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Department of Paediatrics, Faculty of Medicine, University of Tartu, Estonia
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Paper II: Peet A, Hämäläinen AM, Kool P, Ilonen J, Knip M, Tillmann V; DIABIMMUNE Study Group. Early postnatal growth in children with HLA-conferred susceptibility to type 1 diabetes. Diabetes Metab Res Rev 2014;30:60–68.

Paper III: Peet A, Hämäläinen AM, Kool P, Ilonen J, Knip M, Tillmann V; DIABIMMUNE Study Group. Circulating IGF-I and IGFBP-3 in relation to the development of β-cell autoimmunity in young children. Eur J Endocrinol 2015;173:129–137.

Contribution of the author to the preparation of the original publications: study design, examination of part of the patients, collection of clinical data, statistical data analysis, and writing the first manuscript version for all three original publications.

ABBREVIATIONS

ALS	acid labile subunit
APC	antigen presenting cell
BMI	body mass index
CI	confidence interval
FPIR	first-phase insulin response
GADA	glutamic acid decarboxylase autoantibodies
HLA	human leukocyte antigen
HOMA-IR	homeostatic model assessment-insulin resistance
IAA	insulin autoantibodies
IA-2A	insulinoma associated-2 antibodies
ICA	islet cell antibody
IGF-I	insulin-like growth factor I
IGFBP-3	insulin-like growth factor binding protein type 3
IGF-IR	insulin-like growth factor I receptor
NOD	non-obese diabetic
OR	odds ratio
SD	standard deviation
SDS	standard deviation score
STZ	steptotozin
Tregs	regulatory T cells
T1D	type 1 diabetes mellitus
T2D	type 2 diabetes mellitus
WHO	World Health Organization
ZnT8A	zinc transporter 8 autoantibodies

1. INTRODUCTION

The incidence of type 1 diabetes (T1D) is increasing by 3–4% annually in most countries all over the world (Patterson et al., 2009). The background of such an increase is largely unknown (Knip et al., 2005; Onkamo et al., 1999). It is widely accepted, that the aetiology of T1D is multifactorial, where the genetic disease susceptibility and environmental triggers both play an important role. The class II human leukocyte antigen (HLA class II) genes contribute to about 50% of the overall genetic risk for T1D (Noble et al., 1996; Concannon et al., 2005). However, in a scenario, where about 20% of Caucasians carry those T1D risk conferring HLA genotypes, only 5–10% of such individuals develop clinical T1D (Knip et al., 2005). Even in the geographically close area around the Baltic Sea, the incidence of T1D varies between five populations 2 to 6-fold (Tuomilehto J et al., 1992; Teeäär et al., 2010; Harjutsalo et al, 2013). Those observations support the view that the reasons for the fast and continuous increase in incidence rate must be due to environmental changes (Knip et al., 2005; Onkamo et al., 1999).

In many studies, an increased linear growth both in height and weight (Bloom et al., 1992; Larsson et al., 2008; Ljungkrantz et al., 2008;) and increased BMI (EURODIAB Substudy 2 Study Group, 2002; Hyppönen et al., 2000; Knerr et al., 2005) have been observed in children who later presented with T1D. Those observations led to the acceleration hypothesis postulated by Wilkin in 2001. The hypothesis states that in conditions of food overload, obesity, and accelerated early childhood growth, which are common features in modern societies, the demand of insulin production increases, and the overactive β-cells become more susceptible to stress, leading to their apoptosis and destruction (Wilkin, 2001). According to the accelerator hypothesis, insulin resistance plays an important role, to which excessive weight gain undoubtedly contributes substantially (Wilkin, 2008). Insulin resistance promotes metabolic upregulation of β-cells with the resulting increase in vulnerability (Wilkin, 2001) to stress and increased antigenity. The latter could also lead to the increased susceptibility to the autoimmune destruction of islets (Gale, 2005), accelerating β-cell loss (Wilkin, 2009). In 2006 Dahlquist expanded the accelerator hypothesis to the overload hypothesis stating that any stress will make the β-cell more vulnerable to cell damage and initiation of autoimmunity. Moreover an almost linear correlation has been shown between increasing birth weight (BW) and the subsequent risk for T1D (Cardwell et al., 2010; Harder et al, 2009), indicating that accelerated growth as well as other environmental determinants of T1D could be important already in intrauterine life.

The role of genetic factors conferring susceptibility to T1D in the association between T1D and increased pre- and postnatal growth is unclear. There are a relatively limited number of studies, in which a possible association between HLA genotypes conferring risk for T1D and growth has been analysed. In previous studies it was found, that some HLA genotypes contribute positively and some others negatively to various growth parameters (Carlsson et al., 2012;

Larsson et al., 2005; Larsson et al., 2008; Stene et al., 2001; Järvinen et al., 2008; Yang et al., 2014). Several studies have clearly indicated, that environmental factors may modify HLA-related growth (Taylor et al., 2006; Hummel et al., 2007; Larsson et al., 2007) Since environmental factors also represent the main reasons for the increase in T1D incidence (Knip et al., 2005; Onkamo et al., 1999), the potential impact of the HLA genotypes on growth could be expected to be strongest in the countries with the highest incidence of T1D and accordingly the highest diabetogenic environmental pressure. However, so far studies have been performed only in countries with relatively similar incidence rates of T1D and probably a relatively similar environment (Yang et al., 2014; Sterner et al., 2011). Accordingly the potential role of HLA genotypes conferring risk for T1D in pre- and/or postnatal growth remains open.

As the growth hormone (GH) – insulin-like growth factor I (IGF-I) axis is the major determinant of childhood growth, accelerated growth in children who later progress to T1D implies a possible role of IGF-I in the pathogenesis of T1D. This suggestion is supported by the large body of evidence indicating a role for the IGF-I (Agudo et al., 2008; Anguela et al., 2013; Castrillo et al., 2000; George et al., 2002) and its major binding protein – IGFBP-3 (Jones and Clemmons, 1995; Butler et al., 1996; Chan et al., 2005; Kim et al., 2007) in the development of T1D. Different animal models have shown, that IGF-I stimulates the replication and proliferation of pre-existing β-cells after damage (Agudo et al., 2008), protects β-cells from apoptosis (Castrillo et al., 2000), protects islets from lymphocytic infiltration (George et al., 2002), and suppresses the progression of autoimmune diabetes (Anguela et al., 2013). IGFBP-3 has also IGF-I independent functions (Kim, 2014; Johnson and Firth 2014), their role in the pathogenesis of diabetes is not clear, but some studies have observed a positive association between circulating IGFBP-3 concentrations and type 2 diabetes (T2D) (Frystyk et al., 1999; Rajpathak et al., 2012). There is only one earlier study on circulating IGF-I and IGFBP-3 concentrations in prediabetic children with early signs of β-cell autoimmunity (Beyerlein et al., 2014). That study did not find any difference in the serum concentrations of IGF-I or IGFBP-3 between subjects developing autoantibodies and autoantibody-negative control children (Beyerlein et al., 2014). The possible combined impact of the IGF-I/IGFBP-3 system and HLA genotypes on postnatal growth has not been studied before.

The aim of the current study was to analyse the association between HLA genotypes conferring risk for T1D and pre- and postnatal growth. Another aim was to search for possible differences in the strength of these associations among populations with contrasting incidence of T1D. Further the possible impact of the IGF-I/IGFBP-3 system on the development of β-cell autoimmunity, and the potential role of the IGF-I/IGFBP-3 system in the HLA- modifying effect on postnatal growth were studied.

2. REVIEW OF THE LITERATURE

2.1. Definition and autoimmune mechanisms of type 1 diabetes

Diabetes mellitus is a group of disorders characterized by chronic hyperglycaemia resulting from defects in insulin secretion and/or insulin action. Most cases of diabetes can be classified into two major subtypes: T1D, characterized by an almost absolute deficiency of insulin secretion; or type 2 diabetes (T2D), which results from a combination of resistance to insulin and relative insulin deficiency due to inadequate compensatory insulin secretion by the pancreatic β-cells. T1D represents only about 5–10% of subjects with diabetes, but in many populations, especially of Caucasian origin, T1D is the most common form of diabetes in young people (Craig et al., 2014).

T1D is characterized by chronic immune-mediated destruction of the β-cells in the pancreatic islets, leading to partial, or in most cases, absolute insulin deficiency. The first detectable sign of emerging β-cell autoimmunity is the appearance of diabetes-associated autoantibodies (Knip et al., 2005). Several studies have shown that autoantibodies often emerge already in very early life (Kimpimäki et al., 2001). There are five disease-related autoantibodies including islet cell antibodies (ICA), insulin autoantibodies (IAA), autoantibodies to the 65 kD isoform of glutamic acid decarboxylase (GADA), the protein tyrosine phosphatase-related IA-2 molecule (IA–2A), and zinc transporter 8 autoantibodies (ZnT8A) (Watkins et al., 2014). Some or all of those autoantibodies are presented in 85–90% of patients with newly diagnosed T1D (Watkins et al., 2014). The appearance and the number of those autoantibodies directly predict the risk of progression of the β-cell autoimmunity to clinical T1D (Knip, 2002). About 70% of subjects testing positive for multiple (≥2) autoantibodies progressed to T1D over the subsequent 10 years whereas only 15% progressed among subjects with a single islet autoantibody (Ziegler et al., 2013).

It is generally accepted that the destruction of β-cells is mediated primarily by cellular immune responses, namely by CD8-positive T cells (Knip and Siljander, 2008). This is supported by many arguments. Most important of them are the facts that CD8-positive T cells are the major cell types present in insulitis, disease progression is delayed by immunosuppressive drugs directed specifically against T cells, circulating autoreactive T cells are detectible in patients at clinical presentation of T1D (Roep, 2003), and most of the autoantibody-negative subjects diagnosed before the age of 40 years have antigen-specific T-cell response to T1D-related autoantigenes (Lohmann et al., 1997). It still remains open whether there is a true autoantibody-negative non-autoimmune form of T1D. It is possible, that antibodies are detectable in the preclinical period but turn negative before diagnosis, or the assays are not sensitive enough to detect low antibody levels (Sabbah et al., 2000).

