FIRST PHASE INSULIN RESPONSE IN CHILDREN WITH ISLET AUTOANTIBODIES

Results from the Finnish Type 1 Diabetes Prediction and Prevention study

Maarit Koskinen
FIRST PHASE INSULIN RESPONSE IN CHILDREN WITH ISLET AUTOANTIBODIES

Results from the Finnish Type 1 Diabetes Prediction and Prevention study

Maarit Koskinen
University of Turku

Faculty of Medicine
Department of Paediatrics
Doctoral programme in Clinical Research

Supervised by

Professor Jorma Toppari
Institute of Biomedicine
University of Turku
Department of Paediatrics
Turku University Hospital

Professor Riitta Veijola
Department of Pediatrics
Oulu University Hospital

Reviewed by

Professor Karl-Heinz Herzig
Department of Physiology
University of Oulu
Oulu University Hospital

Docent Terhi Tapiainen
Department of Pediatrics and Adolescence
University of Oulu
Oulu University Hospital

Opponent

Associate professor Tiinamaija Tuomi
Helsinki University Hospital
Abdominal Center, Department of Endocrinology
University of Helsinki
Institute for Molecular Medicine Finland FIMM
Folkhälsan Research Center, Helsinki
Professor, Lund University Diabetes Center, Sweden

The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-7836-6 (PRINT)
ISBN 978-951-29-7837-3 (PDF)
ISSN 0355-9483 (Painettu/Print)
ISSN 2343-3213 (Sähköinen/Online)
Painosalama, Turku, Finland 2019
ABSTRACT

Type 1 diabetes results from the deficiency of insulin secretion from the pancreatic beta cells. The preclinical period of type 1 diabetes is characterized by the appearance of islet autoantibodies in the peripheral blood and appearance of two or more autoantibodies is highly predictive of clinical type 1 diabetes. The aim of this thesis was to investigate beta cell function in children from the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study. The DIPP study screens newborns for class II HLA associated risk for type 1 diabetes and follows children regularly. One or more intravenous glucose tolerance tests (IVGTTs) were performed for children who had developed islet autoantibodies.

In progressors a declined first phase insulin response (FPIR) to glucose was observed 4-6 years prior to diagnosis as compared to non-progressors. Furthermore, in the age dependent comparison children progressing to T1D had lower FPIR than non-progressors throughout the whole childhood and this difference increased over time. When the association between FPIR and class II HLA was studied, it was observed that the class II HLA association was secondary to the appearance of autoantibodies. Declining pattern of FPIR was associated with multiple autoantibodies irrespective of class II HLA risk. Genetic variants outside the class II HLA region were, however, associated with the longitudinal pattern of FPIR.

This study showed that in children that progress to clinical T1D the beta cell secretory capacity is reduced years before the onset of the disease.

KEYWORDS: type 1 diabetes, beta cell function, insulin, glucose metabolism, HLA, autoantibodies, IVGTT, preclinical
TIIVISTELMÄ

Tyypin 1 diabetes johtuu haiman betasolujen insuliinin erityksen puutteesta. Prekliinisenä aikana ennen tyypin 1 diabetesta perifeeriseen verenkiertoon ilmaantuu autovasta-aineita haiman saarekesoluja vastaan. Tyypin 1 diabeteken riski on selkeästi lisääntynyt, kun lapselle on ilmaantunut kaksi tai useampi erilaista autovasta-ainetta. Tämän väitöskirjan tarkoituksena oli tutkia betasolujen toimintaa suomalaisen DIPP-tutkimuksen lapsilla. Yksi tai useampi suonensisäinen sokerirasituskoe tehtiin lapsille, joille oli ilmaantunut autovasta-aineita.

Kun tutkittiin luokan II HLA alueen ja ensivaiheen insuliinivasteen välisiä yhteyksiä, havaittiin, että yhteytä johtui autovasta-aineista. Ajan myötä heikkenevä insuliinivaste oli yhteydessä kahden tai useamman autovasta-aineen esiintymiseen riippumatta luokan II HLA genotypiin liittyvän riskistä. Muiden luokan II HLA alueen ulkopuolisten geneettisten varianttien ja insuliinivasteen välillä löytyi kuitenkin yhteyksiä.

Tämä tutkimus osoitti, että lapsilla, jotka sairastuvat tyypin 1 diabetesen betasolujen kyky erittää insuliinia on vähentynyt jo vuosia ennen kliinistä diagnoosia.

AVAINSANAT: tyypin 1 diabetes, betasolujen toiminta, insuliini, glukoosi-metabolia, HLA, autovasta-aineet, IVGTT, prekliininen
# Contents

Abbreviations ........................................................................................................... 7
List of original publications ...................................................................................... 9

1  Introduction ............................................................................................................ 10

2  Review of literature .............................................................................................. 11
  2.1  History of type 1 diabetes .............................................................................. 11
  2.1.1  The discovery of insulin ........................................................................... 12
  2.2  Pathophysiology of type 1 diabetes ............................................................... 12
  2.3  Symptoms and diagnosis of type 1 diabetes .................................................. 13
  2.4  Epidemiology of type 1 diabetes ................................................................. 13
  2.5  Prediction of type 1 diabetes ........................................................................ 14
    2.5.1  Environmental risk factors of type 1 diabetes ....................................... 15
    2.5.2  The HLA DR-DQ associated genetic risk of type 1 diabetes .................. 18
    2.5.3  Genetic risk variants outside the HLA DR-DQ region .......................... 19
    2.5.4  Islet autoantibodies .............................................................................. 20
    2.5.5  Changes in glucose metabolism ......................................................... 22
  2.6  Prevention of type 1 diabetes ........................................................................ 23
  2.7  Beta cells of the pancreatic islets ................................................................. 26
    2.7.1  Anatomy and function ......................................................................... 26
    2.7.2  Embryogenesis .................................................................................... 27
    2.7.3  Replication and function after birth .................................................... 28
  2.8  Glucose metabolism ..................................................................................... 29
  2.9  The first phase insulin response ................................................................. 31

3  Aims of the study ................................................................................................... 33

4  Materials and methods .......................................................................................... 34
  4.1  DIPP study ................................................................................................... 34
  4.2  Study subjects ............................................................................................... 34
  4.3  Genetic analyses and risk categorization (study II) ...................................... 36
  4.4  Genetic variants outside the class II HLA DR-DQ region (study III) ............. 38
  4.5  Islet autoantibody measurements ................................................................ 38
  4.6  IVGTT protocol ............................................................................................ 38
  4.7  Laboratory measurements .......................................................................... 38
  4.8  Markers of glucose metabolism ................................................................... 39
5 Results and discussion .................................................................. 41
  5.1 Longitudinal changes of FPIR after the onset of islet autoimmunity (study I) ........................................................................ 41
    5.1.1 Longitudinal changes of FPIR before type 1 diabetes (study I) .................................................................................. 41
    5.1.2 Longitudinal changes of FPIR in an age-dependent comparison .................................................................................. 42
  5.2 Associations between FPIR, class II HLA risk and autoantibodies (study II) ........................................................................ 43
    5.2.1 Associations between class II HLA risk and islet autoantibodies .................................................................................. 43
    5.2.2 Associations between FPIR and the class II HLA risk .............................................................................................. 44
    5.2.3 Associations between FPIR and islet autoantibodies .............................................................................................. 44
  5.3 Associations of FPIR and genetic variants outside class II HLA region (study III) .......................................................... 46
  5.4 Other markers of glucose metabolism (study I) ................................ 47
  5.5 General discussion .................................................................. 48

6 Conclusions .................................................................................. 53

Acknowledgements ........................................................................ 54

References ...................................................................................... 57

Original publications ...................................................................... 79
Abbreviations

ABIS     All Babies in Southeast Sweden
ANOVA    analysis of variance
AUC0-10 min area under the 10-minute curve
CI       confidence interval
CS       caesarean section
DAISY    Diabetes Autoimmunity Study in the Young
DiMe     Childhood Diabetes in Finland
DiPiS    Diabetes Prediction in Skåne
DIPP     Type 1 Diabetes Prediction and Prevention
ER       endoplasmic reticulum
EV       enterovirus
FDR      first-degree relative
FPIR     first phase insulin response
GADA     glutamic acid decarboxylase antibodies
GLUT     glucose transporter
HLA      human leukocyte antigen
HOMA-IR  homeostasis model assessment for insulin resistance
HR       hazard ratio
IA-2A    islet antigen 2 antibodies
IAA      insulin autoantibodies
ICA      islet cell antibodies
IFG      impaired fasting glucose
IGT      impaired glucose tolerance
INS-VNTR insulin gene variable number of tandem repeats
IVGTT    intravenous glucose tolerance test
MODY     maturity onset of the diabetes of the young
mRNA     messenger RNA
OGTT     oral glucose tolerance test
OR       odds ratio
RIA      radioimmunoassay
SD       standard deviation
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEDDY</td>
<td>The Environmental Determinants of Diabetes in the Young</td>
</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>type 2 diabetes</td>
</tr>
<tr>
<td>ZnT8A</td>
<td>zinc transporter 8 autoantibodies</td>
</tr>
</tbody>
</table>
List of original publications

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


The original publications have been reproduced with the kind permission of the copyright holders.
1 Introduction

Type 1 Diabetes (T1D), one of the most common chronic diseases in childhood and adolescence, has the world’s highest incidence in Finland. Type 1 diabetes results from a deficiency in insulin secretion by the pancreatic beta cells. The preclinical period preceding the clinical onset of T1D is characterized by the appearance of islet autoantibodies in the peripheral blood.

In this thesis beta cell function was studied in children who participated in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study. The DIPP study screens for a class II HLA associated risk for type 1 diabetes. Children with specific genotypes are invited to follow-up and repeatedly tested for signs of islet autoimmunity. Intravenous glucose tolerance tests (IVGTTs), a method to assess the beta cell function, were performed to DIPP children with islet autoantibodies.

The incidence of T1D has significantly increased in different populations. Many hypotheses have tried to explain this phenomenon. It has been suggested that beta cells are overloaded. We hypothesized that changes in beta cell function could contribute to the development of islet autoimmunity and T1D. In this study, we aimed to investigate beta cell function in children who develop T1D and those who remain healthy.
2 Review of literature

2.1 History of type 1 diabetes

The etiology of type 1 diabetes (T1D) is still unknown, but the typical symptoms of T1D were identified as early as in the BC era. The disease which was at the time characterized as “passing too much urine” was treated with nutritional management such as wheat grains, grapes, berries and sweet beer. The field of metabolism and diabetes was intensely investigated in the 19th century. Work done by a French physiologist Claude Bernard (1813-1878) included the demonstration that blood glucose levels are regulated by the absorption of dietary carbohydrates. His concept of milieu interieur, i.e., internal environment, was later refined as homeostasis by American physiologist Walter Cannon (1871–1945).

The islets of Langerhans, containing the endocrine, hormone-producing, cells of the pancreas, were named after the discoverer Paul Langerhans (1847-1888). Still an undergraduate, Langerhans was the first to describe them in 1869 (Langerhans, 1869). In his dissertation he described highly innervated pale-stained areas with a rich vascular network throughout the pancreas but did not suggest any function to them. It was later, in 1893 that these cell clusters were named after him by a French pathologist and histologist Edouard Laguesse (1861-1927) (Laguesse, 1893).

Years later, in 1889, Oscar Minkowski (1858-1931) and Joseph von Mering (1849-1908) discovered that it was the pancreas that was important for glucose homeostasis: they had removed dogs’ pancreases, and this resulted in fatal diabetes (von Mering J, Minkowski O., 1890). This finding was the first experimental evidence that diabetes could be of pancreatic origin.

French physician Etienne Lancereaux (1829-1910) had suspected diabetes to be of pancreatic origin and had already made a distinction between two primary forms of diabetes in 1877. Later, in 1936, Harold Himsworth (1905-1993) tested these subtypes: both oral glucose and intravenous insulin were given to patients with diabetes. Two patterns were observed, one with immediate suppression of hyperglycemia after insulin administration (‘insulin-sensitive’) and other where the suppression was attenuated (‘insulin-insensitive’/’insulin-resistant’) (Himsworth, 1936). The autoantibodies associated with T1D were discovered in 1974 (Bottazzo, 1974).
et al., 1974; MacCuish et al., 1974), and the first reports of genetic associations were published in the 1970’s (Nerup et al., 1974; Singal & Blajchman, 1973).

2.1.1 The discovery of insulin

On October 30th, 1920, a 28-year old surgeon named Frederick Banting (1891-1941) was preparing a lecture for medical students in the University of Western Ontario when his attention was caught by article “The relation of the islets of Langerhans to diabetes with special reference to cases of pancreatic lithiasis” (Barron, 1920). The following night, unable to sleep, he came across the idea of ligating pancreatic ducts in dogs and collecting the pancreatic extract. He was advised to present his idea to professor John James Rickard McLeod (1876-1935) at the Department of Physiology in the University of Toronto, and, after two meetings, Banting had convinced him enough. Professor McLeod provided a working space as well as medical student Charles Best (1899-1978) to help him with the investigation.

The collaboration between Banting and Best was successful. They actively tried different methods and proceeded quickly with their work. Although the initial idea of duct ligation was eventually discarded, they had learned how to collect pancreatic extracts. There were problems with purity of the extract, so a visiting biochemist James Collip (1892-1965) later collaborated with them. Collip was able to remove impurities and prepare clean insulin.

On January 1922, insulin, which was later completely purified, was given to Leonard Thompson, a 14-year-old boy with severe diabetic ketoacidosis. Thompson’s previous treatment had been dietetic regulation and he had been in the hospital for over a month. The treatment with insulin was soon proven to be a success. A preliminary report under the title “Pancreatic Extracts in the Treatment of Diabetes Mellitus” was published in March with emphasis on the first patient (Banting et al., 1922). The discovery and use of insulin meant that people with type 1 diabetes could survive. Banting and MacLeod were awarded a Nobel Prize in 1923, and they shared the prize with Best and Collip, respectively.

2.2 Pathophysiology of type 1 diabetes

Glucose homeostasis is the process that keeps blood glucose at a steady-state level, maintaining equilibrium despite ongoing fluctuations. The word homeostasis comes from Greek and essentially means ‘staying the same’.

