt et al., 2018). When they compared methylation between the haplotypes, they found 41 differentially methylated sites in the HLA region, but altogether 196 differentially methylated sites epigenome-wide. This analysis was done with whole blood and PBMC samples, where methylation differences may reflect differences in cell composition between samples. However, it still suggests that although HLA effects may be centered to HLA region, they may have effects on other regions as well.

In our study, most of the differentially methylated sites were discovered when we first compared methylation between the protective genotype DR2-DQ6 and the

combined group of the risk associated genotypes DR3-DQ2 and DR4-DQ8. In this comparison there were seven differentially methylated sites at *HLA-DRB1* locus in total considering both CD4+ T cells and CD19+ B cells and five of them were hypomethylated in DR2-DQ6 group. Hypomethylation in *HLA-DRB1* loci has earlier been associated with MS disease and the association seems to be mediated by the *HLA-DRB1*15:01* allele (Kular et al., 2018) The results from Kular et al. also indicate that methylation changes correlate with expression in *HLA-DRB1*. We also compared DR3-DQ2 and DR4-DQ8 to each other, similarly to Kindt et al. (Kindt et al., 2018), but we discovered only two differentially methylated CpG sites in CD19+ B cells: cg20821042 and cg07180897 in *HLA-DQB2*, whereas Kindt et al. discovered 196 differentially methylated sites, but this difference may be due to their bigger sample size.

These methylation differences may indicate functional differences between these HLA variants, which could at least partly explain their different associations with autoimmune disorders. In their study Kindt et al. compared HLA-DR expression in CD14+ monocytes between DR3-DQ2 and DR4-DQ8 homozygotes and observed that DR3-DQ2 was associated with reduced HLA-DR expression in cord blood monocytes and also in pDCs in adolescent peripheral blood samples (Kindt et al., 2018). The DR2-DQ6 associated *HLA-DRB1* hypomethylation may also be important for immune cell function. Miller et al. have reported that systemic lupus erythematosus (SLE) is associated with hypomethylation in *HLA-DRB1* in CD8+ T cells, along with some type-I interferon response related genes, such as *STAT1* (Miller et al., 2019). When they stimulated CD8+ T cells both from SLE patients and controls using IFN-α, they noticed that *HLA-DRB1* was upregulated in SLE patients but not in controls. These activated CD8+ T cells were also then able to activate CD4+ T cells but this could be blocked using HLA-DR specific antibodies. Their conclusion was that SLE associated methylation differences in these genes made the cells more responsive to IFN-α. Miao et al. examined histone modifications in T1D-associated loci in monocytes and observed T1D-associated changes in upstream regions of *HLA-DRB1* and *-DQB1* (Miao et al., 2012). They also noticed that these changes were associated with increased expression of *HLA-DRB1* and *-DQB1* when the cells were stimulated with IFN-γ and TNF. Although Miller et al. and Miao et al. did not examine whether these methylation changes were associated with genetic variation, their results indicate that epigenetic modifications in these loci may affect how immune cells react to stimulation.

In conclusion, our results show that T1D-associated HLA genotypes DR2-DQ6, DR3-DQ2 and DR4-DQ8 have few systemic effects on methylation and their effects mainly localize to the HLA region and especially the *HLA-DRB1* locus, where the protective genotype DR2-DQ6 is associated with hypomethylation. These effects

may lead to differences in immune responses and in part explain their different associations with autoimmune disorders.

In study III we wanted to examine the long-term impact of early antibiotic exposure on immune system. Early exposure to antibiotics has been associated with development of several health problems in later life, such as obesity, autoimmune disorders and psychiatric disorders (Ainonen et al., 2024; Aversa et al., 2020; Celind et al., 2018; Fishman et al., 2019; Räty et al., 2024), but little is known about the mechanisms behind these associations. We had samples from 29 case children, who were exposed to antibiotics during their first week of life, and their controls, and we examined their immune cell frequencies in peripheral blood and CD4+ and CD8+ T cell epigenomes in samples collected at 3 months of age.