2.2. Epidemiology, genetic and environmental triggers of type 1 diabetes

2.2.1. Epidemiology of type 1 diabetes

Convincing data show that the incidence of T1D has started to increase after the middle of the 20th century all over the world (Gale, 2002) by 3–4% annually in most of the industrialised countries (DIAMOND Project Group, 2006; Onkamo et al., 1999). In Finland, where the incidence now is the highest in the world, the first nationwide incidence estimation in 1953 showed a rate of 13/100,000/year, and since then the rate has almost steadily increased by 2.8 %/year reaching 65/100,000/year in 2006 (Harjutsalo et al., 2013). The increase in rates differs significantly between age groups and regions of the world. For example, among European countries where the epidemiological data are most accurate, the rates of the increase were highest in Central and Eastern European countries. Evidence also clearly shows that the increases in incidence rate during the last decades of the previous century were highest in the youngest age group (EURODIAB ACE Study Group, 2000; Patterson et al., 2009**).**

Now the overall standardized incidence of T1D in the world varies 600-fold from 0.1/100 000 per year in some regions of China to more than 60/100 000 per year in Finland (DIAMOND Project Group, 2006; Harjutsalo et al., 2013). Interestingly, a remarkable variation in the incidence was found even among five populations of a relatively small area around the Baltic Sea (Tuomilehto J et al., 1992), where there is now approximately a 2 to 6-fold difference (Teeäär et al., 2010; Harjutsalo et al, 2013; Kondrashova et al., 2005). The reasons behind the rapid increase in incidence, including the conspicuous difference seen even between neighbouring countries, and the shift in disease manifestation into the younger age (Larsson et al., 2004) are largely unknown, but environmental changes are the most likely explanation (Onkamo et al., 1999).

2.2.2. Genetic determinants of type 1 diabetes

2.2.2.1. HLA class II genes risk alleles

The most important genes contributing to the disease susceptibility to T1D are the variabilities in the human leukocyte antigen (HLA) region that has been estimated to explain at least 50% of the genetic influence in T1D (Noble et al., 1996; Concannon et al., 2005; Pociot et al., 2010). The HLA system is located on chromosome 6p21 and contains many genes related to the immune system. HLA are divided into three classes, of which class II is the major genetic susceptibility determinant for T1D. HLA class II proteins are coded by DP, DQ, and DR loci. The proteins consist of an α and a β peptide chain that are combined to form the heterodimeric class II molecules. Their expression is normally restricted only to antigen-presenting cells (APC), B cells and activated T cells (Kelly et al., 2003). The physiological role of the molecules encoded by HLA

class II genes is the fitting of the immune response by presenting processed antigens by APC to the immune system (Knip et al., 2002).

Susceptibility to T1D is associated with two combinations of *DQA1* and *DQB1* alleles, namely: *DQA1*0501-DQB1*0201* and *DQA1*0301-DQB1*0302*, which encode the HLA-DQ2 and DQ8 molecules, respectively. Two DRB1 alleles, *DRB1*03* and *DRB1*04* (which encode the DR3 and DR4 molecules, respectively), are also associated with an increased risk of disease. *DRB1*03* is in linkage disequilibrium with the *DQA1*0501-DQB1*0201* allelic combination (forming the *DR3-DQ2* haplotype) whereas *DRB1*04* is in linkage disequilibrium with *DQA1*0301-DQB1*0302* (forming the *DR4-DQ8* haplotype) (Kelly et al., 2003; Eringsmark Regnéll and Lernmark, 2013). Up to 90% of patients with diabetes carry one or both of these haplotypes, and the highest genetic risk of the disease is conferred by the combination of *DR3-DQ2* and *DR4-DQ8* or more precisely *DQA1*0501-DQB1*0201-DRB1*0301* and *DQA1*0301-DQB1*0302-DRB1*04*. Since *DR3* and *DR4* are in linkage disequilibrium with *DQ2* and *DQ8* respectively, the heterozygous combination of those haplotypes is commonly designated synonymously as *DR3/DR4* or *DQ2/8* (Kockum et al 1996). Both components of the haplotype are important in relation to the risk of T1D. This is well illustrated by the *DR4-DQ8* haplotype where *DQ8* is the major T1D determinant, but the influence on the disease risk may be significantly modified by the DRB1 subtype (Kockum et al., 1999). Namely, the *DRB1*0401*, *DRB1*0402*, and *DRB1*0405* subtypes increase the risk of diabetes independent of *DQ8*, whereas *DRB1*0403* and *DRB1*0406* confer protection from the disease (She, 1996, Donner et al., 2000; Undlien et al., 1997). The protective effect of *DRB1*0403* can override the susceptibility conferred by *DQ8*, even in subjects carrying the highest risk *DQ2/DQ8* genotype (Kelly et al., 2003). Some other protective alleles in combination with the risk alleles also neutralize the disease risk (Erlich et al., 2008). On the other hand, alleles such as *DQ6.4*, *DQ5.1*, *DQ4* or *DQ6.3*, which are neutral regarding the risk for T1D, are overridden by the risk alleles when combined with the *DQ2* or DQ8 risk alleles.

2.2.2.2. Evidence for a direct role of the HLA-DR and HLA-DQ molecules in disease pathogenesis

The exact mechanisms of HLA-conferred susceptibility to or protection from T1D are not completely understood. Long ago it was suggested that the risk conferring DR and DQ molecules on the surface of APC are more efficient in presenting T1D related autoantigenes to the T lymphocyte in comparison with other HLA class II molecules. *Vise versa*, the protective gene products probably bind and present diabetogenic autoantigenes less effectively (Knip et al., 2002). Some evidence supporting that suggestion came from studies with *x*-ray crystallography and computer modeling, which have found that the HLA molecules associated with susceptibility to T1D probable have similar chemical and

geometric properties in their antigen binding site. These characteristics are markedly different from those of protective HLA molecules, which are also similar to each other (Cucca et al., 2001; Lee et al., 2001). Some very recent transgenic mouse modeling (Skowera *et al*., 2008; Mohan et al., 2011; Nakayama et al., 2005), as well as *in vitro* human studies (Bulek et al., 2012; Kronenberg et al., 2012), have shown evidence indirectly supporting the suggestion of a better peptide-binding activity of DQ2 and DQ8 and consecutively a more efficient peptide recognition by T lymphocytes.

There are also some evidence of other potential mechanisms behind HLA-related susceptibility to T1D. It has been demonstrated, HLA DQ-conferred genetic risk status is significantly associated with CD4+CD25+(high) T-cell apoptosis (Glisic et al., 2009). Those cells are regulatory T-cells (Tregs), normally preventing or delaying onset of T1D in animal models. Therefore increased Treg apoptosis could accelerate T1D by increased T-cell-mediated destruction of β-cells. Subjects with high risk HLA *DQB1* genotypes showed increased CD4+CD25+(high) T-cell apoptosis compared to subjects with low risk HLA *DQB1* genotypes (Glisic et al., 2009).

2.2.2.3. Other genetic determinants of type 1 diabetes

HLA class I alleles also contribute to the risk of T1D, although considerably less than class II (Eringsmark Regnéll and Lernmark, 2013). In addition to HLA, more than 50 other genes outside the HLA region have been found to be associated with the increased risk for T1D (Concannon et al., 2009). These include the insulin gene, *CTLA4*, *PTPN22*, interleukin 2 receptor α (*IL2RA*), the interferon induced with helicase C domain 1 (*IFIH1*), the *CAPSL-IL7R* block, the lectin *CLEC16A*, the Th1 transcription factor *STAT4*, the tyrosine phosphatase *PTPN2*, as well as other loci (Stankov et al., 2013). Their individual effects are substantially smaller compared with HLA- *DQ* and *DR*. Nevertheless, they contribute either to the risk of islet autoimmunity or to the progression to clinical T1D in subjects who have developed islet autoimmunity (Eringsmark Regnéll and Lernmark, 2013; Pociot et al., 2010; Todd et al., 2007).

2.2.2.4. Prevalence of HLA class II risk alleles in the population and environmental pressure

About 20% of Caucasians carry HLA-conferred susceptibility to T1D, whereas the lifetime cumulative incidence of T1D is about 1–2%, indicating that only approximately 5–10% of individuals with HLA-determined risk develop clinical T1D. This clearly indicates that additional factors are needed to trigger β-cell autoimmunity and destruction. The more than 10-fold difference in the incidence of T1D between different Caucasian populations with similar genetic background can hardly be explained only by genetic factors (Knip et al., 2005).

The significant increase in T1D incidence rate, which started from the middle of the 20th century (Gale, 2002), can neither be explained by increased genetic disease susceptibility in the population, but must be due to the changes in life-style and environment (Knip et al., 2005). Moreover, accumulating evidence suggests that the proportion of subjects with high-risk conferring HLA genotypes has decreased over the last decades among patients with newly diagnosed T1D, whereas the proportion of people with low-risk or protective HLA genotypes has increased (Hermann et al., 2003; Gillespie et al., 2004). Available data also indicate that the incidence of T1D has increased in population groups who have moved from a low-incidence region to a high-incidence area (Åkerblom and Knip, 1998). All those data emphasize the role of environmental factors, resulting in the progression to clinical diabetes in genetically susceptible, as well as in non-susceptible subjects.

2.2.3. Environmental triggers of type 1 diabetes

2.2.3.1. Role of the viruses and bacteria

At present, there is no obvious environmental candidate trigger of β-cell and islet autoimmunity (Eringsmark Regnéll and Lernmark, 2013). Analytical epidemiological studies have identified several environmental risk factors, including older maternal age, preeclampsia, delivery by cesarean section, increased birth weight, early introduction of cow's milk proteins, enteroviral infections, accelerated postnatal growth and weight gain. (Soltesz et al., 2007). As direct triggers of β-cell autoimmunity, multiple factors have been proposed starting from viral and bacterial agents, different food components, and a combination of food components and microbial agents (Yoon, 1990; Knip et al., 2005). Observational studies suggest that β-cell autoimmunity may be initiated at any age, although a majority of the processes seems to start very early in childhood (Leslie and Delli Castelli, 2004).