Insulin is needed for glucose to enter the cells. In most of the cells in the body, glucose is the main source of energy through the generation of ATP in the tricarboxylic acid cycle in mitochondria. Due to the deficiency of insulin glucose cannot enter the cell, and this leads to a derangement in the glucose homeostasis.
Physiologically, insulin is secreted from the pancreas into the portal vein in a pulsatile fashion (Hellman et al, 2009; Song et al., 2000). In T1D, the normally occurring insulin pulsatility is absent and the instant feedback loop between the liver and pancreatic beta cells is disturbed. The insulin response to glucose, measured as the first phase insulin response (FPIR) is obliterated.

2.3 Symptoms and diagnosis of type 1 diabetes

Typical symptoms of T1D are polyuria, polydipsia and unexplained weight loss (Table 1). Diabetes is diagnosed according to the WHO and ADA criteria (Alberti & Zimmet, 1998; American Diabetes Association, 2018), and ISPAD recommendations (Craig et al., 2014). When the glucose level in circulation exceeds the renal glucose threshold, the resultant osmotic diuresis causes symptoms of thirst and polyuria. Insulin-sensitive target organs, such as muscle and adipose tissues do not receive adequate glucose without insulin. In the absence of glucose, energy is produced from free fatty acids and amino acids. Protein and fat breakdown causes weight loss and ketone accumulation causing eventually life-threatening ketoacidosis.

Table 1. The diagnostic criteria of type 1 diabetes.

<table>
<thead>
<tr>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose ≥ 7.0 mmol/l, or</td>
</tr>
<tr>
<td>Plasma glucose ≥ 11.1 mmol/l at 2 hours during OGTT, or</td>
</tr>
<tr>
<td>Classic symptoms of diabetes (polyuria, polydipsia, enuresis, unexplained weight loss) with a random plasma glucose of ≥11.1 mmol/l</td>
</tr>
</tbody>
</table>

2.4 Epidemiology of type 1 diabetes

Finland has the world’s highest incidence of T1D. In 2017 the annual incidence rate of T1D was 62/100000/year in children and adolescents under 15 years of age (www.diabetesatlas.org). Other countries with a high incidence of T1D in children under 15 are Sweden and Kuwait (both 42/100 000/year).

During World War II there was a registry of patients in Finland who received insulin or diet supplements. Although this registry was incomplete it reported 250 individuals under the age of 20 who had diabetes (population at the time was 3.6 million) (Vartiainen, 1944).

In 1953 (Somersalo, 1954) a more detailed report was made when a questionnaire was sent to all 2188 Finnish physicians. There were 663 children born since 1939 that developed diabetes under the age of 15 and 169 individuals had died.
with a diagnosis of diabetes. The incidence of new cases in 1953 was estimated to be 12.5/100,000/year (Somersalo, 1954). Since then, the overall incidence has increased and started to plateau in 2011 (Table 2).

Table 2. The incidence of type 1 diabetes in children under 15 years in Finland from 1965 to 2011. Incidence is shown /100,000/year (95% confidence interval).

<table>
<thead>
<tr>
<th>Time</th>
<th>Overall incidence</th>
<th>Boys</th>
<th>Girls</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980-1984*</td>
<td>34 (33-36)</td>
<td>37 (34-39)</td>
<td>32 (30-34)</td>
<td>(Harjutsalo et al., 2008)</td>
</tr>
<tr>
<td>1990-1994*</td>
<td>39 (38-41)</td>
<td>40 (38-43)</td>
<td>39 (36-41)</td>
<td>(Harjutsalo et al., 2008)</td>
</tr>
<tr>
<td>2006</td>
<td>65 (60-70)</td>
<td>69 (62-77)</td>
<td>60 (53-68)</td>
<td>(Harjutsalo et al., 2013)</td>
</tr>
<tr>
<td>2011</td>
<td>64 (60-70)</td>
<td>76 (68-85)</td>
<td>52 (46-60)</td>
<td>(Harjutsalo et al., 2013)</td>
</tr>
</tbody>
</table>

*the mean annual incidence per 100,000 per year (95% CI)

2.5 Prediction of type 1 diabetes

Much of the data currently known about the pathogenesis process of T1D comes from longitudinal studies that have followed selected population based on their estimated genetic risk, family history or location (Table 3).

Besides revealing several risk factors associated with type 1 diabetes, prospective studies have served as a platform for prevention trials and resulted in better metabolic control at diagnosis reducing the frequency of diabetic ketoacidosis (Hekkala, et al, 2017; Lundgren et al., 2014).
Table 3. Some of the prospective studies that have followed selected population and the development of type 1 diabetes.

<table>
<thead>
<tr>
<th>Study (recruitment began/ recruitment period)</th>
<th>Country</th>
<th>Population</th>
<th>Subjects with T1D after follow-up (total)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular Study in Young Finns (1980)</td>
<td>Finland</td>
<td>General population</td>
<td>18 (3475)</td>
<td>(Knip et al., 2010)</td>
</tr>
<tr>
<td>Childhood Diabetes in Finland DiMe (1986-1989)</td>
<td>Finland</td>
<td>Siblings of children with T1D</td>
<td>47 (701)</td>
<td>(Mrena et al., 2006)</td>
</tr>
<tr>
<td>BABYDIAB-BABYDIET (1989-2006)</td>
<td>Germany</td>
<td>Relatives of patients with T1D</td>
<td>115 (2441)</td>
<td>(Hoffmann et al., 2019)</td>
</tr>
<tr>
<td>Australian Babydiab (1993)</td>
<td>Australia</td>
<td>Relatives of patients with T1D</td>
<td>12 (548)</td>
<td>(Couper et al., 2009)</td>
</tr>
<tr>
<td>The Diabetes Autoimmunity Study in the Young DAISY (1993-2004)</td>
<td>USA</td>
<td>Class II HLA risk genotypes and relatives of patients with T1D</td>
<td>94 (2547)</td>
<td>(Frohnert et al., 2018)</td>
</tr>
<tr>
<td>The Finnish Type 1 Diabetes Prediction and Prevention study DIPP (1994-ongoing)</td>
<td>Finland</td>
<td>Class II HLA risk genotypes</td>
<td>388 (15253)</td>
<td>(Bauer et al., 2019)</td>
</tr>
<tr>
<td>Diabetes Prediction in Skåne DiPis (2000-2004)</td>
<td>Sweden</td>
<td>Class II HLA risk genotypes</td>
<td>51 (4340)</td>
<td>(Lundgren et al., 2019)</td>
</tr>
<tr>
<td>The Environmental Determinants of the Diabetes in the Young TEDDY (2004-ongoing)</td>
<td>USA, Finland, Sweden, Germany</td>
<td>Class II HLA risk genotypes</td>
<td>310 (8676)</td>
<td>(Rewers et al., 2018)</td>
</tr>
<tr>
<td>TrialNet Pathway to Prevention (2004-ongoing)</td>
<td>USA, Finland, Australia, UK, NZ, Canada, Sweden, Germany, Italy</td>
<td>Relatives of patients with T1D</td>
<td>549 (4063)</td>
<td>(Evans-Molina et al., 2018)</td>
</tr>
</tbody>
</table>

2.5.1 Environmental risk factors of type 1 diabetes

Several hypotheses have been proposed to explain the observed increase in T1D (Dahlquist et al., 2006; Rook et al., 2012; Wilkin et al., 2001) The frequencies of class II HLA genotypes in patients with T1D has changed over time, which suggests stronger environmental role in the disease process (Hermann et al., 2003).
Many prenatal risk factors, such as maternal age can affect the risk of T1D. The maternal age over 35 increased the risk of T1D in Swedish children under 15 years (OR 1.36, 95% confidence interval [CI] 1.15-1.59) (Dahlquist & Källén, 1992). Caesarean section (CS) was associated with increased risk of T1D (OR 1.32, 95% CI 1.14-1.52) (Dahlquist & Källén, 1992). This finding was not confirmed in a large Norwegian population-based study (Stene et al., 2003) Later study found an initial association between CS and the increased risk of T1D, but the association was lost after adjustments (OR 1.34, 95% CI 1.01-1.78) (Stene et al., 2004). Certain genotypes may modify the risk conferred by the delivery mode (Bonifacio et al., 2011; Stene et al., 2011).

Growth patterns in children have also been linked to the risk of T1D. Increased weight gain in infancy has been reported to be associated with an increased risk of T1D (Baum et al., 1975; Couper et al., 2009; Hyppönen et al., 1999). In the DiMe study where the index children with T1D were retrospectively investigated there were no significant differences in birth weight between children with T1D or their date and sex matched controls (Hyppönen et al., 1999). In young DiMe children the prevalence of obesity has been associated with an increased risk of T1D (OR 2.37, 95% CI 1.07-5.26 in 4-year-old children) (Hyppönen et al., 2000). In the TEDDY study the prevalence of obesity has been reported as being linked to specific HLA genotypes in 2-4-year-old children (Yang et al., 2014). There was an association between weight at 1 year of age and the development of islet autoimmunity in the TEDDY study (HR 1.16, 95% CI 1.06-1.27) (Elding Larsson et al., 2016). In the DiPis study there was no difference between weight gain in subjects with T1D and the controls; children with T1D were taller at birth compared to their non-HLA matched controls, but not when compared to HLA matched controls (Larsson et al., 2008). The relative height increase has been linked to an increased risk of T1D (Dahlquist et al., 1991; Hyppönen et al., 2000). In 0-4-year-old children an increased height over 0.1 standard deviation (SD) has been linked to an increased risk of T1D (OR 3.09, 95% CI 1.21-7.94) (Dahlquist et al., 1991). In the DAISY study, faster growth (in height) (1 SD difference) was associated with the development of islet autoimmunity (HR 1.63, 95% CI 1.31-2.05) and T1D development (adjusted HR 3.34, 95% CI 1.73-6.42). Weight gain velocity (1 SD difference) did not display the same association (HR 0.88, 95% CI 0.69-1.11; HR 1.01, 95% CI 0.58-1.77, respectively) (Lamb et al., 2009).

When children in the age range of 0-15 years with recent onset of T1D were investigated, an association between enteroviruses (EV) and an increased risk of T1D has been observed (OR 7.21, 95% CI 3.27-15.89) (Nairn et al., 1999). In another study EV RNA was found in 20 % (10/50) of subjects with preclinical T1D, in 36 % (17/47) of newly diagnosed T1D patients and in 4% (2/50) of control children, showing increased risk of T1D in patients with EV RNA (OR 6.0, 95% CI 1.2-29 in
preclinical T1D and OR 13.6, 95% CI 2.9-63 in newly diagnosed) (Moya-Suri et al., 2005). In young DIPP study children, there was a temporal aspect to EV infection during the 6-month period preceding the first detection of islet autoantibodies compared to children without islet autoantibodies (evidence of EV infection by serological testing OR 3.7, 95% CI 1.2-11.4) (Lönnrot et al., 2000). The development of autoimmunity was increased in those who had an EV infection before the age of 12 months and were exposed to cow’s milk before the age of 3 months (Lempainen et al., 2012). More specifically, it was observed in the DIPP study that EV RNA was more often found in serum samples before the appearance of islet autoantibodies from children who progressed to T1D than in samples from non-progressors (OR 7.7 (95% CI 1.9-31.5) (Oikarinen et al., 2011). A peak of EV RNA was also observed in stools before appearance of islet autoantibodies (Honkanen et al., 2017). In more detail, coxsackie B1 infections were associated with the appearance of IAA only as the first islet autoantibody (Sioofy-Khojine et al., 2018).

Some associations between T1D risk and vaccinations have been found (Blom et al., 1991; Dahlquist et al., 1991; Elding Larsson et al., 2018). The lack of measles vaccination was associated with an increased risk of T1D in the age group of 5-9 years (OR 3.33, 95% CI 1.28-8.65) (Dahlquist et al., 1991). In Finnish children from the TEDDY study the influenza vaccination was associated with decreased risk of autoimmunity and T1D (HR 0.47, 95% CI 0.29-0.75; HR 0.38, 95% CI 0.20-0.72, respectively)(Elding Larsson et al., 2018). Interestingly, rotavirus vaccinations were introduced in USA in 2006 and Australia in 2007, after which there was a decrease in the incidence of T1D in children aged 0-4 years in both countries (Perrett et al., 2019; Rogers et al., 2019). In the Australian Babydiab study, rotavirus infections and the development of islet autoimmunity have been reported as temporally associated in high risk children (Honeyman et al., 2000).

The duration of breastfeeding did not affect the risk of T1D in the DAISY study even if the child was breastfed at the time of introduction of solid foods (HR 0.97, 95% CI 0.92-1.01; HR 0.70, 95% CI 0.38-1.28, respectively). However, if the child was breastfed at the time of introduction of wheat/barley, protection against T1D was observed (adjusted HR 0.47, 95% CI 0.26-0.86) (Frederiksen et al., 2013). Late introduction of gluten to the diet was linked to an increased risk of islet autoimmunity (HR 1.57, 95% CI 1.07-2.31) and early introduction was modestly associated with a lower risk (HR 0.68; 95% CI 0.47-0.99) when compared to the introduction between 4 and 9 months (Uusitalo et al., 2018). Cow’s milk has been associated with the risk of T1D, but results are contradictory. Early introduction of formula (before the age of 3 months vs. after 3 months) was associated with an increased risk of T1D in a retrospective study (adjusted OR 1.53, 95% CI 1.1-2.2) (Dahlquist et al., 1990). The TRIGR study tested the use of casein hydrolysate formula during the first 8 months of life but there was no effect on the development
of autoimmunity or T1D (Writing Group for the TRIGR Study Group et al., 2018). Genetic background may influence how the exposure to cow’s milk affects T1D risk (Lempainen et al., 2009).

The intake of sugars and carbohydrates has been associated with the risk of T1D. In the DAISY study, sugar intake was linked to an increased progression rate to diabetes in children with multiple autoantibodies (HR 1.75, 95% CI 1.07-2.85) (Lamb et al., 2015). Compared to the lowest quartile, the carbohydrate intake of 25-75th percentiles was not associated with an increased risk of T1D but at over the 75th centile positively carried this association (OR 1.44, 95% CI 0.98-2.12; OR 4.67, 95% CI 3.02-7.23, respectively) (Dahlquist et al., 1991).