First, we compared all cases and controls and then, for additional analysis, we divided the case-control-pairs into two groups based on the duration of the antibiotic course and compared cases and controls within these groups. We did not see significant differences in immune cell frequencies in any of these comparisons after correcting for multiple comparisons, indicating that one early antibiotic course does not have large long-term effects on immune cell frequencies. However, before correction there were differences in some lymphocyte population frequencies: a small increase in the frequency of naïve CD4+ T cells, decrease in CD27+ effector memory (EM) CD4+ T cells and increase in CD27+ effector memory (EM) CD8+ T cells. Based on CD27 expression, memory cells can be divided into two groups: CD27 negative highly differentiated cells and CD27 positive resting population with low cytokine secretion which require costimulatory signals for TCR signaling (Schiött et al., 2004). This suggests that exposure to antibiotics may be associated with changes in T cell activation. Short antibiotic course was associated with a slight decrease in the frequency of plasmablasts and long antibiotic course with a decrease in frequency of total Tregs, naïve Tregs and memory Tregs in case children. This suggests that early antibiotic exposure may cause some subtle changes in lymphocytes and especially in CD4+ T cells. Epigenetic analyses supported this: in the short antibiotic group, there were 4 and 12 differentially methylated regions (DMRs), and in the long antibiotic group, 27 and 44 DMRs, in CD4+ and CD8+ T cells, respectively, associated with antibiotic exposure. It should be noted that the impact of antibiotic exposure is most likely stronger locally in the gut but, due to availability of samples, we examined these cells in peripheral blood.

In earlier research, neonatal antibiotic exposure has been associated with changes in inflammatory markers sVCAM-1, sCD14, sCD19, sCD27, IL-1RII, sVEGF-R1 and HSP70 at one year of age suggesting that even just one antibiotic course can have long-term effects on immune system (Oosterloo et al., 2020). However, there is little research in humans concerning the effects on immune cell frequencies. Support for our results come from animal studies where early antibiotic exposure has

been associated with changes in lymphocyte subsets (Alhasan et al., 2023; Fouhse et al., 2019; X. Zhang et al., 2021). In pigs, antibiotic exposure has been reported to cause increase in the frequency of total CD4+ T cells, decrease in naïve CD4+ T cells and increase in memory CD4+ T cells in peripheral blood (Fouhse et al., 2019). In mice, antibiotics given to both pregnant mice (Alhasan et al., 2023) and nursing mice (X. Zhang et al., 2021) have been associated with alterations in T cell compartments in the offspring. Antibiotics given to pregnant mice were associated with increased CD4+ and decreased GATA3+ CD4+ T cell frequencies in the offspring at 15 days of age and increased frequency of CD45+ cells still at 49 days of age in colonic lamina propria (LP) samples (Alhasan et al., 2023). When antibiotics were given to nursing mice, the offspring had a decreased frequency and numbers of colonic CD4+ and CD8+ T cells with some changes persisting still at 45 days of age (X. Zhang et al., 2021). Some studies have also reported antibiotic treatment-associated changes in Th17 subset in the intestine (Alhasan et al., 2023; Ozkul et al., 2020).

Our results also suggested that early antibiotic exposure may be associated with changes in Tregs: we saw a decrease in the frequency of total Tregs, naïve Tregs and memory Tregs in case children exposed to 7 days of antibiotics. Some antibiotics associated alterations in Tregs have earlier been reported in animal studies (D. Han et al., 2015; X. Zhang et al., 2021). In one study, mice exposed to antibiotics had an increased FoxP3+ Treg frequency in lymphoid organs and small intestine (D. Han et al., 2015). In another study, Zhang et al. saw no change in Tregs when antibiotics were given to newborn mice, but when they gave antibiotics to mice when they were nursing, they saw reduced FoxP3+ Treg frequency in colonic lamina propria (LP) in the offspring and this change persisted into adulthood (X. Zhang et al., 2021). They also isolated dendritic cells from these mice and observed that they failed to induce naïve T cell differentiation into FoxP3+ Tregs ex vivo. Again, it should be noted that we examined immune cells in peripheral blood, whereas most of the reported changes in animal studies were seen in the intestine where the impact is likely stronger.