Because of very clear seasonal variation in the appearance of the first diabetes-associated autoantibodies and seasonal variation in the presentation of clinical T1D, reported in many Northern Hemisphere studies (Kimpimäki et al, 2001; Laron Z, 1999; Lévy-Marchal et al., 1995) and in some Southern Hemisphere studies as well (Glatthaar et al., 1988), viruses have been for long considered as main candidates for the principle environmental factor initiating β-cell autoimmunity (Eringsmark Regnéll and Lernmark, 2013). The peak incidence of T1D is in the fall-winter period and is more pronounced in countries with marked differences between summer and winter temperatures (Dahlquist and Mustonen, 1994). The role of viruses is also supported, for example, by observations of a strong temporal relationship between enterovirus infections and the appearance of the first diabetes-associated autoantibodies that was seen in several Finnish prospective studies (Hiltunen et al., 1997; Lönnrot et al., 2000; Lönnrot et al., 2000; Salminen et al., 2003), but not in prospective studies coming from other countries (Fuchtenbusch et al., 2001; Graves et al., 2003).

However, it should be taken into account that studies where the association between β-cell autoimmunity and enterovirus infections was not found, had relatively limited number of subjects with positive autoantibodies and wide intervals of sampling for enteroviruses. Experimental work has provided evidence that enteroviruses can infect the thymus and alter thymic selection processes. Thus, maternal or early life infections may facilitate impairment of central tolerance leading to islet autoimmunity (Jaidane et al., 2012). Many studies have searched for viruses from the pancreatic tissue. It has been shown that enterovirus infection in subjects with persistent islet autoantibodies accelerates disease progression from subclinical to overt T1D (Stene et al., 2010). Enteroviruses have also proven to be able to cause a direct infection and damage of β-cells, (Foulis et al., 1987; Dotta et al., 2007), which could accelerate inflammation and functional changes in β-cells (Willcox et al., 2011; In't Veld, 2011). The presence of a viral antigen was observed ten times more often in patients with T1D compared with controls (Richardson et al., 2009). However, it still remains open whether enteroviruses are true triggers or only accelerators of islet autoimmunity (Coppieters and von Herrath, 2011).

The intestinal microbiome represents a complex symbiotic community that influences human health, including the teaching and maintenance of the immune system (Wen et al., 2008; Koenig et al., 2011; Arpaia et al., 2013). Some recent studies suggest that gut microbiota may affect the development of islet autoimmunity. For example, the development of diabetes in non-obese diabetic (NOD) mice may be influenced by the microbial environment, or by exposure to some specific microbial stimuli (Okada et al., 2010). In some rat and NOD mouse studies, the decrease of disease risk was shown after antibiotic treatment (Wen et al., 2008; Schwartz et al., 2007). Placing the NOD mice from natural to pathogen-free settings increased the diabetes incidence (Bach, 2002) whereas the exposure to bacterial antigens and infections decreases the risk for T1D (King et al., 2011) suggesting that some microbial exposures may protect against T1D. Regarding human studies, one small Finnish cohort study demonstrated a higher level of *Bacteroidetes* relative to *Firmicutes* species approximately 6 months after birth in those who further developed T1D (Giongo et al., 2011). A more recent study from Spain showed that children with T1D have increased numbers of *Clostridium*, *Bacteroides* and *Veillonella*, and decreased numbers of *Bifidobacterium* and *Lactobacillus* compared to healthy controls (Murri et al., 2013). A detailed study of dynamical changes in gut microbial colonisation of infants genetically predisposed to T1D has shown a significant decrease in the diversity of gut microbiome in the period between seroconversion and T1D diagnosis accompanied by spikes in inflammation-favoring organisms (Kostic et al., 2015). There are also some other studies showing that gut micobiota affects the risk of T1D development (Dunne et al., 2014).

The results of studies investigating the role of viruses and bacteria in the development of β-cell autoimmunity are, in general, in line with the so-called „hygiene hypothesis", formulated for the first time by Strachan in 1989 (Strachan, 1989). This hypothesis proposed that because of modern standards of

hygiene and other changes of lifestyle in industrialized countries, the exposure to microbes and the infectious burden have decreased. As a result, the development of protective immunity throughout childhood is impaired and the susceptibility to allergic diseases is increased. Further, the hypothesis was extended from the field of allergy to autoimmune diseases (Bach, 2002). Interestingly, the so-called polio hypothesis narrows the hygiene hypothesis arguing that T1D incidence rate increases rapidly in countries like Finland and Sweden where the frequency of enterovirus infections has decreased over the last decades (Vikari et al., 2000).

2.2.3.2. Role of vitamin D and other dietary factors

As it was mentioned above, initial autoantibodies appear more frequently in the fall-winter period and show a similar trend from year to year. Based on these observations it could be concluded that the trigger of islet autoimmunity should show similar seasonal variation. Therefore, some exogenous factors with a stable or consistently increasing exposure in early childhood, such as most dietary components implicated as potential triggers of β-cell autoimmunity, could be excluded. (Knip et al., 2005). However, there is a clear seasonal variation in the amount of daylight and sunshine hours that naturally causes seasonal variation in vitamin D levels. Those variations are especially pronounced in Northern Europe, the region with the highest incidence of T1D in the world (Karvonen et al., 2000). Therefore, vitamin D has been implicated as a possible trigger of islet autoimmunity. Observational studies to test this hypothesis were summarized in a meta-analysis (Zipitis et al., 2008) where a decrease in T1D risk was observed in infants supplemented with vitamin D. However, our very recent study showed that vitamin D status did not differ between subjects positive and negative for T1D-related autoantibodies (Reinert-Hartwall et al., 2014). On the other hand, in Finland, were the vitamin D intake increased significantly from year 2003, a plateau in the T1D incidence rate was observed from year 2006, indicating a temporal association between those two events (Mäkinen et al., 2014). Thus, the possible role of vitamin D in the pathogenesis of T1D remains open.

Cow's milk has also been suggested as a trigger of T1D particularly when cross-reactivity was found between bovine and human insulin (Virtanen and Knip, 2003). Since the age of cow's milk introduction depends on breastfeeding duration, it has been proposed, that breastfeeding could potentially protect against β-cell autoimmunity. However, the data from the literature have shown results reporting a protective effect of breastfeeding (Malcova et al., 2006) as well as no effect (Couper et al., 2001) on the development of β-cell autoimmunity. Studies on the effect of cow's milk on the risk of T1D development have also led to contradictory results showing that cow's milk either is a risk factor for T1D (Verge et al., 1994; Virtanen et al., 1998) or there is no association between the risk for T1D and cow's milk exposure (Norris et al., 1996; Couper et al., 2001). A large meta-analysis, setting out to investigate whether there is a

reduced risk for T1D in children being exclusively breastfed for shorter or longer period, suggested very weak protective associations (Cadwell et al., 2012). Data were available from 43 studies including 9,874 patients with T1D. Overall, there was a reduction in the risk of T1D after exclusive breast-feeding for >2 weeks. Unfortunately, a randomized clinical trial aimed at reducing the risk of T1D development in genetically at risk infants (the TRIGR study) showed that the use of a hydrolyzed formula during the first 6–8 months of life did not reduce the cumulative incidence of T1D autoantibodies after 7 years when compared with a conventional formula, (Knip et al., 2014). The trial is, however, still continuing to assess whether the early dietary intervention has any effect on the development of clinical T1D by the age of 10 years.

2.2.3.3. Role of perinatal factors

Multiple studies have reported different perinatal events as risk factors for T1D. Those factors include preeclampsia, high maternal age, birth order, delivery by caesarean section, gestational infections, short duration of breastfeeding, and some others (Larsson et al., 2004). The results of those studies, however, again are inconclusive. Some studies have shown an increased risk for T1D if the mother has had an infection with enteroviruses during pregnancy (Dahlquist et al., 1995; Hyöty et al., H, 1995). Similar results were later reported by another study (Visalli et al., 2003), but not by all studies (Fuchtenbusch et al., 2001). Therefore, it is still open whether enterovirus infection during the pregnancy is associated with an increased risk for T1D in the offspring (Viskari et al., 2002). Delivery by caesarean section and preeclampsia were also reported to increase the risk for T1D (Dahlquist et al., 1999; Jones et al 1998), but was not confirmed in other study (Stene et al., 2003) In large meta-analysis, where individual patient data were used, delivery by caesarean section showed increased risk for T1D after adjustment for gestational age, birth weight, maternal age, birth order, breast-feeding and maternal diabetes (OR 1.19, CI 1.04–1.36) (Cardwell et al., 2008). In a similar meta-analysis investigating the effect of maternal age on T1D risk, a 5-year increase in maternal age increased the risk for childhood T1D by 5% (95% CI 2–9) (Cardwell et al., 2010).

The reason for conflicting results is probably the small effect of perinatal factors on T1D risk. For most of those factors, the odds ratios were between 0.5 and 1.5. The mechanisms, by which gestational infections, maternal age and other prenatal or neonatal events increase the risk for T1D, are not fully understood (Larsson et al., 2004). So far, the most likely perinatal risk factor associated with T1D risk is birth weight. Two large meta-analyses showed significant correlation between increasing birth weight and the risk for development of T1D (Cardwell et al., 2010; Harder et al, 2009). The relationships between birth weight and risk for T1D are discussed in details below.