Vitamin D supplementation has been thought to offer protection against the development of T1D, and some meta-analyses have concluded that vitamin D supplementation may reduce the risk of T1D (Dong et al., 2013; Zipitis & Akobeng, 2008). In the DIPP study there was no association between serum 25-hydroxyvitamin D levels and the development of T1D (Mäkinen et al., 2016); moreover, these results were supported by the DAISY study (Simpson et al., 2011). Still, genetic factors may also play a role in this case (Frederiksen et al., 2013; Norris et al., 2018).

Severe emotional stress has been linked to the onset of T1D in the age group of 5-9 years (OR 1.82, 95% CI 1.09-3.03) (Hägglöf et al., 1991). In the ABIS study (Nygren et al., 2015) where stress was measured prospectively in over 10,000 participants, childhood experience of a serious life event was associated with a higher risk of T1D (HR 3.0, 95% CI 1.6-5.6 adjusted for age and heredity). Living in a war zone for over a month has been associated with an increased incidence of T1D in Israeli children (Zung et al., 2012).

2.5.2 The HLA DR-DQ associated genetic risk of type 1 diabetes

The risk of T1D is known to be especially attributed to the class II HLA DR-DQ region on the short arm of the 6th chromosome. In addition to the association with T1D, the class II HLA is also linked to coeliac disease, birth weight and later obesity (Carlsson et al., 2012; Elding Larsson et al., 2016; Larsson et al., 2005; Yang et al., 2014). Class II HLA DR3 has shown an association with clinical characteristics of T1D, such as a slower onset; it was described to spread more evenly over the years and presenting severe symptoms less often (Ludvigsson et al., 1986).

The class II HLA molecule consists of α and β chain and form a heterodimer. These molecules are encoded by HLA-DR, -DQ and –DP loci. The function of class II HLA molecule is the presentation of antigens to T-cells. These heterodimers are present in antigen presenting cells, such as B-lymphocytes, dendritic cells and
macrophages. The slightly different structure of the different HLA molecules means that there is a different structure in the cleft that binds peptides. The HLA class II susceptibility alleles DQ2 and DQ8 for T1D have been described to form unstable complexes with most epitopes (Lee et al., 2001).

The HLA genetic area is highly polymorphic meaning that there is a large variability of each gene in the population. Both susceptibility and protective DR-DQ associations with T1D are known (OR ranging from 0.02 to over 10) (Erlich et al., 2008; Ilonen et al., 2016). The risk is especially high in DR3/4-DQ8 siblings who share their both HLA haplotypes with their diabetic proband sibling: by the age of 15 years the risk of T1D is 85 % (Aly et al., 2006). HLA-DQB1*0602 allele is associated with dominant protection from diabetes also in islet cell antibody-positive first-degree relatives of patients with T1D (Pugliese et al., 1995).

2.5.3 Genetic risk variants outside the HLA DR-DQ region

In addition to the class II HLA region, multiple other loci have shown effect on the risk of T1D (Barrett et al., 2009; Groop & Pociot, 2014). The associations of other loci have been shown to include the progression rate to clinical onset of T1D or the appearance of autoantibodies (Lempainen et al., 2015). Adjacent to the HLA II region, class I HLA encodes molecules that are expressed in most cells of the human body and their functions include the presentation of peptides derived from the endoplasmic reticulum (ER) lumen. The presence of certain class I alleles has been found to influence T1D risk in the Finnish population (Mikk et al., 2017).

The PTPN22 locus on chromosome 1p13 encodes for a lymphoid protein tyrosine phosphatase, nonreceptor type 22. This phosphatase manages the activity of immune cells and PTPN22 has been associated with the appearance of islet autoantibodies (Lempainen et al., 2015). Other PTPN22 associations include vitiligo (Canton et al., 2005), rheumatoid arthritis (Orozco et al., 2005) and thyroid diseases (Smyth et al., 2004). The protein tyrosine phosphatase, nonreceptor type 2 (PTPN2) (18p11.2) has been associated with the progression rate to clinical T1D after seroconversion in the DIPP study (Lempainen et al., 2015). A deficiency in PTPN2 expression affects beta cell function in mice (Xi et al., 2015).

Insulin gene (11p15) encodes for preproinsulin, a precursor of proinsulin. The association of between insulin gene and T1D was found in the 1980s (Bell et al., 1984). In the Finnish population insulin gene is one of the most strongly T1D associated genes outside the class II HLA region (Laine et al., 2013). Insulin gene did not affect the progression from autoantibody positivity to the clinical onset of T1D (Lempainen et al., 2012). The promotor region of the insulin gene is a highly polymorphic area consisting of variable number of tandem repeats (VNTR) which are grouped into three classes according of their length: class I VNTR consists in approximately 30 to 60 repeats and
class III allele consists in 140-200 repeats. The class I and class III VNTR are the most common alleles in the Caucasian population. Insulin VNTRs have been associated with size at birth (Dunger et al., 1998; Ong et al., 1999) and growth (Bennett et al., 2004; Heude et al., 2006). During fetal development and early childhood, insulin gene expression at higher levels in the human thymus has been shown to be associated with class III alleles (Pugliese et al., 1997; Vafiadis et al., 1997). In the pancreas, class III VNTRs have been shown to be linked to a lower insulin transcription compared to class I alleles (Bennett et al., 1995; Vafiadis et al., 1996).

The FUT2 gene encodes for fucosyltransferase 2, which synthesizes the ABO antigens in secretions and bodily fluids, such as saliva, tears, breast milk, gastric juice and gastrointestinal mucus. A nonfunctional gene (called non-secretors) has been shown to confer increased susceptibility to T1D and resistance to norovirus infection (Laine et al., 2013; Smyth et al., 2011). The prevalence of secretors was increased in rapid progressors with high risk HLA genotypes (Pöllänen et al., 2017). The secretor status has also been associated with the composition of intestinal microbiota: for example, non-secretors were less colonized by Bifidobacteria (Kumar et al., 2015; Wacklin et al., 2011).

The gene encoding for cathepsin protease CTSH, a ubiquitously expressed lysosomal cysteine protease can be involved in overall protein turnover and specific cellular processes, such as apoptosis, antigen presentation, and prohormone processing. The CTSH genotypes are associated with the need for insulin in newly diagnosed T1D patients (Fløyel et al., 2014).

Both ERBB3 and IKZF4 are located in the same region on the long arm of chromosome 12. Both genes have been associated with T1D (Hakonarson et al., 2008; Laine et al., 2013; Todd et al., 2007). In more detail, the association between the IKZF4-ERBB3 region (12q13) and the development of T1D was observed in children with GADA as the first detected autoantibody (Lempainen et al., 2015). Both genes have also shown an inverse association with IAA positivity at diagnosis (Lempainen et al., 2013).

### 2.5.4 Islet autoantibodies

Islet autoantibody is a generic term that is used to describe any autoantibody that is targeted to the islets of Langerhans. Among islet autoantibodies are islet cell cytoplasmic antibodies (ICA) and biochemical autoantibodies (IAA, insulin autoantibodies; GADA, glutamic acid decarboxylase antibodies; IA-2A, insulinoma-2-associated antibodies and zinc transporter 8 antibodies ZnT8A). The unspecific islet cell antibodies (ICA) were the first ones found in 1974 (Bottazzo et al., 1974; MacCuish et al., 1974).
Islet autoantibodies are considered as a marker of beta cell dysfunction, not pathological per se (Ziegler et al., 2013). In the follow up studies there has been a high incidence of appearance of islet autoantibodies already during the first years of life (Krischer et al., 2015; Parikka et al., 2012; Steck et al., 2011; Ziegler et al., 2012). The risk of T1D is low when ICA alone is present (Orban et al., 2009; Siljander et al., 2009). A single temporary ICA positivity was associated with a 5-year risk of 1.4% (Siljander et al., 2009). Multiple islet autoantibodies are associated with strongly increased risk of T1D (Parikka et al., 2012; Siljander et al., 2009; Ziegler et al., 2013). This risk has been estimated to be over 40% in a 5-year follow up and over 80% in a 15-year follow-up (Ziegler et al., 2013). Some studies have also used levels of islet autoantibodies in the prediction of T1D (Achenbach et al., 2004; Mrena et al., 2006; Steck et al., 2011).

The immunofluorescence technique is used to detect ICA and identifies any antibody that binds to islet tissue, therefore it is not specific. The procedure involves sectioning of the pancreas from a human donor with an O blood type. The serum from the patient is incubated on these sections of cryocut pancreas and an antihuman immunoglobulin conjugate is added. Afterwards, when the slide is examined under a fluorescence microscope, the islets fluoresce if there are islet cell cytoplasmic antibodies present in the serum. ICA are expressed in terms of JDF (Juvenile Diabetes Foundation) units.

Insulin autoantibodies (IAA) were found in 1983 in patients with recent onset of T1D (Palmer et al., 1983). As the first appearing autoantibody, the peak period for IAA is during the second year of life in the DIPP and TEDDY studies (Ilonen et al., 2013; Krischer et al., 2015). Genetic associations with IAA have included the HLA-DR4-DQ8 risk haplotype (Kimpimäki et al., 2001; Krischer et al., 2015; Sabbah et al., 1999) and the insulin gene polymorphism (Lempainen et al., 2015).

As the major autoantigen of T1D, GAD65 (65 kDa isoform of glutamate decarboxylase GAD65) antibodies were identified in 1990 (Baekkeskov et al., 1990). As the first appearing autoantibody in DIPP and TEDDY children, GADA has been shown to peak between 3 and 5 years of age (Ilonen et al., 2013; Krischer et al., 2015). The genetic associations with GADA have included the HLA-DR3-DQ2 haplotype (Ilonen et al., 2017) and polymorphism in the IKZF4-ERBB3 region (Lempainen et al., 2015). An increase in the prevalence of GADA was observed by number of DQ2 haplotype in young age groups (Graham et al., 2002).

The autoantibodies to IA-2 were initially characterized in 1995 in patients with recent onset T1D (Payton et al., 1995). IA-2 is a membrane protein found in the secretory granules of endocrine cells and neurons (Solimena et al., 1996). IA-2A has been associated with the HLA-DR4-DQ8 genotype (Gullstrand et al., 2008).

ZnT8 is a recent major islet autoantigen discovered in 2007 (Wenzlau et al., 2008). It belongs to a family of zinc transporters and is localized in the insulin-containing
secretory granules where zinc forms a complex with insulin. ZnT8 plays an important role in this accumulation of insulin (Chimienti et al., 2006). ZnT8A were detected in 63% of a group of Finnish children with T1D, with an inverse correlation with age at diagnosis: children diagnosed under the age of 5 were less likely to be positive for ZnT8 at the time of diagnosis than children in older age groups (Salonen et al., 2013). The frequency of ZnT8A was highest in those that carried neither DR3 nor DR4, but there was a positive relationship between ZnT8A and the class II HLA haplotype not associated with T1D ((DR13) -DQB1*0604) (Salonen et al., 2013).

2.5.5 Changes in glucose metabolism

When ICA positive relatives of T1D patients were followed for 7 years, a dysglycemic OGTT occurred at least once in 60% of the subjects during the follow-up (Sosenko et al., 2009). Dysglycemia at the 6-month visit was highly predictive of T1D, especially in those aged less than 13 years (HR 5.4, 95% CI 3.6-8.1) (Sosenko et al., 2009).

Most rapid rise in 2 h glucose OGTT in relatives of T1D patients (with either ICA and IAA or reduced FPIR) and rise of HbA1c as well as 2 h glucose in OGTT has been estimated to appear mostly during the last before diagnosis of T1D (Ferrannini et al., 2010).

Prior studies in DIPP study children with multiple autoantibodies observed an increase in 2-hour glucose in OGTT 1.5 years before diagnosis of T1D (Helminen et al., 2015). Change in HbA1c was observed 2 years before the diagnosis T1D (Helminen et al., 2015). Random plasma glucose values started to rise 1.5 years before diagnosis and value ≥7.8 mmol/l was predictive of T1D in DIPP study children (HR 6.0, 95% CI 4.3-8.6) (Helminen et al., 2015). Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) were predictive of T1D (HR 3.2, 95% 1.5-7.0; HR 8.3, 95% CI 6.0-11.5, respectively) (Helminen et al., 2015).

There has not been much longitudinal data on the change of FPIR in preclinical period, although low FPIR value based on a single sample is clearly predictive of T1D (Bingley et al., 1996; Bingley et al., 2006; Siljander et al., 2013; Vardi et al., 1991). In the T1D model (Eisenbarth, 1986), an initially normal insulin secretion capacity, has been suggested to decline linearly before diagnosis of T1D. In two earlier studies with small study population (Colman et al., 1998; Srikanta et al., 1984) a linear decrease in FPIR was observed before the onset of T1D. More recently, using longitudinal data on 74 high- risk subjects, Sosenko et al. reported an accelerating decline in FPIR about 1.5 to 0.5 years before the diagnosis (Sosenko et al., 2013).

A single value of insulin resistance index determined by the homeostasis model assessment (HOMA-IR index) measuring insulin sensitivity (Matthews et al., 1985)
did not predict T1D but HOMA-IR adjusted to FPIR levels has been shown to be predictive of T1D (Fourlanos et al., 2004; Siljander et al., 2013; Xu et al., 2010).

2.6 Prevention of type 1 diabetes

Already in the 1980s it was observed that immune intervention in the form of cyclosporin (Feutren et al., 1986), anti-thymocyte globulin and prednisone (Eisenbarth et al., 1985), or with azathioprine and prednisone (Silverstein et al., 1988) prolonged remission in T1D. However, the serious side effects of immune suppressants prevented their long-term use. In the 1990s, several small studies that included the use of cyclosporine, prophylactic insulin and nicotinamide (component of vitamin B3) showed effects in delaying the onset of T1D (Carel et al., 1996; Elliott et al., 1993; Ziegler et al., 1993). Modern primary and secondary prevention studies (Table 4) have included diet modifications or supplementations, antigen specific therapies and immunomodulators.

Primary prevention studies are performed before there are signs of islet autoimmunity. Encouraging results were obtained from the FINDIA study with the use of bovine insulin free formula during the first 6 months of life (Vaarala et al., 2012). The use of FINDIA formula was associated with a decreased risk of islet autoimmunity when compared to cow’s milk formula in 3 years and 6 years of follow-up (HR 0.24, 95% CI 0.08-0.72; HR 0.39, 95% CI 0.16-0.93, respectively). TRIGR (Trial to Reduce IDDM in the Genetically at Risk) study later investigated the use of hydrolyzed formula and the development of islet autoimmunity or T1D. Initially, hydrolyzed formula presented possible protection from the development of islet autoimmunity (Knip et al., 2010) but subsequent study results were negative for protection against the development of autoimmunity and T1D (Writing Group for the TRIGR Study Group et al., 2018).