Studies have also reported antibiotics associated changes in immune responses (Fouhse et al., 2019; Lynn et al., 2018; Madany et al., 2022). Fouhse et al. observed that pigs, who were exposed to antibiotics, produced more IFN-γ than controls when their cells were stimulated with T cell mitogen. They also produced more IFN-γ, IL-2 and IL-6 after being challenged with a heat-killed *S. Typhimurium*. In another study early antibiotic exposure in pigs was associated with a decreased amount of IFN-γ in serum but in this study they did not stimulate the cells (Xu et al., 2020). Maternal antibiotics have also been associated with changes in the offspring: Lynn et al. observed that offspring from the antibiotic exposed pregnant mice had weaker immune responses to five different vaccines and their booster shots than controls.

Similarly, as in the study by Fouhse et al., they also observed that CD4+ T cells extracted from these mice produced more IFN-γ when the cells were stimulated. Another group noticed that maternal antibiotic exposure did not affect serum cytokine levels but led to increased levels of pro-inflammatory cytokines when the offspring were challenged with lipopolysaccharide (LPS) (Madany et al., 2022). These results indicate that antibiotic exposure may affect later immune responses in a long-term manner.

In our study, we did not stimulate the cells but we examined their long-term gene expression regulation by analyzing CD4+ and CD8+ T cell epigenomes. We found only four differentially methylated regions when all samples were analyzed, but when short and long antibiotic groups were analyzed separately, we saw more differences especially in the long antibiotic group. We also conducted an enrichment analysis for the significant DMRs and found that they were associated with cell secretion, regulation of translation and amide metabolism (Pahkuri et al., manuscript in preparation). There was no direct connection to immune system functions. Longer antibiotic exposure had a bigger impact on methylation but these case children had a confirmed infection, which may have had an impact as well, making interpretation of the results harder. The weaknesses of our study were that our sample size was small and we used peripheral blood samples. Based on animal studies, many antibiotic exposure associated changes may be specific to the gut immune system (Alhasan et al., 2023; Ozkul et al., 2020; X. Zhang et al., 2021).

In conclusion, we aimed to examine whether early antibiotic exposure affects immune system in humans by examining immune cell frequencies and CD4+ and CD8+ T cell methylome. We saw only small differences mainly in children who received longer antibiotic course. However, our results together with other research indicate that early antibiotic exposure may affect lymphocytes, especially CD4+ and CD8+ T cells and their activation or differentiation into Treg cells. This could lead to changes in immune responses against later infections. Insufficient differentiation into Tregs might also increase the likelihood of autoimmunity.

7 Conclusions

The main conclusions of this thesis include:

1. Type 1 diabetes-susceptibility associated SNPs rs689 in *INS* and rs12722495 in *IL2RA* were associated with DNA methylation in *INS* and *IL2RA* gene promoters in lymphocytes, indicating that methylation changes may mediate their effect on T1D risk. This supports previous observations and confirms the association in Finnish population.

2. Type 1 diabetes was associated with DNA methylation in only one CpG site in *INS* promoter in CD8+ T cells. We did not see any T1D-associated methylation in *IL2RA* or *PTPN22* promoters in the examined lymphocyte subsets. This indicates that T1D may have some effects on DNA methylation, but they may be cell specific and with small effects.

3. These results highlight the importance of taking genetic variation into account when examining T1D-associated DNA methylation.

4. T1D susceptibility-associated HLA class II haplotypes, *DRB1*03-DQA1*05-DQB1*02* (DR3-DQ2) and *DRB1*04:01-DQA1*03-DQB1*03:02* (DR4-DQ8), and protection associated haplotype, *DRB1*15-DQA1*01-DQB1*06:02* (DR2-DQ6), had few systemic effects on epigenome-wide DNA methylation and their effects mainly localized to the HLA region and especially the *HLA-DRB1* locus, where the protective haplotype DR2-DQ6 was associated with hypomethylation.

5. Antibiotic exposure during the first week of life was not associated with changes in immune cell frequencies in PBMC at the age of 3 months.

6. Early antibiotic exposure was associated with epigenome-wide DNA methylation changes in CD4+ and CD8+ T cells at the age of 3 months, especially in cases who received longer 7-day antibiotic course and who had a confirmed infection.

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