2.3. Growth in children developing type 1 diabetes later

2.3.1. Prenatal growth

Increased prenatal growth resulting in an increased birth weight has been observed to be associated with subsequent risk for development of T1D in many studies (Cardwell et al., 2005; Haynes et al., 2007; Stene et al., 2001) whereas in some studies no such an association have been seen (Bock et al., 1994; Malcova et al., 2006; Wadsworth et al., 1997), and even a reduced risk has been reported (Wei et al., 2006). The interpretation of those diverse results is difficult because many studies did not have enough statistical power and they used many different groupings or categorisations of birth weight (Stene et al., 2001; Cardwell et al., 2005; Ievins et al., 2007; McKinney et al., 1999; Sipetic et al., 2005), or reported findings only for extreme birth weights (Dahlquist et al., 1999; Patterson et al., 1994). In a series of studies birth weight was just a secondary characteristic among many other factors**,** without exact description of methodology (Marshall et al., 2004; Polanska et al., 2007; Sadauskaite-Kuehne et al., 2004; Tenconi et al., 2007; Visalli et al., 2003). Nevertheless two recent comprehensive meta-analyses showed almost linear correlation between increasing birth weight and the subsequent risk for development of T1D (Harder et al., 2009; Cardwell et al., 2010). The meta-analysis by Harder et al (2009) included data from 2,398,150 individuals, 7,491 of whom had T1D. The study by Carwdell et al (2010) included 12,807 cases with T1D. Both meta-analyses showed similar results. In the latter a consistent, but a small increase in the risk of T1D was seen in children who were heavier at birth. Children with a birth weight of 3.5–4 kg had an increased risk of diabetes of 6%, and children with a birth weight more than 4 kg had an increased risk of 10% compared to children weighting 3.0–3.5 kg. This corresponds to a linear increase in diabetes risk of 3% per 500 g increase in birth weight (OR 1.03 (CI 1.00–1.06). After adjustment for maternal age, gestational age, birth order, breastfeeding, caesarean section, and maternal diabetes, the results remained the same (Cardwell et al., 2010). In a big Swedish study (Dahlquist et al., 1996) with 4,584 patients with T1D, not included into those two meta-analyses, higher birth weight was also associated with increased risk for T1D. Most of the other similar studies, but with smaller number of patients (less than 1000), have showed a modest, and not always statistically significant, positive correlation between birth weight and risk for T1D (Podar, et al., 1999; Soltesz et al., 1994; Hathout et al., 2006).

It is unlikely that birth weight plays a direct causal role in the pathogenesis of T1D (Cardwell et al., 2010). However, it is possible, that birth weight may be a marker of some unknown exposures that influence risk for T1D, for example maternal nutrition or maternal diseases (Gluckman et al., 2008). It has been also shown that birth weight may predict an earlier onset of the disease without increasing the risk itself (Betts et al., 2005). As fetal insulin is an important growth factor, the children with greater intrauterine growth and consequently higher birth weight have more active β-cells, producing more insulin (Cardwell et al., 2010). Simultaneously, as reviewed by Brown et al, the β-cells actively

secreting insulin are more prone to destruction by various mechanisms (Brown et al., 2008). Thus, the association of an increased birth weight with the risk of T1D development fits well with the acceleration hypothesis postulated by Wilkin in 2001 (Wilkin, 2001). The possible mechanisms behind the acceleration hypothesis are discussed below.

2.3.2. Early childhood growth

The association between increased weight gain and the risk of T1D can be operating also in childhood (Harder et al., 2009) where an increased linear growth (Blom et al., 1992; Larsson et al., 2008; Ljungkrantz et al., 2008; Hyppönen et al., 2000), weight or BMI (EURODIAB Substudy 2 Study Group, 2002; Hyppönen et al., 2000; Knerr et al., 2005) were observed in children who later presented with T1D. However, there are some inconsistency and ambiguity in the results of the studies that have analyzed the associations between linear growth and the risk of T1D. Some studies have demonstrated a positive association between T1D and height only in specific subpopulations (Brown et al., 1994; Japan and Pittsburgh Childhood Diabetes Research Groups, 1989; Songer et al., 1986). Brown et al. found a positive association between increased height and T1D only in those who had their T1D diagnosed between the age of 5–10 years (Brown et al., 1994). Those diagnosed under the age of 5 years were shorter, and those diagnosed after the age of 10 years were similar in height compared to the controls (Brown et al., 1994). Similar results were described by Songer and colleagues (Songer et al., 1986). In a family-based case-control study, the association of increased growth with the development of T1D was limited only to the first year of life (Kharagjitsingh et al., 2010). Some other authors have demonstrated a positive association only for girls (Pond, 1970), while others only for boys (Delsten et al., 1981). There are also surveys where no differences in height were observed between patients developing T1D and the controls (Draminsky Petersen et al., 1978; Choudhury et al., 2000). One of the largest studies that assessed the stature of 451 children with T1D at diagnosis and more than 10 000 controls, reported that patients less than 1 year of age were shorter by 1 standard deviation (SD) and children who developed T1D between the age of 3 years and up to puberty were taller by 0.3 SD than the controls, but after adjustment for parental height the latter difference vanished (DiLiberti et al., 2002). The meta-analysis of 38 earlier publications (DiLiberti et al., 2002) paralleled these observations and showed, that children with T1D were taller than their controls, except those who developed the disease during their first years of life, the latter tended to be shorter, and those who presented with T1D in adolescence were of similar height as the controls (DiLiberti et al, 2002). In the largest existing report where 9248 subjects with T1D were compared with relevant controls, the mean height SD score (SDS) at the time of diagnosis was +0.15 ($p<0.0001$). The vanishing of this difference from early childhood to adulthood was also demonstrated in that study (Knerr et al., 2005).

There are two interesting studies where subjects from countries with low incidence of T1D were included. One of them, where more than 500 subjects with T1D were compared with controls, has demonstrated that only children from USA (the country with a relatively high incidence of T1D), and only those developing T1D before puberty were taller than the controls, whereas newly diagnosed children with T1D in Japan had similar height compared to the non-diabetic population (Japan and Pittsburgh Childhood Diabetes Research Groups, 1989). In India, where the incidence of T1D is similar to that in Japan, no difference in height was seen between subjects with newly diagnosed T1D and controls (Ramachandran et al., 1994). These results indicate that in countries with different incidence rate of T1D environmental factors may influence differently the growth of subjects later developing T1D.

As well as increased height, increased weight and/or BMI have also been found to be associated with enhanced risk for T1D (EURODIAB Substudy 2 Study Group, 2002; Hyppönen et al., 2000; Knerr et al., 2005). The EURODIAB multicentre study with approximately 500 T1D subjects and 1,500 controls observed that the weight SDS was significantly increased among patients with T1D from the age of 1 month after birth and achieved the maximum difference from the controls at the age of 1 to 2 years (EURODIAB Substudy 2 Study Group, 2002). The analysis of growth in 600 patients with T1D and 600 controls in Finland demonstrated that children developing T1D are consistently heavier throughout childhood compared to controls. A 10% increment in relative weight was associated with a 20–60% increase in the risk of T1D (depending on age). Obesity after 3 years of age was associated with a more than two-fold risk of developing T1D (Hyppönen et al., 2000). In the largest study from Germany and Austria, 9248 children developing T1D had a significantly higher mean BMI than the controls in all age groups (Knerr et al., 2005). In the patient cohort as a whole, the weight SDS was +0.33 and BMI SDS +0.34 ($p<0.00001$) compared to the reference population. There was also a clear association between a higher BMI SDS and a younger age at diabetes manifestation, and the children diagnosed with T1D under the age of 5 years presented with higher weight and BMI SDS than the older patients. A slightly smaller recent German study that included about 1000 children with T1D did not, however, find excessive weight gain in any of the examined age intervals before the presentation of T1D. Only high birth weight was significantly correlated with increased risk for T1D (Kuchlbauer et al., 2014). A recent study, where individual BMI trajectories of 1011 infants were followed from birth to the age of 2 years, showed that an earlier age of the infant BMI peak was associated with the development of islet autoimmunity whereas other early growth parameters such as peak height velocity, weight velocity, and BMI itself were not (Beyerlein et al., 2014).

Thus, based on the above studies, it seems that increased BMI and/or weight play some role in the development of T1D, at least at a younger age. Many large-cohort studies have demonstrated that the strongest association between increased BMI and risk of T1D is in the youngest age group (Knerr et al., 2005). The studies that did not confirm the overall association, often observed a

significant association at least in the youngest age group (EURODIAB Substudy 2 Study Group, 2002; Ljungkrantz et al., 2008). It has been also shown that weight gain in early life may predict the risk of development of β-cell autoimmunity in children with first-degree relatives affected by T1D (Couper et al., 2009).

There are also studies where correlations between increasing prevalence of obesity (and/or increasing BMI), an increasing incidence of T1D and the age at diagnosis have been analyzed. For example, a retrospective analysis from the USA showed an inverse correlation between the age at diagnosis of T1D and BMI (Evertsen et al., 2009). However, the study by Vehik et al. reported that only an increased linear growth, but not increased BMI or weight, accounts for the younger age at diagnosis of T1D (Vehik et al., 2009). The absence of any correlation between BMI SDS and the age at diagnosis has been observed by several other studies as well (O'Connell et al., 2007; Dabelea et al., 2006). A metanalysis summarizing the results of many studies has concluded that there is overall evidence for an association between childhood obesity or higher BMI, and increased risk of subsequent T1D (Verbeeten et al., 2011)

2.3.3. The acceleration or overload hypothesis; the role of insulin resistance

An explanation for the mechanism(s) behind the association of T1D with a higher birth weight, an increased postnatal linear growth, and an increased BMI was introduced by Wilkin in 2001. This is the acceleration hypothesis stating, that in conditions of food overload, obesity, and accelerated early childhood growth, which are common features in modern societies, the demand of insulin production increases, and the overactive β-cells become more susceptible to stress, leading to their apoptosis and destruction. This could also result in an increased antigenicity of β-cells and an increased vulnerability to autoimmune destruction (Dahlquist, 2006; Gale, 2005). The acceleration hypothesis assumes that there is only one form of diabetes. Age at onset, incidence, and the particular clinical picture of diabetes, either type 1 or type 2, are all determined by the rate of β-cell destruction. The dominating type of destruction, i.e either mostly autoimmune or caused more by the insulin resistance, lead to the consecutive increased stress of β-cell and their death. The rate of destruction depends on environmental factors and pressure (insulin resistance) and the genetic response to that pressure (Wilkin, 2009).