The TrialNet Nutritional Intervention to Prevent (NIP) Type 1 Diabetes Study Group investigated the benefits of supplementation with docosahexaenoic acid (DHA) in children at high genetic risk for T1D (Chase et al., 2009). Supplementation of diets with DHA has resulted in increased DHA levels in red blood cells by at least 20% at the age of 12 months (Chase et al., 2015).

The German BABYDIET study analyzed whether a delay in the introduction of gluten would affect the development of islet autoimmunity in infants with genetic risk. The first gluten exposure was at the age of 6 months (control group) or at the age of 12 months (intervention group); during the 3 years of follow-up the intervention did not reduce the risk of islet autoimmunity (Hummel et al., 2011). A gluten-free diet for 18 months is currently being studied in subjects positive to at least one autoantibody and with signs of dysglycemia in the TEFA (TEDDY Family Prevention) study (NCT02605148).
Despite negative results, the large-scale multicenter secondary prevention trials with oral/parenteral insulin or nicotinamide have increased the knowledge of T1D disease pathogenesis. The European Nicotinamide Diabetes Intervention Trial (ENDIT) study screened more than 30,000 relatives of patients with T1D (3-40 years) for ICA in 354 clinical centers in 18 European countries, Canada and in USA. A total of 552 participants (252 participants under 15 years) were randomized to receive oral nicotinamide or placebo, but the intervention was ineffective (Gale et al., 2004).

In the parenteral arm of the Diabetes Prevention Trial-Type 1 Diabetes (DPT-1) study, the effect of subcutaneous insulin was investigated in a randomized controlled nonblinded trial (Diabetes Prevention Trial-Type 1 Diabetes Study Group, 2002). Over 80,000 first- and second-degree relatives (3-45 years) of T1D patients were initially screened for ICA. After metabolic testing including OGTT and IVGTT and estimation of overall T1D risk, a total of 339 participants were randomly assigned to subcutaneous insulin and yearly 4-day continuous infusions of insulin. During 3.7 years of follow up, a total of 139 subjects were diagnosed with T1D. The intervention was not effective (HR 0.96, 95% CI 0.69-1.34).
Table 4. Type 1 diabetes intervention trials based on primary or secondary prevention strategies.

<table>
<thead>
<tr>
<th>Study (site)</th>
<th>Subjects</th>
<th>Intervention</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIGR (Finland)</td>
<td>newborn up to 6-8 months of age</td>
<td>casein hydrolysate formula</td>
<td>pilot study showed effect in the development of autoimmunity</td>
<td>(Knip et al., 2010)</td>
</tr>
<tr>
<td>TRIGR (International)</td>
<td>newborn up to 6-8 months of age</td>
<td>casein hydrolysate formula</td>
<td>no effect on autoimmunity or T1D</td>
<td>(Knip et al., 2014; Writing Group for the TRIGR Study Group et al., 2018)</td>
</tr>
<tr>
<td>FINDIA (Finland)</td>
<td>first 6 months of life</td>
<td>whey based formula free of bovine insulin</td>
<td>protection from the development of autoimmunity</td>
<td>(Vaarala et al., 2012).</td>
</tr>
<tr>
<td>TrialNet (international)</td>
<td>third semester or during the first 5 months of life</td>
<td>docosahexanoic acid DHA</td>
<td>pilot study where safety and effect on RBC DHA levels were investigated</td>
<td>(Chase et al., 2009; Chase et al., 2015)</td>
</tr>
<tr>
<td>BABYDIET (Germany)</td>
<td>children &lt;2 months</td>
<td>delay of introduction of gluten</td>
<td>no effect</td>
<td>(Hummel et al., 2011)</td>
</tr>
<tr>
<td>DIPP (Finland)</td>
<td>young children at increased risk</td>
<td>nasal insulin</td>
<td>no effect on progression rate</td>
<td>(Näntö-Salonen et al., 2008)</td>
</tr>
<tr>
<td>DPT-1 (USA, Canada)</td>
<td>relatives of patients with T1D (3-45 yrs)</td>
<td>parenteral insulin</td>
<td>no effect on progression rate</td>
<td>(Diabetes Prevention Trial–Type 1 Diabetes Study Group, 2002)</td>
</tr>
<tr>
<td>DPT-1 (USA, Canada)</td>
<td>relatives of patients with T1D (3-45 yrs)</td>
<td>oral insulin</td>
<td>post hoc analysis suggested protective effect in a subgroup (with high IAA titers)</td>
<td>(Skyler et al., 2005)</td>
</tr>
<tr>
<td>TrialNet (International)</td>
<td>subjects with IAA (median age 8 years)</td>
<td>oral insulin</td>
<td>there was suggestive protective effect in subjects with low FPIR</td>
<td>(Writing Committee for the Type 1 Diabetes TrialNet Oral Insulin Study Group et al., 2017)</td>
</tr>
<tr>
<td>ENDIT (International)</td>
<td>ICA positive relatives of T1D patients (3-40 years)</td>
<td>nicotinamide</td>
<td>no effect on progression rate</td>
<td>(Gale et al., 2004)</td>
</tr>
<tr>
<td>Pre-POINT (Germany, Austria, USA, UK)</td>
<td>children at high risk (2-7 yrs)</td>
<td>oral insulin</td>
<td>pilot study investigated immune responses to insulin and adverse events</td>
<td>(Bonifacio et al., 2015)</td>
</tr>
<tr>
<td>DiAPREV-IT (Sweden)</td>
<td>subjects aged 4-18 with GADA and another islet aab</td>
<td>GAD-alum</td>
<td>no effect on progression rate</td>
<td>(Elding Larsson et al., 2018)</td>
</tr>
<tr>
<td>TrialNet (International)</td>
<td>8-50 years with ≥2 islet aab</td>
<td>teplizumab</td>
<td>effect on progression rate</td>
<td>(Herold et al., 2019)</td>
</tr>
</tbody>
</table>
In the oral arm of the DPT-1 study, over 100,000 relatives of patients were screened and a total of 372 subjects with estimated 26-50% risk of progression to T1D in 5 years of follow-up received oral insulin or placebo. During the median follow-up of 4.3 years, 97 participants were diagnosed with T1D. The intervention did not affect the progression to T1D (HR 0.76, 95% CI 0.51-1.14). However, an effect in a subcohort was observed, as among participants with confirmed high IAA titers (n=263) there were less progressors in the oral insulin group (HR 0.57, 95%CI 0.36-0.89). The TrialNet study could not confirm this finding, but there was a protective effect in subjects (n=55) with low FPIR (HR 0.45, 95% CI 0-0.82) (Writing Committee for the Type 1 Diabetes TrialNet Oral Insulin Study Group et al., 2017).

Oral insulin has also been studied in the PREPOINT study in young children without islet autoantibodies but with genetic risk (Bonifacio et al., 2015). Nasal insulin in young autoantibody positive children from the DIPP study did not prevent or delay the onset of T1D (Näntö-Salonen et al., 2008). A nasal insulin trial in 4-30 years old first- or second-degree relatives of T1D patients with two or more islet autoantibodies and a FPIR above the threshold is still ongoing in Australia (NCT00336674).

Antigen-specific therapies have also included treatment with GAD. Children and adolescents (4-18 years) who were positive for GADA and at least one other autoantibody received two injections of GAD formulated in alum or placebo, but it did not affect the progression to the disease (Elding Larsson et al., 2018).

More recently, teplizumab (an anti-CD3 monoclonal antibody) showed delaying effect of diabetes in 76 participants (8-50 years) with two or more islet autoantibodies (HR 0.41, (95% CI 0.22-0.78) with the median follow up 745 (74-2683) days. The presence of HLA-DR4 and absence of DR3 or ZnT8 autoantibodies were associated with more robust responses to teplizumab (Herold et al., 2019). Teplizumab, as well as abatacept, have also showed beneficial effects in patients with recent onset T1D (Herold et al., 2002; Orban et al., 2011). A secondary prevention trial with abatacept is still ongoing in 6-45 year old subjects with two islet autoantibodies excluding IAA (NCT01773707). Other ongoing trials include methyldopa (NCT03396484) and the use of incretin liraglutide (NCT02898506).

### 2.7 Beta cells of the pancreatic islets

#### 2.7.1 Anatomy and function

In human pancreas (in Greek pan”all”, kreas ”flesh”) there has been estimated to be approximately 1.5 million of islets: the estimates have ranged a lot (from 1 million to 14.8 million) depending partly if the small islets are also included (Korsgren et al., 2005). In adults, pancreas weighs approximately 50-125 g and the exocrine
pancreas which takes part in digestion accounts for 98% of the total mass, thus the weight of the islets is only 1-2 grams. In addition to beta cells, islets contain four other cell types: alpha, delta, PP and more recently found epsilon cells (Gray et al., 2019).

Beta cells secrete insulin in a pulsatile fashion in 5-min cycles (Hellman et al., 2009; Song et al., 2000). These insulin pulses may account for as much as 70% of total insulin secretion in the basal state (Hellman et al., 2009; Pørksen et al., 1995). The gap junctions between beta cells are important in the electrical coupling and regulation of synchronous insulin secretion (Bavamian et al., 2007). While the main stimulus for beta cells to release insulin is elevated blood glucose, there are multiple extracellular factors including amino and fatty acids, neurotransmitters, hormones that can amplify insulin secretion (Henquin et al., 2003).

The pancreas receives blood from branches of both the coeliac and superior mesenteric arteries (Jansson, 1994). Pancreatic islets are especially highly vascularized. The blood that leaves the pancreas goes to the splenic and the superior mesenteric veins, eventually leading to the portal vein. The thin-walled lymphatic vessels of the pancreas have a relatively large lumen and drain into the lymph nodes that are located around the organ and its surroundings (O'Morchoe, 1997).

The islet structure in humans is different from that of a rodent (Barbieux et al., 2016). In humans, beta cells are distributed, not cluttered and there is likely more paracrine interaction with other endocrine cells (Cabrera et al., 2006). Islets are innervated by parasympathetic (Rossi et al., 2005) and sympathetic fibers (Borden et al., 2013). The innervation of human islets has been described to be sparse and constituting mostly of sympathetic fibers (Rodriguez-Diaz et al., 2011).

### 2.7.2 Embryogenesis

During embryogenesis, beta cells are derived from the endoderm layer, which is one of the three layers forming the embryo, the other two being ectoderm and mesoderm. In humans, at approximately 5 weeks after conception two separate buds, dorsal and ventral, are formed from the endoderm. The expression of Pdx1 operates as a marker for the pancreatic bud development (Kim & MacDonald, 2002; Pan & Wright, 2011). The two buds fuse together later during gut rotation. The tail and body of the pancreas are derived from the dorsal part whereas the head is originated from both parts. Extracellular signals from neighboring cells contribute to this, and many of the transcription factors that regulate differentiation of beta cells have been identified (Cerf, 2006). The developing aorta and notochord give signals that induce the endodermal epithelium into pancreatic endoderm. Notch signaling in mice has been described to take part in the differentiation of progenitor cells into endocrine or exocrine cells (Apelqvist et al., 1999).
The beta cells have been the first endocrine cells detected during the development of human embryo (Piper et al., 2004). The vasculature and lymphatic network have been shown to be complete during the second trimester of the pregnancy (Roost et al., 2014).

Intrinsic regulator Neurog 3 is essential for endocrine development in the pancreatic endoderm (Salisbury et al., 2014) and its expression increases rapidly after the embryonic period and coincides with the appearance of fetal beta cells (Jennings et al., 2013; Lyttle et al., 2008; Piper et al., 2004). A mutation in the Neurog3 gene has caused neonatal diabetes with severe congenital diarrhea (Rubio-Cabezas et al., 2011) or isolated diarrhea with later T1D by 9 years of age (Wang et al., 2006). Thus, Neurog3 appears to play a role in the enteroendocrine cellular development. NEUROG3 activation through a mutation in STAT3 causes premature differentiation into endocrine cells (Saarimäki-Vire et al., 2017). Glis3 is a transcriptional regulator of endocrine cell progenitors (Kang et al., 2009) and mutations in GLIS3 have been associated with neonatal diabetes and congenital hypothyroidism (Senee et al., 2006).

The human fetal pancreas has been shown to be responsive to glucose already during the first half of gestation, although the pulsatility of insulin only happens at a later stage of development (Otonkoski et al., 1988). A more recent study has observed that beta cells show endocrine functions already during the first trimester (Piper et al., 2004). The beta cell replication rate was high starting from the 9th week of gestation (Meier et al., 2010). The frequency of beta cell apoptosis was shown to be relatively high during prenatal life.

2.7.3 Replication and function after birth

Beta-cell replication is the primary mechanism used in the postnatal expansion of beta-cell mass in humans (Meier et al., 2008). Neogenesis happens mostly during the fetal period but has been shown to continue up to the first two years of life (Gregg et al., 2012). The baseline population of beta cells and their connections with other islet cells is set up before the age of 5 years (Gregg et al., 2012). However, in adults, new beta cells mainly originate from pre-existing beta cells by duplication (Dor et al., 2004). There is adaptive beta cell increase after pregnancy or body weight increase (Butler et al., 2010; Butler et al., 2007). It has been estimated that beta cell replication is mainly limited to the first three decades of life (Perl et al., 2010).

In mice, it has been shown that a short-term glucose stimulus increases beta cell hypertrophy and hyperplasia (Bonner-Weir et al., 1989). Beta cell replication showed increase after immune therapy in mice, but the recovery of the beta cell area was mostly attributed to the regranulation of beta cells with insulin (Sherry et al., 2006).
The exact quantification of beta cell mass in humans is still difficult. Early studies have noted that beta cells are still present in T1D patients; moreover, islet hyperplasia was also observed (Maclean & Ogilvie, 1959). Autopsy data from young recent onset T1D cases revealed that most of them (14 out of 16) had beta cells; moreover, in 25% of cases, it was described that there was a relatively large number of beta cells still present (Gepts & De Mey, 1978). Signs of islet regeneration were found in many T1D cases and beta cells were described as hyperactive with high RNA but low insulin content in the cytoplasm (Gepts & De Mey, 1978).