One of the key issues of the acceleration hypothesis is insulin resistance that is postulated to be as important in the pathogenesis of T1D as in T2D. Insulin resistance leads to metabolic upregulation of β-cells resulting in stress, apoptosis and increased antigenity. Subjects with susceptible genotypes will subsequently establish an autoimmune response, further accelerating β-cell loss (Wilkin, 2009). Weight gain undoubtedly contributes to the insulin resistance (Wilkin, 2008). As it was described in details above (Harder et al., 2009; Card-

well et al., 2010; EURODIAB Substudy 2 Study Group, 2002; Hyppönen et al., 2000; Knerr et al., 2005), there is a large body of evidence confirming an increase in linear growth and BMI in children later developing T1D. Furthermore, there are some indications that children with higher BMI develop T1D at a younger age (Kibirige et al., 2003; Betts et al., 2005; Clarke et al., 2006; Evertsen et al., 2009), although all studies were not able to endorse that finding (Vehik et al., 2009; O'Connell et al., 2007; Dabelea et al., 2006). Moreover, there are several observational studies demonstrating that prediabetic children are more insulin-resistant compared to their non-diabetic controls (Fourlanos et al., 2004; Xu et al., 2007). Fourlanos and colleagues compared the insulin resistance in autoantibody-positive subjects who progressed to T1D with those who did not develop T1D. They concluded that insulin resistance is an independent risk factor for progression to overt T1D. Progressors had higher insulin resistance relative to their insulin secretion at baseline, higher fasting glucose, and fasting insulin. The progressors were characterized by an increased relative insulin resistance, i.e. the ratio between insulin resistance assessed based on the homeostatic model (HOMA-IR) and the first-phase insulin response (FPIR) (Fourlanos et al., 2004). In other study, the HOMA-IR and FPIR-to-HOMA-IR ratio were also associated with progression to T1D (Xu P et al., 2007). However, a large study where intravenous glucose tolerance tests, were performed in more than 200 children with HLA-DQB1-conferred susceptibility to T1D and advanced β-cell autoimmunity (characterized by the presence of multiple autoantibodies), showed that the role of insulin resistance in the progression from β-cell autoimmunity to overt T1D is minor, although FPIR was confirmed to be a strong predictior of clinical T1D (Siljander et al., 2013). Several other studies showed no association between the insulin resistance markers and the risk of progression to T1D (Bingley et al., 2008; Winkler et al., 2009). In the work of Gardner et al., the correlation between insulin resistance and anthropometrics were poor at 5 years of age, but strengthened by the age of 8 years (Gardner et al., 2008).

2.4. Role of HLA risk genotypes in pre- and postnatal growth in children developing type 1 diabetes

2.4.1. HLA genotypes and prenatal growth

Since most patients with T1D have particular HLA DQB1 genotypes, it has been proposed that those genotypes could, at least partly, explain the association between increased growth and risk of T1D. The first study aimed at assessing this idea was performed in 2001 by Stene and colleagues. In that study, the association between birth weight and HLA *DQ* genotypes conferring risk for T1D was tested among 969 Norwegian non-diabetic children. The investigators observed that in contrast to the initial assumption, the HLA genotypes conferring risk for T1D diabetes were associated with reduced birth weight. The mean difference in birth weight between subjects with the protective *DQB1*0602/DQB1*0602* genotype (n=31) and subjects with high-risk heterozygous DQ2/DQ8 genotype (n=46) was 354 g (CI 105–604) (Stene et al., 2001). The next study came from Finland where the association of HLA *DRB1* alleles with birth weight was analyzed in 1263 children and an association between birth weight and the HLA *DRB1*13* allele was found. Infants positive for HLA *DRB1*13* (n=319) had higher birth weight than infants negative for this allele (n = 944; median 3690 g *vs.* 3650 g, respectively; p=0.044). There was no association between other HLA *DRB1* alleles and birth weight. However, only such *DRB1* alleles, the frequency of which exceeded 3% in the screening population were analyzed (Aroviita et al., 2004). Subsequently a large study came from Sweden, where the association of T1D risk HLA genotypes with birth weight was analyzed in 16,709 children born to healthy mothers. Birth weight was expressed in relative units (Z-scores) adjusted for gestational age and divided into quartiles. The distribution between the quartiles in children with different HLA genotypes was assessed. The highest quartile was defined as high relative birth weight and the lowest quartile as low relative birth weight. Genotypes conferring risk for T1D were observed to be strongly associated with relative birth weight. Children with the high-risk genotypes, defined as those carrying HLA *DQ2/DQ8*, HLA *DQ8/0604* and HLA *DQ8/X* genotypes (X was not a *DQB1*02*, *0301*, *0302*, *0602*, *0603*, *0604*), had higher relative birth weight (28% vs 24% in the rest of the population; OR=1.20 (CI 1.08–1.33), p=0.0006). The HLA *DQB1*0603* allele, which is protective against T1D, was also associated with high relative birth weight (OR 1.13 (CI 1.02–1.28), p=0.025). No other HLA risk genotype was associated with high or low relative birth weight (Larsson et al., 2005). Those finding confirmed a previous report on an association between *DQB1*0603*-linked HLA *DR13* (Aroviita et al., 2004) and increased relative birth weight, and partially confirmed the finding by Stene et al., where the HLA *DQB1*0602* allele, closely related to *DQB1*0603* was shown to be associated with increased birth weight (Stene et al., 2001). However, in 2006 Stene et al. published a new report, where they performed a population-based case-control study including 471 cases with T1D and 1,369

control subjects. In a logistic regression analysis, no risk conferring HLA genotype contributing to birth weight was identified (Stene et al., 2006). In a recent Finnish study involving 342 children (Järvinen et al., 2008), only the population-specific diabetogenic extended haplotype HLA *A2,Cw1,B56,DR4,DQ8* was associated with high birth weight (p=0.028) in families with a diabetic offspring. Other T1D risk conferring HLA haplotypes showed only nonsignificant tendency for a higher birth weight (Järvinen et al., 2008).

2.4.2. HLA genotypes and environmental interactions

Many studies have analysed the impact of different environmental factors on the relationships between HLA genotypes and birth weight. The study by Taylor et al. showed that the interactions between specific fetal HLA *DQA1* and *DQB1* alleles and maternal smoking can influence birth weight, thus clearly demonstrating that environment is important and may be even a key determinator of the HLA genotype effects on birth weight. The authors compared the mean birth weights of 552 newborn infants in UK typed for HLA *DQ* whose mothers had either smoked or not smoked during pregnancy. Maternal smoking resulted in significant birth weight reduction, but furthermore, the combined effects of maternal smoking and fetal *DQA1*0101* or *DQB1*0501* alleles resulted in reduction of about 200 g in mean birth weight. In contrast, *DQA1*0201* and *DQB1*0201* (*DQ2*) alleles showed a protective effect on smoking-associated birth weight reduction (Taylor et al., 2006).

Other study, emphasizing the importance of environmental effects on genetic background, investigated whether the HLA *DR4* (i.e. the *DR4-DQ8* haplotype) is associated with increased birth weight in a maternal diabetes environment, and whether these effects could persist during early childhood. Birth weight was obtained and the HLA *DR* typing was performed in singleton births from mothers with T1D (n = 1161), and children whose fathers or siblings have T1D (n = 872). In addition, the weights and heights of all those children were measured at the age of 2 and 5 years. In children born by mothers with T1D, the birth weight percentile was positively and independently related to HLA *DR4* alleles ($p < 0.0001$), while in children from non-diabetic mothers, the HLA *DR4* was not associated with an increased birth weight. Interestingly, the high birth weight, but not HLA *DR4*, was associated with the increased weight and BMI at the age of 2 and 5 years ($p < 0.0001$) (Hummel et al., 2007). As a continuation of the study published in 2005, where 16,709 children were tested for HLA alleles conferring risk for T1D (Larsson et al., 2005), Larsson with colleges studied in a part of those newborn infants whether maternal infections during pregnancy have any impact on birth weight. The study showed that the children born by mothers reporting fever or gastroenteritis during pregnancy had an increased risk of high relative birth weight ($p = 0.0003$). More importantly, the effect of the high-risk HLA *DQ2/DQ8* genotype on birth weight was aggravated by infections in more than one trimester (OR= 5.24; $p = 0.003$) indicating again

the interaction of HLA DQ-DR alleles with environmental factors, and their combined effect on growth (Larsson et al., 2007). Last, but not least, in a study investigating 4349 newborn infants typed for HLA alleles conferring risk for T1D, a shorter duration of pregnancy was observed in mothers of children with high-risk HLA genotypes compared to the low-risk groups (271.2 ± 11.6 days vs. 274.6 ± 12.5 days; $p < 0.05$) (Locatelli et al., 2007). However, birth weight was not observed to be related to HLA risk genotypes, similar to some other studies (Aroviita et al., 2004; Stene et al., 2006).

Since the environment plays an important role in HLA-related differences in birth weight, it would be interesting to investigate these associations in countries with low and high incidence of T1D. To our best knowledge, there has been only one study that examined the T1D risk-conferring HLA genotypes effect on birth weight in different countries (Sterner et al., 2011). That study was performed on the basis of populations from Finland, Germany, Sweden, and the United States, i. e. in countries with relatively high T1D incidence (DIAMOND Project Group, 2006). The study involved 1495 children and it did not reveal differences in birth weight between the HLA risk genotypes. There were some unadjusted differences, but most of them disappeared after adjustment for parental characteristics. Children with the *DQ2/DQ2* genotype compared to those with HLA *DQ4/DQ8* were heavier only in the US (p=0.028) and not in the other counties. This difference, however, was mostly explained by parental weight. Regarding length, the children with *DQ2/DQ8* in the US were taller compared to *DQ4/DQ8*-positive infants (p=0.023), but the difference disappeared after adjustment for parental height. Only the Swedish children with *DQ2/DQ8* (p=0.023) and *DQ8/DQ8* (p=0.046) genotype were taller independent of parental height compared to *DQ4/DQ8*. There were no other significant differences in the anthropometric parameters between newborn infants from different countries with various HLA risk genotypes (Sterner et al., 2011).

2.4.3. HLA genotypes and postnatal growth

So far four studies investigating the associations between HLA genotype and postnatal growth has been published. The first study, already mentioned above, did not find any association between *DR4* (or the *DR4-DQ8* haplotype) and weight or BMI at the age of 2 and 5 years (Hummel et al., 2007). A small cohort study from Sweden, where the early postnatal growth data of 58 T1D children and 155 controls matched for HLA were analyzed, reported that children developing diabetes were significantly taller from 6 to 18 months of age than both the non-HLA-matched and the HLA-matched controls, i.e the postnatal growth was independent of the HLA genotypes (Larsson et al., 2008). However, a recent large study from Sweden (Carlsson et al., 2012), setting out to test the hypothesis that the HLA-*DQ* risk genotypes may be associated with an increased BMI, observed that in overall the HLA *DQ* risk was inversely associated with BMI ($p < 0.0008$). BMI was compared between 2278 children with

newly diagnosed T1D and 2000 HLA-matched controls. Among the patients carrying the HLA genotypes associated with the highest risk for T1D (*DQA1*0501-DQB1*0201/DQA1*X-DQB1*0302* and *DQA1*X-DQB1*0302/Z*) the proportion of overweight or obese subjects was not higher than among the controls. Moreover, the proportion of subjects with the highest risk genotype decreased along with increasing BMI. *Vice versa,* the lower risk *DQ* genotype (*DQA1*0501-DQB1*0201/ DQA1*0501-DQB1*0201*) was associated with an increased proportion of patients who were overweight or obese. The odds ratio in patients with this genotype of being obese as compared to controls was 1.80 (CI 1.21–2.61; $p<0.006$). The highest proportion of overweight patients were seen within the group of subjects with the HLA *DQA1*0501-DQB1*0201* haplotype when T1D was diagnosed between 5 and 9 years of age (Carlsson et al., 2012).

The TEDDY Study Group has performed a prospective study comprising 5969 children with the HLA-conferred susceptibility to T1D from Finland, Sweden, Germany and USA, with repeated measurements of weight and height between the age of 2 to 4 years. According to their HLA genotypes, the children were divided into four groups: those with the *DQ2/DQ2*, *DQ8/DQ8*, *DQ8/X*, or *DQ2/DQ8* genotypes. Multivariable logistic regression models were used to estimate the risk of being overweight or obese by comparing the proportion of those subjects with the HLA genotypes *DQ2/DQ2*, *DQ8/DQ8* and *DQ8/X* against the proportion of obese or overweight subjects with the *DQ2/DQ8* genotype. The average BMI was comparable across specific HLA genotypes at every age point. The proportion of overweight was not significantly different by age, HLA genotypes, or country of residence, but a decreased frequency of obesity was observed among those carrying the *DQ2/DQ8* genotype (p=0.0315). Simultaneously, *DQ2/DQ2* was independently associated with a significantly higher risk of obesity at age 4 years (n=3252, odds ratio=2.41, CI 1.21–4.80) (Yang et al., 2014). Both findings are, in general, close to those in the study by Carlsson et al (Carlsson et al., 2012). Last but not least, within each country of the TEDDY study, the prevalence of overweight and obesity was lower than the country-specific data from similar study cohorts, indicating that all children at increased genetic risk for T1D were leaner compared to the general population (Yang et al., 2014). A recent study on adults with a slowly progressive form of T1D demonstrated that in the lower risk HLA group (all T1D risk conferring genotypes excluding HLA *DQ2/DQ8* heterozygotes) the median BMI of 27.6 kg/m^2 was higher compared to 24.7 kg/m^2 in the high risk HLA group (HLA *DQ2/DQ8* heterozygotes) (p=0.03). In the lower HLA risk group, 44% of individuals were obese (BMI $\geq$ 30 kg/m^2) compared to 17% in the high HLA risk group (p =0.04) (Fourolanos et al., 2014).

2.4.4. Conclusions: HLA genotypes and pre- and postnatal growth

The specific role of HLA genotypes conferring susceptibility to T1D in the association between T1D and an increased pre- and postnatal growth is unclear. At the moment, there is a relatively limited number of investigations analyzing these associations (Aroviita et al., 2004; Stene et al., Stene et al., 2006; Taylor et al., 2006; Larsson et al., 2005; Larsson et al., 2007; Sterner et al., 2011; Hummel et al., 2007; Carlsson et al., 2012; Larsson et al., 2008; Järvinen et al., 2008; Yang et al., 2014; Locatelli et al., 2007). Regarding birth weight, only particular genotypes or alleles, such as the *DQB1*0602/DQB1*0602* genotype (Stene et al., 2001), the *DQB1*0603*-linked HLA *DR13* allele (Aroviita et al., 2004), or the *HLA-DQB1*0603* allele **(**Larsson et al., 2005), all conferring protection against T1D, were shown to be associated with increased birth weight. Other studies have demonstrated that most of the HLA genotypes conferring high risk for T1D (HLA *DQ2/DQ8*, *DQ8/0604* and *DQ8/X*) are associated with increased birth weight (Larsson et al., 2005; partially in Hummel et al., 2007), whereas some other studies have failed to observe such an association (Aroviita et al., 2004; Stene et al., 2001; Stene et al., 2006; Locatelli et al., 2007; Sterner et al., 2011).

Studies on postnatal growth have shown an association between HLA risk genotypes and BMI. Two large studies demonstrated very similar results. The HLA *DQ2/DQ8* genotype linked to the highest T1D risk was associated with a decreased BMI, whereas the HLA DQ2/DQ2 genotype linked to a lower risk of T1D was associated with increased BMI (Carlsson et al., 2012; Yang et al., 2014). But there have been also studies where no significant associations were seen between HLA genotypes and BMI (Hummel et al., 2007; Larsson et al., 2008). Recently, Fourlanos with colleagues proposed that the increased BMI in adults simply promotes the development of T1D in subjects carrying lower-risk HLA risk genotypes by increasing the penetrance of lower risk HLA genes, increasing thus the proportion of lower risk HLA genes among subjects with higher BMI (Fourolanos et al., 2014). The presence of HLA genotype-related differences in BMI in children without any sings of T1D, as reported by Yang et al in 2014, speaks against that suggestion.

Although the specific role of HLA genotypes conferring risk for T1D in pre- and postnatal growth remains controversial, it is obvious that HLA genotypes may have some distinct impact. The variable and conflicting results may be explained by the differences in the incidence of T1D in the background population and in the study cohort sizes, but also by different methodologies applied in these studies. Moreover, the possible environmental impact on HLA-related growth should also be taken into account. As clearly shown in the studies on maternal smoking (Taylor et al., 2006), maternal diabetic environment (Hummel et al., 2007), and maternal infections (Larsson et al., 2007), the intrauterine environment effects on birth weight are significantly modified by the HLA risk genotypes. Many investigators hypothesize that the difference in birth weight might be due to the variation in the interactions between the developing fetal

immune system and infections defined by differences in HLA genotypes (Järvinen et al., 2008; Larsson et al., 2005; Larsson et al., 2007). The same suggestion could be applied on postnatal growth, since various HLA genotypes may influence the microbial colonization of the newborn infant, probably due to differences in the interactions of microbiota with the immune system (Palma et al., 2010; Olivares et al., 2014; Palma et al., 2012; Sánchez et al., 2011).

As environmental factors have been considered to be the main reason for the increase in the incidence of T1D (Knip et al., 2005), the potential impact of the HLA genotypes on growth could be defined more closley in studies aimed at testing the association between HLA risk genotypes and growth, conducted in countries with a contrasting difference in the rate of T1D. However, only two such studies have been performed so far, but both in countries with a relatively high incidence of T1D (Finland, Sweden, Germany, and USA) (Sterner et al., 2011; Yang et al., 2014).

2.5. Role of the IGF-I/IGFBP-3 system in the pathogenesis of type 1 diabetes

2.5.1. Physiology of the GH-IGF-I/IGBP-3 system

2.5.1.1. Physiological role of IGF-I

Growth hormone (GH) is the main regulator of postnatal growth (Ohlsson et al., 1998). The growth-promoting effect of GH is, to a large extent, mediated through stimulation of the expression of insulin-like growth factor I (IGF-I) in liver and in other tissues. The liver is the principal source of IGF-I producing up to 80% of circulating IGF-I (Sjogren et al., 1999**)** while the other 20–25% are synthesized locally in various tissues (Ohlsson et al., 2000). GH, insulin, and the nutritional status are the three main factors regulating the hepatic IGF-I biosynthesis, GH being the main regulator (LeRoith et al., 2001). In extrahepatic tissues, the IGF-I gene expression is regulated by multiple additional factors. For example, parathyroid hormone (PTH) (Pfeilschifter et al., 1995) and estradiol (Ernst and Rodan, 1991) increase the IGF-I mRNA levels in osteoblasts, angiotensin II regulates the IGF-I expression in the cardiovascular system (Brink et al., 1999), and thyroid-stimulating hormone (TSH) induces the IGF-I expression in thyroid cells (Hofbauer et al., 1995).

IGF-I is a multipotent growth factor controlling cell proliferation, differentiation, apoptosis, tissue growth and have many organ-specific functions (Kooijman, 2006; Frystyk et al., 2010; Dupont and Le Roith, 2001; Kofidis et al., 2004) such as bone formation, protein synthesis, glucose uptake in muscle, and neuronal survival (LeRoith, 1997). One of the major effects of IGF-I is to promote cell survival (Baxter, 2000). The biological functions of IGF-1 are mediated through its binding to the type I IGF receptor (IGF-1R), which is a transmembrane tyrosine kinase receptor (Siddle et al., 2001; Baserga 2003) ubiquitously expressed virtually in all human tissues (LeRoith et al., 1995).

2.5.1.2. Physiological role of IGFBP-3 and IGF-I/IGFBP-3 interaction

The vast majority of circulating IGF-I is bound to a family of six structurally and evolutionally related insulin growth factors binding proteins (labeled as IGFBP 1–6), more than 95 percent being bound to IGFBP-3 (Jones et al., 1995; LeRoith, **1997**) which protects IGF-I from destruction prolonging thus its circulating half-life (Butler et al., 1996). More precisely, the majority of IGF-I is bound to a large 150-kDa ternary complex that includes IGFBP-3 and the acid-labile subunit (ALS) (Butler et al., 1996). The circulating concentrations of IGFBP-3 are predominantly regulated post-translationally through stabilization by its GH-dependent binding partners IGF-I and ALS. Thus, at the serum level, IGFBP-3 is GH-dependent (Baxter and Martin 1986), but is GH-independent at the transcriptional level (Olivecrona et al., 1999). IGFBP-3 is produced primarily in the liver, and also locally by most tissues (LeRoith, 1997).