In very young children with an acute onset and ketoacidosis there were only a few beta cells present, and the degree of insulitis differed between subjects (Lernmark et al., 1995). The beta cell area and mass were significantly higher in T1D donors who had insulitis, compared to those without insulitis (Campbell-Thompson et al., 2016). Insulitis means lymphocytic infiltration of islets and is now defined by the presence of a minimum of 15 CD45+ cells in at least three pancreatic islets (Campbell-Thompson et al., 2013).

2.8 Glucose metabolism

Throughout the regulation of glucose metabolism, the pancreas interacts with many other organs. The functions of each organ in glucose metabolism are different and depend on many factors, such as the duration of fasting, activity levels, as well as the circadian rhythm (Colberg et al., 2010; Mattson et al., 2014). In the anticipation of a meal, a cephalic phase of insulin secretion occurs even before food is introduced (Caumo & Luzi, 2004). This phase has been shown to be important in postprandial glucose management (Ahrén & Holst, 2001).

Approximately 20% of the energy derived from glucose is used by the brain (Mergenthaler et al., 2013). Glucose sensors in the brain and in the hepatic portal system help maintaining glucose homeostasis and correcting possible hypoglycemia. Glucose sensing neurons in the brainstem and hypothalamus react to decreased glucose availability by, for example, activating the sympathetic nervous system (Myers & Olson, 2012). The glucose sensing neurons in the hypothalamus may be particularly important in controlling the first phase of insulin secretion (Osundiji et al., 2012).

Glucose molecules (molecular formula C₆H₁₂O₆) can be found in sucrose (the “common” sugar), where they are bound to fructose and in starch where they form straight amylose chains or branched amylopectin chains. Fructose has the same molecular formula as glucose, but with a five-membered ring structure. In human digestion, carbohydrates are first hydrolyzed to monosaccharides, which starts in the mouth, but mostly occurs in the small intestine. Carbohydrates that are not digested in the small intestine are anaerobically metabolized by bacterial flora in the large intestine.
intestine. Despite having the same chemical formula as starch, cellulose has a different structural formula (each successive glucose monomer is positioned at 180 degrees around the axis), making it indigestible in humans (Cummings, 1984). Gastrin, secreted by the stomach, may play a significant role in the stimulation of insulin release during protein rich meals (Rehfeld & Stadil, 1973).

Glucose is transported from the intestinal lumen into enterocytes by the Na+/glucose cotransporter (SGLT1), although glucose transporter type 2 (GLUT2) may also play a role. Enzymes that hydrolyze carbohydrates are secreted by the intestinal cells that line the villi. The gastrointestinal tract also releases hormones, called incretins that stimulate insulin secretion (Bradley et al., 2011; Rehfeld, 2018).

The circulating glucose is then taken up by a glucose transporter, and beta cells in the pancreatic islets have been shown to use mainly GLUT 1 and GLUT 3 transporters whereas GLUT 2 is the main transporter in rodents (De Vos et al., 1995; McCulloch et al., 2011; Rorsman & Braun, 2013). However, mutations in SLC2A2 gene that encodes GLUT2 can cause neonatal diabetes, thus GLUT2 probably also has a role in human insulin secretion (Sansbury et al., 2012).

When glucose enters beta cells, it is phosphorylated by glucokinase (GCK). This is the first step in the glycolytic pathway as the glucose is converted to glucose 6-phosphate. Type 2 of MODY (maturity onset diabetes of the young) is caused by a mutation in the GCK gene (Froguel et al., 1992). In MODY2 the threshold for initiation of glucose-stimulated insulin secretion is altered and fasting glucose levels are usually slightly elevated.

By releasing insulin, the pancreas regulates the storage of glucose in the liver. In glycogenesis glucose molecules are joined to the glycogen chains of multibranched glucose polysaccharides. The liver maintains glucose homeostasis by absorbing and storing glucose after meals. The absorbed glucose in liver is converted into glucose 6-phosphate by specific kinases. HNF1α is a transcription factor highly expressed in the liver and regulates the expression of many hepatic genes. HNF1α is also expressed in the pancreas, and HNF1α-deficient mice have impaired insulin secretion (Pontoglio et al., 1998). Importantly, a mutation in the gene that encodes this protein is associated with one type of MODY (HNF1A-MODY) (Vaxillaire et al., 1997).

The liver also releases glucose in a controlled manner between meals (through glycogenolysis, the removal of glucose residues in the form of glucose-6-phosphatase from glycogen). By releasing glucagon from alpha cells, the pancreas regulates the release of glucose by the liver. Gluconeogenesis, glucose production from non-carbohydrate sources such as glucogenic amino acids or triglycerides occurs in the liver and kidney. Muscles have a large storage of glycogen and GLUT4 facilitates glucose uptake in this tissue. In adults with type 2 diabetes (T2D), GLUT4 protein content and glucose disposal were increased after seven days of exercise training (O’Gorman et al., 2006). The secretion of glucocorticoids that can be a result
of stress and hypothalamic-pituitary-adrenal axis activation seems to decrease glucose transport in skeletal muscle by lowering insulin-stimulated GLUT4 translocation and that may induce insulin resistance (Dimitriadis et al., 1997; Qi & Rodrigues, 2007).

2.9 The first phase insulin response

After removal of the signal peptide from preproinsulin in the ER, the proinsulin is packaged into small granules in the Golgi complex. These granules are located at least in two different intracellular pools within the beta cell. Apart from the reserve pool, a small fraction of the granules locates in the proximity of the cell membrane in the readily releasable pool. These granules contribute to FPIR (Daniel et al., 1999; Olofsson et al., 2002) representing the increase of insulin values during the first ten minutes in IVGTT. In addition to glucose, other pharmacological agents such as tolbutamide and arginine have been used to trigger insulin secretion (Henquin et al., 2003).

An increase in extracellular glucose concentrations leads to a cascade that results in an influx of Ca\textsuperscript{2+} in beta cells which, in turn, triggers the fusion of insulin granules to the plasma membrane and the activation of exocytotic machinery (Henquin et al., 2003). The increased ATP/ADP ratio in the cell due to the glycolytic process closes ATP-dependent K\textsuperscript{+} channels, the subsequent depolarization of the membrane opens the voltage-dependent Ca\textsuperscript{2+} channels (Henquin et al., 2003). The maximum exocytotic rate in a single beta cell has been shown to be reached in 26 ms after depolarization (Barg et al., 2001).

The mechanism of transportation of insulin granules to the proximity of the plasma membrane is not completely known, however, it apparently involves specific motor proteins and translocation along microtubules (Varadi et al., 2003). The fusion of insulin granules to the membrane is mediated by members of the SNARE protein superfamily (Hastoy et al., 2017). In mice approximately 7% of a total of 9000 insulin granules have been estimated to be docked below the plasma membrane (Olofsson et al., 2002). Glucose stimulates docking of the granules to the membrane (Straub, et al., 2004). Dissociation of C-peptide and mature insulin occurs after the release of the insulin granules from the beta cell as proteases split proinsulin into equal amounts of insulin and C-peptide. The FPIR is followed by a gradually developing ATP-dependent second phase (5-60 min) to the same release site that requires translocation of the reserved granule pool to the plasma membrane. A more prolonged glucose stimulation with the second phase release has been described to compensate for a low FPIR (Cerasi & Luft, 1967).

Age, pubertal status and ethnicity affect FPIR (Lorini & Vanelli, 1996; Smith et al., 1988, Arslanian & Suprasonsgin, 1996; Gower et al., 2002; Xie et al., 2010).
Normally, FPIR increases over time in healthy children and adolescents (Allen et al., 1993), and it has shown correlation with body mass index (Allen et al., 1993; Gerich, 2002; Gungor et al., 2004). Cardiorespiratory fitness has also been found to be associated with insulin response in healthy children, depending on total adiposity (Lee et al., 2006). Estimates of beta cell mass have been correlated with FPIR (Hao et al., 2017; Larsen et al., 2006; McCulloch et al., 1991).

There is variation in FPIR between and within subjects (Smith et al., 1988). The area under the 0-10 min curve showed less between-subject variation in healthy adults (Smith et al., 1988). The within-subject variation between two tests that were taken 2 weeks to 4 months apart was only 4% in FPIR and 6.7% in the area under the 0-10 min insulin curve (Rayman et al., 1990). Good reproducibility associated with low values may be a sign of “non-normality” (Soeldner, 1988) and the importance of more than one low FPIR value has been emphasized (Chase et al., 1991; Ismail et al., 2015). A standardization of IVGTT was developed in 1992 to overcome problems regarding the heterogeneity in the methods such as glucose dose and infusion time (Bingley et al., 1992). In general, the correlation of FPIR values in young subjects has been moderate-good, ranging from 0.74 to 0.87 (Keskinen et al., 2002; Robert et al., 1991; Vialettes et al., 1988).

The relationship between FPIR and insulin sensitivity has been described as hyperbolic (Kahn et al., 1993). Thus, any changes in insulin sensitivity are associated with small or large changes in insulin secretion depending on insulin sensitivity (Kahn et al., 1993). The correlation coefficient between insulin response and insulin sensitivity has ranged from 0.21 to 0.39 depending on the degree of glucose tolerance (Ferrannini et al., 2011).
3 Aims of the study

The main aims of this study were

1. To study beta cell function, measured as first phase insulin response (FPIR), from the onset of islet autoimmunity in a longitudinal setting with several measurements in DIPP children that progressed to T1D (progressors) and in DIPP children with ICA only and remaining healthy (non-progressors) (study I)

2. To compare FPIR values during the childhood between progressors and non-progressors (study I)

3. To study the associations between FPIR, autoantibodies and the class II HLA DR DQ genotypes (study II)

4. To study the associations between FPIR and genetic variants outside the class II HLA DR DQ region (study III)
4 Materials and methods

4.1 DIPP study

The Type 1 Diabetes Prediction and Prevention (DIPP) study was launched in 1994 in Turku and during the following years also in Oulu and Tampere.

The DIPP study protocol includes the screening of the newborn for class II HLA-conferred genetic T1D risk (chapter 4.3). Eligible children are invited to the study and followed regularly. During the first two years of life, study visits are performed every 3-6 months. After that, visits are performed every 6-12 months. However, if islet autoantibodies are detected, the frequency of study is returned to every 3 months.

Since 1994, over 230,000 newborn children have been screened for the genetic risk of T1D (J. Ilonen, personal communication). The HLA screening criteria have slightly changed during the DIPP study (Chapter 4.3).

4.2 Study subjects

Study children (Figure 1) were participants of DIPP study from Turku, Oulu and Tampere centers. Originally, a total of 685 children had undergone an IVGTT after the appearance of autoantibodies during years 1996-2012.

Study I included altogether 402 children. Of these, a total of 192 children (116 male, 60.4%) had developed only classical ICA at the median age at seroconversion of 4.6 (0.7-12.6) years and remained healthy during the follow-up (non-progressors). The remaining 210 children had developed T1D during the follow-up (progressors). Of the 210 progressors (117 male, 55.7%), 17 children had been originally recruited as siblings (they had been recruited when the index child entered the study). In 193 index progressors the median (min-max) age at seroconversion was 1.6 (0.5-10.6) years and the median (min-max) age at the time of appearance of multiple islet autoantibodies was 2.0 (0.8-10.6) years. ICA was included in the definition of multiple autoantibodies. The age at diagnosis in 210 children was 6.6 years (range 1.6-16.1 years). A total of 155 children had been involved in the nasal insulin trial (Näntö-Salonen et al., 2008). There were no differences in the baseline FPIR or in the change from the baseline FPIR to the last FPIR between the placebo and nasal insulin groups.
**Materials and methods**

**Study Flowchart**

Over 230,000 children screened by April 2018

> Over 15,000 children in follow-up

> 685 children with IVGTT 1996-2012 after appearance of islet autoantibodies

**Studies II and III** originally included 574 children that had been fully genotyped for class II HLA DR-DQ conferred T1D risk. Those subjects who had their first IVGTT performed during the last 2 years prior to diagnosis were excluded from the analysis in order to avoid the strong influence of a late preclinical decrease in FPIR. This resulted in 514 children as 60 progressors from the initial data set were not included since these children developed T1D in less than 2 years from their first IVGTT.

The 514 children were categorized according to their islet autoantibody status based on the presence of biochemical islet autoantibodies (IAA, IA-2A, GADA or ZnT8A) or ICA only (later referred to as no biochemical autoantibodies) at the time of the first IVGTT. Data on ZnT8A was available from 509 (99.0%) of the subjects. Children initially with zero or one biochemical islet autoantibody but with additional biochemical autoantibody at other age points were analyzed separately (76 children). Thus, a total of 438 children were included in the hierarchical linear mixed model in *studies II and III*. Of these, 205 children were persistently negative for biochemical autoantibodies and 38 children developed T1D with one biochemical autoantibody only at later points in follow-up.

**Figure 1.** The study flowchart.

* Includes also 17 siblings with T1D
* Analyses were performed without last two years prior to diagnosis (except when the time before T1D was specifically investigated)
autoantibodies, 38 were positive for only a single biochemical autoantibody and a total of 195 children had two or more biochemical autoantibodies at the time of the first IVGTT. In the study cohort of 438 children who remained in the initial autoantibody group (268 males, 61.2%), the median (IQR) age at the first IVGTT was 4.6 (2.7-7.3) years. The total number of IVGTTs in these children was 1149.

4.3 Genetic analyses and risk categorization (study II)

HLA-DR/DQ genotyping was performed stepwise beginning from definition of HLA-DQB1 alleles and expanded thereafter to cover informative DQA1 alleles and in the case of DQA1*03-DQB1*03:01 and DQB1*03:02 haplotypes also various DRB1*04 alleles. The details of the methods and procedure have been described earlier (Kiviniemi et al., 2007; Ilonen et al., 2013).

As the knowledge of the HLA-risk associated DR/DQ haplotypes and screening criteria have evolved it was learned that some of the DIPP children invited to regular follow-up carry genotypes associated with neutral or even decreased T1D risk despite being initially classified as carrying increased risk-associated DQB1 alleles. However, children with the most common strongly protective HLA-DQB1*06:02 allele (Pugliese et al., 1995) have not been eligible for the DIPP follow-up.

The risk of T1D can now be divided into six different categories: highly, moderately or slightly increased risk, neutral risk and groups with slightly decreased or strongly decreased risk (Ilonen et al., 2016). This categorization is based on the presence of DR/DQ haplotypes associated with the disease risk observed in the series of the Finnish Pediatric Diabetes Register (Ilonen et al., 2016, Table 5a-b).

The highly increased risk is the combination of the DR3 ((DR3)-DQA1*05-DQB1*02) and DR4 (DRB1*04:01/2/4/5-DQA1*03-DQB1*03:02) haplotypes. The moderately increased risk means a combination of DRB1*04:01/2/5-DQA1*03-DQB1*03:02, the stronger risk haplotype together with a neutral haplotype. The slightly increased risk means combinations of lower risk haplotypes DRB1*04:04-DQA1*03-DQB1*03:02 or (DR3)-DQA1*05-DQB1*02 with a neutral haplotype or a combination of the strong risk haplotype with a weakly protective haplotype. Neutral risk associated genotypes are various combinations of neutral haplotypes or combinations of a risk and a protective haplotype, and decreased risk associated genotypes are combinations of neutral and protective haplotypes or various protective haplotypes. In study II, the groups with a neutral risk, slightly decreased risk and strongly decreased risk were combined for analyses, thus resulting altogether in four different risk groups.
**Materials and methods**

**Table 5 a-b.** The risk of type 1 diabetes in different HLA DR-DQ haplotypes (a) and combinations of haplotype risk in genotype risk groups (b). ↑↑, strong susceptibility; ↑, weak susceptibility; ↔, neutral; ↓, weak protection; ↓↓, strong protection. Modified from Ilonen et al., 2016.

<table>
<thead>
<tr>
<th>Class II HLA DR-DQ haplotype</th>
<th>Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1<em>04:01-DQA1</em>03-DQB1*03:02</td>
<td>↑↑</td>
</tr>
<tr>
<td>DRB1<em>04:05-DQA1</em>03-DQB1*03:02</td>
<td>↑↑</td>
</tr>
<tr>
<td>DRB1<em>04:02-DQA1</em>03-DQB1*03:02</td>
<td>↑↑</td>
</tr>
<tr>
<td>DRB1<em>04:04-DQA1</em>03-DQB1*03:02</td>
<td>↑</td>
</tr>
<tr>
<td>(DR3)-DQA1<em>05-DQB1</em>02</td>
<td>↑</td>
</tr>
<tr>
<td>(DR13)-DQB1*06:04</td>
<td>↔</td>
</tr>
<tr>
<td>(DR9)-DQA1<em>03-DQB1</em>03:03</td>
<td>↔</td>
</tr>
<tr>
<td>(DR8)-DQB1*04</td>
<td>↔</td>
</tr>
<tr>
<td>(DR16)-DQB1*05:02</td>
<td>↔</td>
</tr>
<tr>
<td>(DR7)-DQA1<em>0201-DQB1</em>02</td>
<td>↔</td>
</tr>
<tr>
<td>(DR1/10)-DQB1*05:01</td>
<td>↔</td>
</tr>
<tr>
<td>(DR4)-DQA1<em>03-DQB1</em>03:01</td>
<td>↔</td>
</tr>
<tr>
<td>(DR13)-DQB1*06:09</td>
<td>↔</td>
</tr>
<tr>
<td>DRB1<em>0403-DQA1</em>03-DQB1*03:02</td>
<td>↓</td>
</tr>
<tr>
<td>(DR13)-DQB1*06:03</td>
<td>↓</td>
</tr>
<tr>
<td>(DR11/12/13)-DQA1<em>05-DQB1</em>0:301</td>
<td>↓</td>
</tr>
<tr>
<td>(DR7)-DQA1<em>02:01-DQB1</em>03:03</td>
<td>↓↓</td>
</tr>
<tr>
<td>(DR15)-DQB1*06:01</td>
<td>↓↓</td>
</tr>
<tr>
<td>(DR15)-DQB1*06:02</td>
<td>↓↓</td>
</tr>
<tr>
<td>(DR14)-DQB1*05:03</td>
<td>↓↓</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Haplotype risk combinations</th>
<th>Class II HLA genotype risk group</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑↑/↑↑/↑↑/↑↑ (if different, both DR3 and DR4 haplotypes)</td>
<td>High risk</td>
</tr>
<tr>
<td>↑↑/↑↑/↑↑/↑ (if same haplotypes, two DR3 or two DR4), ↑↑/↔ and ↑↑/↔</td>
<td>Moderately increased risk</td>
</tr>
<tr>
<td>↑/↔ and ↑↑/↓</td>
<td>Slightly increased risk</td>
</tr>
<tr>
<td>↔/↔, ↑↑/↓↓, ↑/↓↓ and ↑/↓↓</td>
<td>Neutral</td>
</tr>
<tr>
<td>↓/↔</td>
<td>Slightly decreased risk</td>
</tr>
<tr>
<td>↓↓/↔, ↓/↓, ↓↓/↓ and ↓↓/↓↓</td>
<td>Strongly decreased risk</td>
</tr>
</tbody>
</table>
4.4 Genetic variants outside the class II HLA DR-DQ region (study III)

The eight SNP markers **IFIH1** (rs1990760), **ERBB3** (rs2292239), **INS** (rs689), **IKZF4** (rs1701704), **PTPN2** (rs45450798), **FUT2** (rs601338) **CTSH** (rs3825932) and **PTPN22** (rs2476601) were genotyped by using the Sequenom (San Diego, California, USA) platform (University of Eastern Finland, Kuopio, Finland) (Lempainen et al., 2015) or Taqman SNP Genotyping Assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA) (**CTSH rs3825932**). The assays of class I HLA alleles (**B*39, A*24**) were analyzed on the DELFIA platform (Mikk et al., 2014; Mikk et al., 2017).

4.5 Islet autoantibody measurements

The islet autoantibody measurements in the DIPP study has been performed in the Diabetes Research Laboratory, University of Oulu and includes the detection of IAA, GADA, IA-2A and ICA as described earlier (Siljander et al., 2009). In addition, ZnT8A have been measured from all subjects positive for other islet autoantibodies by a radiobinding assay in the University of Helsinki (Salonen et al., 2013).

4.6 IVGTT protocol

A standard protocol was followed (Bingley et al., 1992) Children underwent IVGTTs after overnight fasting. A local anesthetic (EMLA, AstraZeneca, Södertälje, Sweden) was used before the cannulation. The fasting samples were drawn through an intravenous cannula 5 and 0 min before the start of the glucose infusion. The glucose dose was 0.5 g/kg (maximum 35 g). Glucose was infused through the cannula in 3 min ± 15 s, and samples were subsequently taken at 1, 3, 5, and 10 min (in Turku center also at 30 and 60 minutes) after the end of the infusion (Keskinen et al., 2002).

4.7 Laboratory measurements

Plasma glucose concentrations were measured by an enzymatic method (Beach & Turner , 1958). For the quantitative serum insulin measurements an ELISA (Insulin ELISA Kit, Dako, Glostrup, Denmark) method (Andersen et al., 1993) was used in the Diabetes Research Laboratory, University of Oulu for samples obtained in Oulu and Tampere. In Turku, the method for serum insulin measurement has changed twice during the DIPP study (described in detail in the study I). The current method is an electrochemiluminescence immunoassay (ECLIA, Roche Diagnostics). The insulin assays in the Oulu and Turku laboratories have been compared before
(Keskinen et al., 2002) and a regression transformation equation was used to make the values from the Oulu laboratory comparable with the Turku radioimmunoassay (RIA) values. The insulin concentrations obtained with the more recent Turku assays were also corrected to make them comparable with the concentrations generated with the initial Turku RIA.

4.8 Markers of glucose metabolism

Beta cell function was evaluated by FPIR (study I-III) and the area under the 0 to 10 min insulin curve (AUC_{0-10 min} for insulin, study I). As surrogate markers for insulin sensitivity (study I), fasting insulin concentrations, the HOMA-IR index and the HOMA-IR to FPIR -ratio (relative insulin resistance) were used. HOMA-IR index is calculated as (fasting glucose x fasting insulin)/22,5. Fasting values for insulin and glucose (study I) were calculated as the mean of 5- and 0-min values from the IVGTT. When the fasting insulin values were below the detection limit, that limit was used as an actual value. Glucose at 60-minute values from Turku data set were analyzed to investigate the late-phase glucose values (study I).

The first phase insulin response (FPIR) was calculated as the sum of insulin concentrations at 1 and 3 minutes during IVGTT. We also calculated as an additional variable ΔFPIR based on the change in FPIR: the last FPIR minus the first FPIR divided by the time between the samples (in years).

4.9 Statistical analyses

The response variables (glucose metabolism markers) were log-transformed for the analyses to achieve the normality assumption in the statistical model.

In study I, the scatterplots between age and response variables were noisy, so the data was explored using cubic splines (Reinsch, 1967). The time periods of 0-2 years, 2-4 years and 4-6 years before diagnosis (progressors) or the last IVGTT (non-progressors) were analysed separately. The progressors’ last response variable before the diagnosis was compared with the previous one by a paired samples t-test. Non-parametric tests were used when appropriate.

The effect of age on response variables was assessed by a linear mixed model. Predictor variables were age, group and their interaction. Given estimates for age represent how response variables change when age is increased by one year. Study variables between the study groups were compared at the ages of 2, 4, 6, 8, and 10 years. In the age-dependent comparison, the difference between the study groups describes how many percent the response variable has changed in non-progressors compared to progressors. Analyses were performed excluding data from the last 2 years prior to diagnosis in the progressors.
One-way ANOVA was used to compare markers of glucose metabolism at the time of 2 (±1) years prior to diagnosis in children categorized by their first appearing biochemical islet autoantibody (IAA, IA-2A or GADA).

In study II, the relationship between autoantibody status and HLA risk was analyzed with Chi Square test. Class II HLA risk was treated as a categorical variable 1-4. Cochrane Armitage test was applied to study the trends of two-categorical autoantibody groups in different class II HLA groups. Hierarchical linear models were used to analyze repeated measurements of FPIR. The statistical models included HLA and autoantibody status groups, as well as interaction terms HLA by time and autoantibody group by time. The number of IVGTTs decreased notably after 5 years from the first test, so the time period of 0-5 years from the first IVGTT was selected. The total number of IVGTTs in this period was 1023 (89.0% of all).

Study III also used hierarchical linear mixed model. The model included islet autoantibody status, age, and the genotype for each of the loci analyzed (three/two groups). Three types of models (additive, recessive or dominant) were applied.

Statistical analyses were performed with JMP Pro version 11.2 and SAS version 9.4. $P$ values of less than 0.05 (two-tailed) were considered statistically significant.

4.10 Ethical aspects

The study was conducted according to the guidelines of the Declaration of Helsinki. It was approved by the local Ethics Committees and a written informed consent from the families was provided for the study.
5 Results and discussion

5.1 Longitudinal changes of FPIR after the onset of islet autoimmunity (study I)

The aim of study I was to investigate FPIR during the preclinical period of T1D from the onset of islet autoimmunity. A total of 210 DIPP study children had progressed to T1D during the follow-up (progressors) while 192 children had only ICA and remained healthy (non-progressors).

5.1.1 Longitudinal changes of FPIR before type 1 diabetes (study I)

The declining median values of FPIR before the diagnosis of T1D are shown in Figure 2 (black solid line). When comparing the last two samples before diagnosis of T1D, there was a significant decrease of FPIR ($p<0.005$). Compared to non-progressors (dotted line in Figure 2), FPIR was decreased in progressors up to the time period of 4-6 years before the diagnosis of T1D ($p<0.005$). We could not detect a difference in FPIR or other studied markers between the groups at the time of 2 ($\pm 1$) years before T1D based on the first appearing autoantibody.

![Figure 2](image.png)

Figure 2. The median values of FPIR before the diagnosis of type 1 diabetes in the progressors (black line) and in the non-progressors until the last IVGTT (dotted line). The x axis indicates years before the diagnosis or the last IVGTT (point 0). Figure from Koskinen et al., 2016, used under the Creative Commons CC by 3.0 licence.
These results showed the overall declining pattern of FPIR before the diagnosis which is in line with previous studies (Chase et al., 1991; Colman et al., 1998; Srikanta et al., 1984). The decline of FPIR may be more rapid approximately year before diagnosis which has also been observed in the DPT-1 study (Sosenko et al., 2013). It has been speculated that the loss could be more gradual (Sosenko et al., 2013). Similarly, in our study the change in FPIR between the last two samples was significant. Because of the long follow up time, however, both the timing of the last sample (median time 2.9 years before diagnosis; range 5 days-14 years) and time between the last two samples (median time 1.1 years; range 0.2-4.7 years) varied a lot so there can be subjects with more gradual loss in FPIR.

We were able to obtain new information when we could compare the progressors to children with only ICA (non-progressors). Compared to non-progressors we could observe a difference in FPIR between the groups up to the time period of 4-6 years before the diagnosis.

5.1.2 Longitudinal changes of FPIR in an age-dependent comparison

We then investigated FPIR in the longitudinal age-dependent comparison (Figure 3). The difference in FPIR between the progressors and non-progressors was significant in all age groups ($p<0.001$). FPIR was significantly higher in the non-progressors than in the progressors, and the difference between the groups increased with age. At 2 years the difference was 50% (95% CI 28%-75%) and at 10 years difference was 172% (95% CI 128%-224%).

![Figure 3. Mean values of FPIR shown in cubic splines between the non-progressors and progressors as a function of age (years). The solid lines show the values of the progressors with the last 2 years prior to diagnosis included (grey line) or excluded (black line). The black dotted line represents the non-progressors. Figure from Koskinen et al., 2016, used under the Creative Commons CC by 3.0 licence.](image-url)
Taken together, children progressing to T1D had significantly lower mean values of FPIR throughout the childhood. The difference between the groups increased by age as the mean FPIR was almost three times greater in the non-progressors than in the progressors at the age of 10 years.

In children presenting with only islet cell antibodies the risk of T1D is known to be low and these children had increasing FPIR over time. This is in line with the results from a previous study in healthy children (Allen et al., 1993). The difference between the groups at the age of 2 years was already 1.5-fold between the progressors and the non-progressors. In the DIPP study it has previously been observed that the increase of FPIR was seen less often in very young autoantibody positive children with FPIR below the 5th percentile compared to those who had FPIR above the 5th percentile (Keskinen et al., 2002) which could imply decreased capacity to increase insulin secretion over time in these children.