The fact that the circulating IGF-I concentration in adults is approximately 100 nM and the IGF receptors mostly approach saturation at IGF-I concentrations of 5 nM or lower implies that the regulation of circulating IGF bioavailability is a key function of the IGFBPs (Baxter, 2000), probably mostly through controlling the access of IGF-I to its receptor (Kim, 2013; Zapf, 1995). This role of IGFBP-3 is well illustrated by studies that investigated the actions of IGF-I in diabetic nephropathy (LeRoith, 2003). GH and IGF-I were shown to affect the diabetic kidney by increasing the renal blood flow and glomerular filtration, resulting in an enlarged kidney typical for patients with recent onset of diabetes (Flyvbjerg, 2000). In animal studies such kidney enlargement was associated with an increased IGF-I concentration in renal tissue, but a significant reduction in the overall IGF-I mRNA expression. This resulted in the suggestion that IGF-I is trapped in the kidney from the peripheral circulation (LeRoith, 2003). Subsequent studies demonstrated that some IGFBPs are expressed in the kidney more intensively. This led to the understanding that IGFBPs play an important role in the presentation of IGF-I to its receptor (Landau et al., 1995). Furthermore, a huge complexity of the regulatory actions of IGFBPs, including stimulatory, as well as inhibitory effects, on IGF function became more evident. The exact mechanisms are still poorly understood, but there is some evidence that the binding of IGF-I to its receptors is modulated by IGFBP modifications, such as phosphorylation, glycosylation, and proteolysis (Baxter, 2000).

During the past decade a multiple biological functions of IGFBP-3, that are considered to be IGF-I independent, became evident (Baxter, 2013; Kim, 2013). As first demonstrated in mouse studies, IGFBP-3 inhibits fibroblast growth factor (FGF)-stimulated DNA synthesis independently of IGF-I and the effect is reversed by IGF-I (Valentinis et al., 1995; Villaudy et al., 1991; Zadeh and Binoux, 1997). An independent apoptotic effect of IGFBP-3 was demonstrated also in other tissues (Buckbinder et al., 1995; Rechler and Clemmons, 1998, Gill et al., 1997). An IGF-independent IGFBP-3 action was observed in the IGF-unresponsive breast cancer cell line where IGFBP-3 markedly enhanced

the apoptotic effect of the ceramide analog C2 (Gill et al., 1997). Other studies revealed that IGFBP-3 is one of the factors preventing cell replication and promoting cell death when genomic integrity is compromised (Kinzler and Vogelstein, 1997), and contributes to the repair of damaged DNA. At least some actions of IGFBP-3 are assumed to be mediated through signaling receptors located on the plasma membrane of the target cells (Baxter, 2000) but the direct transport of IGFBP-3 to the cell nucleus (Schedlich et al., 1998) and IGFBP-3 interaction with the retinoid X receptor RXRα, belonging to the family of nuclear hormone receptors, have also been demonstrated (Liu et al., 2000).

2.5.1.3. Total and free IGF-I

The measurement of total IGF-I concentration in serum is common clinical practice (Shalet et al., 1998; Patel et al., 2000). One of the reasons for using the total IGF-I is a lack of reliable assays for the measurement of free IGF-I (Bang et al., 2001). However, the judgments cannot rely only on the determination of serum total IGF-I, since its level is related to many modulatory factors (Frystyk, 2004). For example, in a considerable proportion of patients diagnosed as GH-deficient, and in some patients with acromegaly, the levels of total IGF-I remains normal (Juul et al., 1997; Peacey and Shalet, 2001; Hoffman et al., 1994). Furthermore, in some pathophysiological conditions, such as T1D, obesity, and chronic renal failure (Thissen et al., 1994; Feld et al., 1996; Scacchi et al., 1999; Bereket et al., 1999) the level of serum total IGF-I is normal, whereas the GH level is high, reflecting some degree of disintegration of the GH/IGF-I axis caused, in part, by an altered GH sensitivity (Frystyk, 2004). The interpretation of any assay, either for free or for total IGF-I, is also complicated due to the fact that IGFBPs are able to alter IGF-I bioavailability and activity even without changing the extractable free concentrations of IGF-I (Frystyk, 2004).

The IGF-I/IGFBP-3 molar ratio is one of the proposed markers of bioavailable IGF-I (Juul et al., 1995) and has been widely used in many studies (Frystyk, 2004). The usefulness of this ratio, roughly representing the free, also called bioavailable, IGF-I, has been demonstrated in many studies (Cortizo et a., 1998; Smyczynska et al., 2013; Lee et al., 2013, Jung et al., 2014). However, it has been actually questioned whether the IGF-I/IGFBP-3 molar ratio is a reliable marker of a free bioactive IGF-I as this has not been validated neither proven experimentally (Baxter, 2014). In conditions with primary abnormalities in GH secretion, the IGF-I/IGFBP-3 ratio changes in the same direction as free IGF-I, but with secondary abnormalities in GH secretion, the ratio shows relatively week association with free IGF-I. Therefore, the usefulness of the IGF-I/IGFBP-3 molar ratio depends on the clinical situation (Frystyk, 2004).

2.5.2. Evidence of IGF-I involvement into the pathogenesis of type 1 diabetes

As the GH - IGF-I axis is the major determinant of childhood growth, the accelerated growth of children later developing T1D (Cardwell et al., 2010; DiLiberti et al., 2002; Knerr et al., 2005; Ljungkrantz et al., 2008) suggests a possible role of IGF-I in the pathogenesis of T1D. This assumption is supported by the growing number of evidence indicating an important role of IGF-I in the regulation of islet β-cell growth, survival and metabolism, mostly providing protection against T1D (Smith et al., 1991; Hayakawa et al., 1996; Agudo et al., 2008; Castrillo et al., 2000; George et al., 2002).

The ability of pancreas to generate new β-cells in response to an increased metabolic demand or after injury has been described in an experimental diabetes mouse model (Dor et al., 2004). Moreover, lineage trace analysis provided a compelling evidence for β-cell replication as a major pathway for the renewal of adult β-cells in mice, both in normal conditions as well as after partial pancreatectomy (Dor et al., 2004). Importantly, expansion of β-cell mass after birth has also been demonstrated in humans (Meier et al., 2008). The presence of IGF-I receptors in islet cells was shown already in 1987 (Van Schravendijk et al., 1987). The possible role of IGF-I in the regeneration of β-cells first came from studies demonstrating an increased IGF-I expression in the focal areas of β-cell regeneration in rats and dogs with partial pancreatectomy (Smith et al., 1991; Hayakawa et al., 1996; Calvo et al., 1997). IGF-I is able to increase the replication and proliferation of β-cells (Agudo et al., 2008; Lingohr et al., 2002) and to protect β-cells from apoptosis (Castrillo et al., 2000; Liu et al., 2002). Interestingly, in transgenic mouse the liver-specific IGF-I gene deficiency caused GH hypersecretion, insulin resistance and accelerated diabetes, whereas the pancreatic-specific IGF-I gene deficiency led to a pro-islet environment where normal islet growth was stimulated (Lu et al., 2004). However, in a mouse model with enriched β-cell-specific IGF-I overexpression, the lack of islet hyperplasia was observed in parallel with significant resistance from streptozotocin-induced β-cell damage and diabetes (Robertson et al., 2008). The inactivation of either the IGF-1 receptor or IGF-I did not alter the development of endocrine pancreas in animals (Kido et al., 2002; Kulkarni et al., 2002). Those findings led to the concept that IGF-I does not promote β-cell growth or replication, but inhibits β-cell apoptosis (Robertson et al., 2008).

It has also been demonstrated that IGF-I can be directly involved in the regulation of immune tolerance. Animal studies showed that the expression of IGF-I in the pancreatic islets of transgenic mice protects islets from lymphocytic infiltration, reduces cytotoxicity and insulitis induced by low doses of streptozotocin (STZ) (George et al., 2002), IGF-I overexpression in the liver suppresses the progression of autoimmune diabetes and is associated with a decreased islet inflammation (Anguela et al., 2013). In transgenic mice, the expression of interferon (IFN)-β in β-cells led to increased islet lymphocytic infiltration compared to the controls when treated with low doses of STZ

(Casellas et al., 2006). The IGF-I expression in IFN-β–expressing β-cells in double-transgenic mice decreased islet infiltration and apoptotic β-cell death and in this way protected them from the development of diabetes (Casellas et al., 2006). Human *in vitro* studies showed that the adenoviral gene transfer of IGF-I to human islets blocked interleukin-1β (IL-1 β) induced impairment of β-cell function and apoptosis, and suppressed the recruitment of lymphocytes to the islets (Giannoukakis et al., 2000).

The role of circulating IGF-I in paracrine and autocrine regulation of pancreatic endocrine function has not been fully understood. Nevertheless, it has been shown that the systemic administration of recombinant IGF-I to NOD mice reduced the severity of insulitis (Mabley et al., 1997; Bergerot et al., 1995; Kaino et al., 1996), reduced the T-cell invasion of islets and delayed the onset and incidence of diabetes (Bergerot et al., 1995), implying an important role of circulating IGF-I in the development of diabetes. There have also been studies in NOD mice demonstrating that treatment with IGF-I protects islets against the cytokine-mediated inhibition of insulin secretion and cell death by apoptosis (Chen et al., 2004).

2.5.3. Role of IGF-I/IGFBP-3 in glucose homeostasis and insulin resistance

Increasing evidence indicates that the GH/IGF-I axis plays an important role in glucose homeostasis. While insulin is a key short-term regulator of glucose homeostasis, IGF-I is involved in the longer term glucose homeostasis, most likely by modulating insulin sensitivity (Murphy, 2003). IGF-I has significant structural homology with insulin and shares downstream the signaling pathways with insulin. Similar to insulin, IGF-I can promote the glucose and fatty acid uptake in peripheral tissues (Murphy, 2003; Holt, 2003). A substantial part of the non-hepatic circulating IGF-I and IGFBPs originates from adipose tissue (Wabitsch et al., 2000). Experimental animal studies and human data demonstrate that the IGF-I gene mutation results in a state of insulin resistance that improves with IGF-I therapy (Yakar et al., 2001; Camacho-Hubner et al., 1999). Administration of recombinant IGF-I decreases serum glucose levels and improves insulin sensitivity in healthy individuals as well as in subjects with diabetes (Boulware et al., 1994; Moses et al., 1996).