Furthermore, ICA-negative siblings of patients with T1D were described to have significantly lower FPIR from 8 years onwards when compared to control children which could implicate a defect in the growth of beta cell mass or insulin secretion (Carel et al., 1993). Indeed, the difference between the progressors and non-progressors was significant already at an early age and the difference in the capacity to secrete insulin became more pronounced over time. It would be reasonable to search for a specific defect in the beta cell function in children that progress to T1D.

5.2 Associations between FPIR, class II HLA risk and autoantibodies (study II)

The class II HLA region is known to have the strongest association with T1D risk so in study II we aimed to search for the associations with class II HLA and FPIR. Children (n=438) were categorized by the presence of autoantibodies at the time of the first IVGTT and by the class II HLA conferred T1D risk. It was hypothesized that the effect of HLA risk group on FPIR would be explained by the appearance of islet autoantibodies.

5.2.1 Associations between class II HLA risk and islet autoantibodies

First, the relationship between islet autoantibodies and class II HLA genotype conferred genetic risk was studied. There was an association between class II HLA risk group and autoantibody status ($p<0.0001$ in Chi-Square test). Furthermore, there was a significant difference in the proportions of the HLA risk groups when comparing children with no biochemical autoantibodies to those with a single biochemical autoantibody or those with multiple biochemical autoantibodies.
(\(p<0.02\) and \(p<0.0001\), respectively, Cochrane Armitage trend test). There was no statistically significant difference in the HLA risk group distribution when comparing children with single autoantibody to those with multiple biochemical autoantibodies (\(p=0.08\)).

### 5.2.2 Associations between FPIR and the class II HLA risk

When the change of FPIR over time was analyzed, the class II HLA risk was initially investigated alone in the model. The median FPIR over time was different between HLA groups (\(p=0.008\)), but not when effect of time was included in the model (\(p=0.50\)). When the autoantibody group with time interaction were added to the model, HLA risk grading did not anymore significantly associate with FPIR (\(p=0.26\)) nor FPIR change over time was significant between the HLA groups (\(p=0.35\)).

The class II HLA genotypes are associated with the initiation of autoimmunity (Ilonen et al., 2016). Previous studies have observed an association between class II HLA conferred strong genetic risk and multiple islet autoantibodies (Ilonen et al., 2016; Knip et al., 2002; Siljander et al., 2009). Furthermore, the genotypes conferring decreased risk are less common among the autoantibody positive subjects (Knip et al., 2002).

Thus, the class II HLA genotypes are associated with the initial levels of FPIR but the change of FPIR over time is further associated with the number of autoantibodies. An earlier study has also shown that the HLA DQB1 genotypes conferring increased T1D risk were associated with lower levels of FPIR (Veijola et al., 1995). The increase of FPIR over time was slower in DQB1 risk genotype carriers and these changes were also associated with the presence of autoantibodies (Veijola et al., 1995).

### 5.2.3 Associations between FPIR and islet autoantibodies

When the autoantibody status was included in the model in the current study, lower FPIR was strongly associated with the positivity for multiple biochemical autoantibodies in both HLA-adjusted and HLA unadjusted models. (\(p<0.0001\) for both). There was a significant interaction by time as the change of FPIR over time was significantly different between children with multiple autoantibodies and those with single or no autoantibodies (\(p<0.0001\)). Children with multiple autoantibodies had decreasing FPIR and children with 0 or 1 autoantibody had increasing FPIR (Figure 4).
Results and discussion

Figure 4. FPIR over time in children with various number of biochemical islet autoantibodies (0, single or multiple). The Y-axis shows FPIR on a logarithmic scale. X-axis shows time from the first FPIR. Coefficient estimates are -0.00020 SE 0.000046 p<0.0001 in children with multiple autoantibodies, 0.000188 SE 0.000101 p=0.063 in children with single autoantibody and 0.000195 SE 0.000060 p=0.0013 in children with no autoantibodies. Figure from Koskinen et al., 2018, used under the Creative Commons CC by 4.0 licence.

We compared children who had one biochemical autoantibody at the baseline and additional biochemical autoantibodies after or before the first IVGTT. The median of the first FPIR in children with one biochemical autoantibody at the baseline and an additional biochemical autoantibody after the first IVGTT was 85.0 mU/l (95% CI 52.1 mU/l-110.2 mU/l). In children with one biochemical autoantibody at the baseline and the second biochemical autoantibody before the first IVGTT the median of the first FPIR was 50.3 mU/l (95% CI 32.6 mU/l-89.8 mU/l). The difference between the groups did not reach significance (p=0.16, unpublished result).

We found that there is an association between decreased FPIR and multiple autoantibodies. The presence of multiple autoantibodies multiplies the risk of T1D (Ziegler et al., 2013). The presence of two autoantibodies with dysglycemia marks a different stage in the process of T1D development (Insel et al., 2015). The decreasing capacity to secrete insulin was also associated with multiple autoantibodies independent of the class II HLA risk group indicating that presence of multiple autoantibodies may be secondary to the initial beta cell injury.
The increase in FPIR over time was seen also in children with no biochemical autoantibodies. This is similar to the findings in the study I where an increase in FPIR over time was seen in children with ICA only. However, study II reports a new observation that also children with one biochemical autoantibody had increasing FPIR over time. Furthermore, difference between FPIR in children with 0 or 1 biochemical autoantibody was not statistically significant ($p=0.3$).

Overall, the beta cell failure was associated with the presence of multiple autoantibodies. The declining pattern in FPIR was associated with multiple autoantibodies irrespective of HLA risk group. This result was corroborated by our finding that the difference between the last and the baseline FPIR per time in years (ΔFPIR) was significant in all groups of children with multiple autoantibodies regardless of the HLA risk group, indicating the possible role of other genetic factors than class II HLA in the longitudinal pattern of FPIR. In the following analyses we searched for the associations between FPIR and specific genetic variants outside class II HLA region.

5.3 Associations of FPIR and genetic variants outside class II HLA region (study III)

In study III, we investigated whether genetic variants outside the class II region associate with the change of FPIR over time. Children had been categorised according to the presence of multiple biochemical islet autoantibodies.

In children with multiple autoantibodies, the change of FPIR over time was significantly different between subjects with various PTPN2 (rs45450798), FUT2 (rs601338), CTSH (rs3825932) and IKZF4 (rs1701704) genotypes in at least one of the analytical models. In general, children carrying susceptibility alleles for T1D had steeper decline of FPIR compared to children without susceptibility alleles. The presence of the class I HLA $A^{*}24$ allele was also associated with steeper decline of FPIR over time in children with multiple autoantibodies.

Thus, a more rapid decline in insulin secretion was observed in children carrying susceptibility alleles in these genes compared to children without susceptibility alleles. CTSH has been linked with beta cell function (Fløyel et al., 2014). PTPN2 has been shown to take part in the beta cell apoptosis (Moore et al., 2009). The effect of class I HLA $A^{*}24$ allele on the development of T1D was observed in a previous study analyzing Finnish subjects, although the effect was restricted to specific HLA-DR/DQ haplotypes (Mikk et al., 2017).
5.4 Other markers of glucose metabolism (study I)

In study I we investigated also other markers of glucose metabolism from the onset of islet autoimmunity. Overall, the findings using the area under the 10-minute insulin curve (AUC\textsubscript{0-10\,min} for insulin) were similar to the results of FPIR. The change in AUC\textsubscript{0-10\,min} for insulin in the last two samples before diagnosis was significant ($p<0.0005$) and compared to non-progressors AUC\textsubscript{0-10\,min} for insulin was also decreased up to the time of 4-6 years before the diagnosis ($p<0.005$). In the age dependent comparison, there was a difference in AUC\textsubscript{0-10\,min} for insulin between the groups ($p<0.001$), the difference between the groups was increased slightly more over time compared to FPIR: at 2 years the estimate for the difference was 36\% (95\% CI 17\%-58\%) and at 10 years the difference was 186\% (95\% CI 143\%-237\%). Thus, according to these results, FPIR and the area under the 10-minute curve are similar markers of beta cell function. FPIR is a good marker for the initial 10-minute insulin response, although it can slightly underestimate the difference compared to AUC\textsubscript{0-10\,min} for insulin curve.

Before diagnosis, the change of fasting insulin did not reach significance when the last two time points were analyzed ($p=0.07$). The change of fasting glucose, HOMA-IR and HOMA-IR to FPIR -ratio in the last two time points before diagnosis were significant ($p<0.005$ for all). HOMA to FPIR -ratio was increased in the progressors up to the time of 4-6 years before the diagnosis and fasting glucose was increased during 0-2 years prior to diagnosis ($p<0.01$ for both). HOMA-IR or fasting insulin did not differ between the groups before diagnosis. Occasionally HOMA to FPIR -ratio has been used as a marker of insulin resistance in the prediction of T1D (Fourlanos et al., 2004), but it should be acknowledged that the low FPIR significantly contributes to this value.

In the whole study cohort of study I, fasting insulin, fasting glucose and HOMA-IR increased over time in both groups: fasting insulin increased 3.9\% (95\% CI 2.5\%-5.2\%) per year, fasting glucose 0.50\% (95\% CI 0.17\%-0.83\%) per year and HOMA-IR index increased 4.4\% (95\% CI 2.9\%-5.9\%) per year ($p<0.005$). When glucose at 60 minutes during the IVGTT was analyzed separately from the Turku cohort, the change in glucose at 60 minutes was significant in the last two samples before diagnosis ($p<0.001$). Glucose at 60 minutes was increased in the progressors 0-2 years and 2-4 years before the diagnosis ($p<0.001$, $p=0.03$, respectively). Glucose at 60 minutes was higher in the progressors from the age of 6 years onwards ($p<0.001$) and the difference increased with age: at 6 years the difference was 11\% (95\% CI 7\%-15\%) and at 10 years the difference was 24\% (95\% CI 18\%-29\%).

Taken together, a deterioration of glucose tolerance can be observed over time in the cohort of DIPP children with any islet autoantibodies, including ICA only. Indeed, this is known to occur with age also in healthy individuals (Ferrannini et al., 2010). In the DIPP study a rise in HOMA and its components fasting insulin and
Fasting glucose was observed over time in both non-progressors and progressors. The fasting glucose may show more rapid increase before diagnosis after earlier linear increase (Ferrannini et al., 2010). In the DIPP study we could not observe differences in glucose metabolism based on the initiating autoantibody in the time period of 2 ±1 year prior to diagnosis, but this also could be due to the relatively low number within the subgroups.

5.5 General discussion

Our main finding of this study was that FPIR, a measure of beta cell function that represents the release of insulin granules located in the proximity of beta cell membrane, is decreased several years before diagnosis of T1D. When compared to non-progressors, we could observe a difference in the beta cell secretory capacity of progressor children 4-6 years prior to diagnosis of T1D. In the age dependent comparison, progressors had constantly lower insulin secretory capacity throughout the whole childhood and the difference was observed already at the age of 2 years. In addition, we found that the declining pattern of FPIR was associated with multiple biochemical islet autoantibodies.

In the age dependent comparison, the difference between the groups increased over time: mean FPIR values were almost three times greater in the non-progressors at the age of 10 years compared to progressors. The difference between the groups was mostly due to the growth of FPIR over time in non-progressors, which did not occur in progressors. Although there was an overall declining pattern of FPIR before T1D diagnosis, the fastest decline appears to occur approximately one year before disease onset. The pattern of FPIR in the progressors can potentially be more stable or even have periods of recovery over time (Srikanta et al., 1984).

In very young (mean age 2.5 years) DIPP study children most (11 out of 13) of the progressors had a FPIR below the fifth percentile already in the first test (Keskinen et al., 2001). Still, 11 out of 22 with low values did not progress to T1D during the study follow-up. Some of the study children had a second IVGTT and in most cases FPIR was clearly higher in the second test when the first test resulted over the fifth percentile, suggesting the importance of beta cell function growth during the first years of life.

Beta cell dysfunction, defined as differences in C-peptide levels in OGTT, has been observed in the DPT-1 study already at the study baseline over five years before clinical onset of T1D and a faster change in C-peptide levels occurred 2 years prior to the disease onset (Evans-Molina et al., 2018). In a former small study postprandial C-peptide was low up to 74 months prior to the onset of T1D (Ginsberg-Fellner et al., 1982). It was also noted that once C-peptide levels fell 2 SD below normal, they never returned to the normal range (Ginsberg-Fellner et al., 1982). In contrast,
among 15 ICA-positive non-progressors who were twins of T1D patients were followed for 17 years by IVGTTs, natural fluctuation of FPIR was observed, yet none of them ever had a FPIR ever below the first percentile (Srikanta et al., 1984).

Even with low insulin secretory capacity, there are children who do not progress to T1D for a considerably long time. The DIPP study investigated children with at least one islet biochemical autoantibody, 151 of them defined as progressors during the study follow-up, and found that a reduced FPIR <24 mU/l was clearly associated with T1D risk (5-year progression rate over 80%) (Siljander et al., 2013). Children with FPIR <59 mU/l remained healthy much longer (5-year progression rate 21%) (Siljander et al., 2013). Among DPT-1 participants (median age of 11 years at randomization for oral insulin and 12 years for parenteral insulin) there were subjects that were initially classified at having a low FPIR value at the time of the initial test but remained healthy during the next 5-6 years (Barker et al., 2007). In 12 identical twins of T1D patients that developed diabetes after a mean follow up of 3 years, the initial sample of FPIR was already significantly lower in preclinical twins compared to control children (Hawa et al., 2005). In twins of T1D patients that had not developed T1D, there was no significant difference to control subjects although FPIR values were more stable (mean values of 465 mIU/ml·10 min⁻¹ and 499 mIU/ml·10 min⁻¹ in low risk twins against 796 mU/ml·10min⁻¹ and 433 mIU/ml·10 min⁻¹ in controls) (Hawa et al., 2005).

Although in children progressing to T1D the insulin secretory capacity was significantly lower throughout the childhood than in those who did not progress to T1D a difference in 60-minute glucose values was significant from the age of 6 years onwards and from the time period of 2 to 4 years prior to diagnosis, implying that subjects progressing to T1D somehow adapt or compensate for the low insulin secretory capacity before the rise in glucose values is observable. In the DIPP study it was observed that 2-hour OGTT plasma glucose starts to rise approximately 2 years before diagnosis and a random plasma glucose value of over 7.8 mmol/l was very predictive of future T1D (Helminen et al., 2015). Changes in Hba1c preceded T1D diagnosis approximately 2 years (Helminen et al., 2015). When compared to autoantibody negative controls DIPP study children with multiple autoantibodies had delicate signs of change in glucose metabolism, such as a higher variation in glucose levels and increased evening glucose values (Helminen et al., 2016).

In this study we observed an increase over time in markers of insulin resistance in both non-progressors and progressors. Glucose tolerance is known to deteriorate over time even in healthy subjects (Ferrannini et al., 2011). As there is an overall increase in insulin resistance over time, and occasional periods of increased insulin resistance, children who progress to T1D may at that time fail to increase beta cell function enough to maintain glucose homeostasis. Fluctuations between dysglycemia and normoglycemia have been observed before T1D during a long
follow-up (Sosenko et al., 2009). However, the manner in which normoglycemia is achieved in these subjects remains unknown.

The correlation between FPIR and pancreatic insulin content in baboons has been described as strong and crossing the y-axis at zero (McCulloch et al., 1991). However, the correlation between FPIR and beta cell mass has been shown to intercept the y-axis at 0.19 g suggesting there would be histologically detectable beta cells even when insulin response approaches zero (McCulloch et al., 1991). This is in line with the many observations that beta cells are still present in T1D patients (Gepts & De Mey, 1978; Maclean & Ogilvie, 1959). Some insulin secretion has been shown to exist years after T1D diagnosis (Keenan et al., 2010; Oram et al., 2014). The remaining insulin secretion could be a result of persisting beta cells (Lam et al., 2017). When subjects with insulitis or autoantibody positivity were investigated, a decrease in beta cell mass was not observed, and moreover, in some of the islets there were signs of beta cell proliferation (In't Veld et al., 2007). Surprisingly, when beta cell mass was estimated by the proinsulin area the organ donors with islet autoantibodies had larger beta cell mass compared to control donors (Rodriguez-Calvo et al., 2017).

Overall, factors that could contribute to low insulin secretory capacity include viruses (Henderson et al., 1991; Nghahi & Walsh, 2017), free fatty acids (Kashyap et al., 2003; Paolisso et al., 1995), metals and pollutants (Alonso-Magdalena et al., 2010; Diaz-Villasenor et al., 2006; Henriksen et al., 1997; Lee et al., 2017). In mice, the postnatal maternal diet was not linked to beta cell mass in offspring, but glucose intolerance was observed with dysfunction of the hypothalamic neurocircuitry (Vogt et al., 2014). The prenatal maternal high fat diet in rodents has also been associated with insulin values of the offspring; insulin levels were low at birth and hyperinsulinemia was present in adult age (Howie et al., 2009).

The class II HLA genotypes that are mostly known to be associated with a T1D risk exert their effect in the development of islet autoimmunity. The time between the development of autoimmunity and the onset of T1D can range from weeks to decades. In the DIPP study the median time from the appearance of islet autoantibodies to T1D was 2.5 years with a maximum period of 12 years (Parikka et al., 2012), while in Finnish subjects recruited from the general population this period reached 20 years (Knip et al., 2010). In the DIPP study the decreasing capacity of insulin secretion was associated with multiple biochemical autoantibodies regardless of the class II HLA risk group. HLA-independent associations have also been observed in siblings of patients with T1D when fasting proinsulin values were described to be higher than in control subjects (Hartling et al., 1989; Lindgren et al., 1991). With decreased level of immediately available insulin, this could increase beta cell stress. Years before insulin treatment was needed, the insulin response to oral glucose was reported to be above normal (Ilonen et al., 1980).
The class II HLA risk genotypes may also be associated with more high-affinity immunity and maturation of the permanent autoantibody response (Gullstrand et al., 2008). In mice, B cells produced autoantibodies that activated T cells, and this was shown to promote glucose intolerance (Winer et al., 2011). Presence of islet autoantibodies also predicted worsening of beta cell function in T2D patients (Turner et al., 1997). LADA patients have shared genes in common with T1D but also with T2D patients (Cousminer et al., 2018).

As the decreased capacity to secrete insulin was not independently associated with the class II HLA risk genotype it is still possible that other genetic factors can potentially influence the longitudinal pattern of FPIR. Indeed, the results of study III showed that certain genetic variants outside the class II HLA region can affect the longitudinal pattern of insulin response. The presence of the class I A*24 allele was associated with steeper decline in insulin secretion in the current study and also the rate of progression to T1D in an earlier analysis (Mikk et al., 2017). Beta cells can express autoantigens in the context of class I HLA molecule (Richardson et al., 2016).

The CTS H CC genotype was associated with a faster decline in insulin secretion in children with multiple autoantibodies when compared with other genotypes. Interestingly, in an earlier study, the CTS H CC genotype has been associated with CTS H overexpression which increased chronic insulin release and decreased cytokine-induced apoptosis (Floyel et al., 2014). The proinflammatory cytokines were shown to decrease the CTS H expression in human islets (Floyel et al., 2014).

PTPN2 can sensitize beta cells to surrounding proapoptotic cytokines especially in the vicinity of local inflammation (Santin et al., 2011). T_{reg} cells that were reprogrammed by the transcription factor Eos have been shown to respond rapidly to the inflammation site (Sharma et al., 2013). The finding that FUT2 was associated with a change in insulin secretory capacity was surprising. Still, the secretor status has been associated with intestinal microbiota composition (Kumar et al., 2015; Wacklin et al., 2011) and there are associations between autoimmunity and the microbiome (Davis-Richardson et al., 2014; Vatanen et al., 2016). The growth and development of beta cells is likely to be optimal in the presence of healthy microbiome.

Compared to other stimuli, the beta cell insensitivity to glucose has been shown to be more severe in early T1D or in remission without insulin treatment (Ganda et al., 1984). A defect in beta cell glucose recognition has been estimated to be present years before diagnosis (Ferrannini et al., 2010). The impaired glucose sensing of the beta cell instead of a defect in the compensation for insulin resistance has been suggested to be the key determinant in glucose intolerance (Mari et al., 2011). Dysregulation of insulin response is known to occur when glucose levels are above normal (Bruzell et al., 1976). The increase in metabolic flux can lead to chronic
beta cell stress and potentially irreversible dysfunction of the cell (Eizirik et al., 2008; Robertson et al., 2003). This is accompanied by the accumulation of unfolded proteins and diminished activity of insulin gene activator (Eizirik et al., 2008; Poitout et al., 1996).

The strength of this study is that it includes data about young children representing a population that has the highest global incidence of T1D. The long follow-up and repeated monitoring of autoantibodies and IVGTTs in a large cohort of children who were observed from birth has built a unique dataset for this study. However, our study subjects were young children and results may not be generalizable to adults. The protective genotypes are common in the general population but were present in a minority of subjects in our study.

In children progressing to T1D the capacity to secrete insulin is impaired several years before the onset of T1D. The impairment of the beta cells can be due to extra burden or specific environmental factors. The failure to increase the insulin secretory capacity over time implies that there can be problems in the development of beta cell mass or function. If we were able to pinpoint the specific defect or learn the compensatory mechanisms that delay the disease onset despite of the low insulin secretion capacity we might find opportunities to prevent the development of T1D.
6 Conclusions

The main conclusions of this thesis include:

1. FPIR is decreased 4–6 years before the onset of T1D in children that progressed to T1D (progressors) compared to children who did not progress to T1D during the follow-up (non-progressors).

2. Progressors have significantly lower mean FPIR values throughout childhood compared to non-progressors and the difference between the groups increases with age.

3. The association between the class II HLA T1D risk group and FPIR is explained by the appearance of islet autoantibodies. The declining FPIR is associated with multiple autoantibodies irrespective of the class II HLA risk.

4. The longitudinal pattern of FPIR values may be modified by genetic variants outside the class II HLA region.
Acknowledgements

This work would not have been possible unless the contribution of numerous people that have been involved in some way in the DIPP study during the years. The overall work carried by you is immense, and I sincerely thank all of you that have contributed to this study.

There are some people I would like to especially thank.

When I started my journey in T1D research in 2009, I was guided by Professor Olli Simell. I felt welcomed by you and the whole DIPP study personnel, and I still remember our inspiring discussions in Datacity. I express my sincere gratitude to you Olli for giving me the opportunity, trust and support.

I express my sincere gratitude to my supervisors Professor Jorma Toppari and Professor Riitta Veijola, who have supported and encouraged me during this long process. I thank Jorma especially for his politeness and for reacting quickly to my astonishments. I thank Riitta especially for the times we spent thinking and wondering, and you also taught me to pay attention to details, something which I am particularly grateful.

Professor Jorma Ilonen is warmly thanked for sharing his knowledge and expertise in questions related to genetic analyses.

I have very much appreciated the feedback offered by the pre-examiners of this thesis. I thank Professor Karl-Heinz Herzig and Docent Terhi Tapiainen for making great comments and suggestions that really improved the content of this thesis. I also thank Associate Professor Tiinamaija Tuomi for accepting to be my opponent in the defence of this thesis.

The members of the follow-up committee, Professor Markus Juonala and Professor Olli Raitakari are thankfully acknowledged.

The assistance given by Esko Pakarinen, Jari Hakalax and Mika Riikonen in data management and general IT help was an absolute necessity during this work. Thank you!

Jaakko Matomäki and Eliisa Löyttyniemi are warmly thanked for their expertise in statistical analyses.

All my coauthors and coworkers are sincerely thanked for their efforts during these years.
Professor Mikael Knip is thanked especially for his inspiring approach in T1D study. Docent Robert Hermann† is thanked for his enthusiastic supervision in the beginning of my PhD thesis project. Docent Johanna Lempainen, Docent Juha Mykkänen, Mari Vähä-Mäkilä, Annika Adamsson, Marjaana Mäkinen (especially for the time in Medicity), Mirja Nurmio, Docent Jukka Kero, Leena Karlsson, Susanna Aspholm, Docent Paula Vähäsalo, Anne Hekkala, Olli Helminen, Tuula Simell, Ville Simell, Taina Härkönen, Docent Heli Siljander, Minna Kiviniemi, Mari-Liis Mikk, Antti-Pekka Laine, Jaakko Koskenniemi (especially Fig.3 in study II) and Sinikka Heikkilä are thankfully acknowledged for their contribution.

The personnel in laboratories and all study centers are gratefully acknowledged. In particular, I thank Minde (especially for the help with IVGTT data), Elina, Tiina, Zhian, Satu, Maria, Maija, Petra, Sini, Eija, Eeva and Sanna. I think you are doing a marvelous job.

Turku Brain and Mind Center is acknowledged for providing me a great working space. Overall, the people in the second floor of Teutori open office are thanked for creating such a nice atmosphere to work in and for the occasional gym sessions. I also thank Henriette Acosta for the help with the translation work.

I have been very fortunate in having many wonderful friends. I thank especially my friends from medical studies, and also my friends from chemical engineering in TKK, years that I still cherish. Special thanks go to Heidi who has been a true friend for over three decades. I would not be the person I am without you. I also thank Sini for her contribution.

I would like to thank my parents Matti† and Marja-Leena for their love. I am grateful for all of my siblings: Annamari, Katariina, Mikko and Karri†. I especially thank my mother for her kindness, sense of humor and the love of art we both share. I also thank Kari and Riitta for being there for us, and particularly for your bond with Kunto.

My dear Juho (Kusti), I am grateful for your love and support. I really appreciate that we are so different in many ways. Thank you for being you. My darling son Kunto, you are my treasure and my sunshine. You have grown along with this project, you don’t probably remember me doing anything else for a work. There is no doubt you have inspired me from the very beginning of this study.

Finally, I would like to express my special gratitude to all families who have participated in the DIPP study and given their time and effort. Without your contribution, this study would not have been possible in the first place.

This work was funded by the JDRF, the Special Research Funds for Turku, Oulu and Tampere University Hospitals in Finland, the Academy of Finland, the Turku University Foundation, the University of Turku Graduate School Doctoral Programme, Turku Centre for Lifespan Research, the Diabetes Research Foundation in Finland, the Sigrid Juselius Foundation, the Päivikki and Sakari Sohlberg
Maarit Koskinen

Foundation, the Emil Aaltonen Foundation, Kyllikki and Uolevi Lehikoinen Foundation, the Signe and Ane Gyllenberg Foundation, the European Union Biomed and the Foundation for Paediatric Research in Finland.

September 19th, 2019 in Turku

Maarit Koskinen
References


Ahren, B., & Holst, J. J. (2001). The cephalic insulin response to meal ingestion in humans is dependent on both cholinergic and noncholinergic mechanisms and is important for postprandial glycemia. *Diabetes, 50*(5), 1030-1038.


References


Groop, L., & Pociot, F. (2014). Genetics of diabetes—are we missing the genes or the disease? *Molecular and Cellular Endocrinology, 382*(1), 726-739.


Helminen, O., Aspholm, S., Pokka, T., Ilonen, J., Simell, O., Veijola, R., & Knip, M. OGTT and random plasma glucose in the prediction of type 1 diabetes and time to diagnosis. Diabetologia, 58(8), 1787-1796.


autoimmunity by several months: possible evidence for slowly operating mechanisms in virus-induced autoimmunity. *Diabetologia*, 60(3), 424-431.


References


Mikk, M. L., Kiviniemi, M., Laine, A. P., Härkönen, T., Veijola, R., Simell, O., Knip, M., Ilonen, J., & Finnish Paediatric Diabetes Register. (2014). The HLA-B*39 allele increases type 1 diabetes risk conferred by HLA-DRB1*04:04-DQB1*03:02 and HLA-DRB1*08-DQB1*04 class II haplotypes. *Human Immunology, 75*(1), 65-70.


Childhood Diabetes in Finland Study Group. Clinical and Experimental Immunology, 116(1), 78-83.
patients with type 1 diabetes: The diabetes prevention trial–type 1. Diabetes Care, 28(5), 1068-1076.


FIRST PHASE
INSULIN RESPONSE IN CHILDREN WITH ISLET AUTOANTIBODIES
Results from the Finnish Type 1 Diabetes Prediction and Prevention study
Maarit Koskinen