It has been hypothesized that the interindividual heterogeneity in endogenous IGF-I and IGFBP levels may influence the risk of developing T2D (Rajpathak et al., 2008). Polymorphism in the promoter region of the IGF-I gene, present in 12% of Caucasians, was shown to be associated with low IGF-I levels and an increased relative risk for T2D (RR 1.7 (CI 1.1–2.7) (Vaessen, et al., 2001). Many studies have shown that in obese individuals the total IGF-I concentration is normal, whereas free IGF-I is significantly elevated (Frystyk et al., 1995; Nam et al., 1997). Unfortunately, there are only few studies investigating the association of serum IGF-I level with the risk of developing

diabetes. Small-cohort studies have generated contradictory results (Rajpathak et al., 2012; Head, et al., 2001; Frystyk et al., 1999). A large cross-sectional study in 922 adults older than 65 years, did not find any significant association between total IGF-I and increased prevalence of glucose intolerance or diabetes (Rajpathak et al., 2008). In a prospective study including 615 subjects aged 45–65 years a higher IGF-I concentration was associated with a reduced risk of developing impaired glucose tolerance or diabetes during follow-up for 4 years (Sandhu et al., 2002). In another prospective nested case-control study (742 subjects matched 1:1 to control subjects) diabetes was not associated with baseline total IGF-I levels. However, free IGF-I was associated with diabetes. Interestingly, in subjects with high insulin levels, free IGF-I was inversely correlated with diabetes development, whereas in subjects with low insulin level, a positive correlation was seen (Rajpathak et al., 2012).

As evident from the studies described above, the precise role of IGF-I in maintaining normal glucose homeostasis and insulin sensitivity is not well defined. It is, however, clear that IGF-I is necessary for normal insulin sensitivity (Clemmons, 2004). Studies have raised the possibility that the relation between IGF-I and the risk of diabetes may be bimodal. Among patients with a pre-existing insulin resistance, the insulin-like activity of IGF-I on glucose uptake may attenuate the insulin resistance (Rajpathak et al., 2012). Laboratory studies have confirmed this assumption. In parallel with insulin resistance, the insulin/IGF hybrid receptor expression is upregulated, thus facilitating the attenuating effect of IGF-I on insulin resistance (Federici et al., 1998; Federici et al., 1997). On the other hand, prior to the development of insulin resistance, the adverse metabolic effects of IGF-I may predominate. For example, IGF-I accelerated the preadipocyte differentiation and proliferation (Boney et al., 1998; Kawai and Rosen, 2010). Transgenic mouse models have shown that the reduction of IGF-I receptor expression in adipose tissue leads to a reduction in fat mass (Boney et al., 1998; Holzenberger et al., 2001). Accordingly, in non-obese subjects the IGF-I signaling might promote adiposity and increase the risk of diabetes (Rajpathak et al., 2012).

2.5.4. IGF-I independent functions of IGFBP-3

IGFBP-3 has multiple biological functions that are considered to be IGF-I independent (Baxter, 2013; Kim, 2013). The possible role of those actions on glucose homeostasis and the pathogenesis of diabetes (either type 1 or type 2) is not clear, but many studies imply possible causal associations. First of all, IGFBP-3 binds to and co-localizes with retinoid X receptor in the cell nucleus (Liu et al., 2000) that is an important transcription factor in glucose homeostasis (Rajpathak et al., 2012). Retinoid X receptor is a binding partner for the peroxisome proliferator-activated receptor-γ (PPARγ), a nuclear protein that is involved in the transcriptional regulation of enzymes involved in glucose and lipid metabolism (Juge-Aubry et al., 1997). Moreover, PPAR-γ plays a role in

the regulation of genes that control the differentiation of preadipocytes (Morrison and Farmer, 2000) and insulin sensitivity (Lehmann et al., 1995; Steppan et al., 2001). In transgenic mice, the overexpression of IGFBP-3 results in fasting hyperglycemia, glucose intolerance and insulin resistance (Silha et al., 2002). It has also been shown that IGFBP-3 leads to insulin resistance in adipocytes (Chan et al., 2005; Kim et al., 2007) and inhibits the differentiation of adipocytes (Penkov et al., 2013). In contrast, in a study of IGFBP-3 knockout mice the fasting hyperglycemia and hyperinsulinemia suggesting insulin resistance were shown (Yamada et al., 2010). Moreover, a recent study reported that IGFBP-3 can inhibit cytokine-induced insulin resistance in an IGF-independent manner (Mohanraj et al., 2013). There are also some human studies exploring the association of IGFBPs with insulin resistance and diabetes. Most of them have shown decreased IGFBP-I level in obese persons, but with no significant association between IGFBP-3 and the severity of obesity or diabetes (Rajpathak et al., 2008; Sandhu et al., 2002). However, in a small study involving 80 subjects, an increased IGFBP-3 level was seen in obese subjects compared to lean subjects (Frystyk et al., 1999). A large prospective case-control study by Rajpathak with colleagues showed that the baseline serum IGFBP-3 level was positively associated with diabetes risk (Rajpathak et al., 2012). Taking into account the complicated regulatory system of IGFBP-3, it has been suggested that in disease state IGFBP-3 may have different functions from those seen in healthy subjects (Kim, 2013). As mentioned before, IGF-I is likely to have contrasting actions in the pathogenesis of diabetes (Rajpathak et al., 2012). The role of the entire IGF-I/IGFBP-3 system in the development of insulin resistance and diabetes may be bimodal and strongly depend on the particular clinical condition.

According to the acceleration hypothesis proposed by Wilkin, insulin resistance and obesity play an important role also in the pathogenesis of T1D similar to T2D (Wilkin, 2001). IGF-I and IGBP-3, both involved in glucose homeostasis, insulin resistance, β-cell survival, and inhibition of islet autoimmune damage (George et al., 2002; Anguela et al., 2013; Casellas et al., 2006; Murphy, 2003; Holt, 2003; Robertson et al., 2008), could have an important role in the context of the acceleration hypothesis. There are plenty of studies investigating the role of IGF-I/IFBP-3 system in patients with established T1D (reviewed by Murphy, 2003; Holt, 2003; Frystyk, 2004; Frystyk, 2014). Despite of the large number of studies indicating an important role of the IGF-I/IGFBP-3 system in the development of T1D (Smith et al., 1991; Hayakawa et al., 1996; Anguela et al., 2013; Agudo et al., 2008; Castrillo et al., 2000; George et al., 2002), there is only one study on circulating IGF-I and IGFBP-3 concentrations in children with early signs of β-cell autoimmunity i.e. in those with diabetes-associated autoantibodies (Beyerlein et al., 2014). In that study, the differences in the serum concentrations of IGF-I and IGFBP-3 at 0, 9, and 24 months measured in 1011 subject were not associated with the risk of development of β-cell autoimmunity. The possible combined impact of the IGF-I/IGFBP-3 system and HLA genotypes on growth has never been studied before.

3. AIMS OF THE STUDY

1. To investigate the associations between HLA genotypes conferring risk for T1D and birth weight, and search for possible differences in the strength of these associations among populations with contrasting incidence of T1D.
2. To investigate the potential contribution of T1D HLA risk genotypes to the observed association between early postnatal growth and risk for T1D in two countries with contrasting disease rate.
3. To investigate the role of IGF-I and IGFBP-3 in the development of β-cell autoimmunity (i.e. appearance of autoantibodies) and their potential impact on early postnatal growth in children with HLA-conferred susceptibility to T1D.

4. SUBJECTS AND METHODS

4.1. Study population

All study subjects were derived from the international DIABÌMMUNE study, aimed at testing the hygiene hypothesis in three neighboring countries with conspicuous contrasts in the standard of living and hygiene: Estonia, Finland and Russian Karelia. For that purpose the intention was to test between September 2008 and August 2010 about 2500 newborn infants in each country for HLA-conferred susceptibility to autoimmunity including T1D and to recruit at least 300 predisposed newborn infants for prospective observation up to the age of 36 months. The local Ethics committees in all three countries approved the study. The parents of the participating subjects gave written informed consent for participation in the study, which was carried out in accordance with the Principles of the Declaration of Helsinki.

For the first study data from 8368 newborns tested for HLA DR-DQ alleles conferring risk for T1D was used, including 3103 from Espoo, Finland, 2713 from Tartu and Põlva, Estonia, and 2553 from Petrozavodsk, Karelian Republic of Russia. A questionnaire asking about the course of pregnancy, gestational age, delivery, child condition at birth, birth weight and length was completed by the study nurses during the first 3 days after delivery. At the same time, the parents completed a questionnaire on family history. For the subsequent analysis, infants born by mothers with any type of diabetes, from multiple pregnancies, or born before 35 week of gestation were excluded. Late-preterm infants (35–36 gestational weeks) were included into the analysis in order to increase the statistical power of the study. Overall, the data of 7931 newborns, 2931 from Finland, 2521 from Estonia and 2479 from Russia were included in the analyses.

For the second study the data of 496 newborn infants with HLA-conferred susceptibility to T1D, participating in the follow-up visits at the ages of 3, 6, 12, 18, and 24 months (± 1 month), was used. In total, there were 235 infants from Estonia and 261 from Finland. In addition to those 496 subjects, 191 children with a neutral or protective HLA genotype were randomly selected from the initial Estonian DIABIMMUNE cohort tested for HLA risk DR-DQ alleles. They formed the control group and their linear growth and weight gain data were collected from the case records of general practitioners involved in the primary care of those children. Data on parental heights were collected for all children involved in the study. Children born from multiple pregnancies or gestational age of less than 32 weeks were excluded. However, children born by mothers with any type of diabetes were included (six mothers with T1D or T2D, and 41 mothers with gestational diabetes or impaired glucose tolerance). Such a decision was made since in Estonia only risk groups are screened for gestational diabetes, which means that up to 50% of the cases remain undiagnosed (Wilcox et al., 1996).

In the third study subjects were derived from 258 Estonian and 305 Finnish children with HLA-conferred susceptibility to T1D as a part of the DIABIMMUNE study monitored for the development of diabetes-associated autoantibodies from birth up to the age of 36 months. We included all 17 autoantibody- positive cases from Estonia (four with multiple autoantibodies including one case who developed T1D by the age of 2.4 years) and all 23 autoantibody-positive cases from Finland (eight with multiple antibodies including three cases who developed T1D by the age of 2.6, 3.2, and 3.7 years). In addition, for every case we selected from the same DIABIMMUNE cohort two controls with no diabetes-associated autoantibodies matched for sex, country of origin, and as close as possible for HLA genotype and date of birth. In this way 80 controls were selected for 40 autoantibody-positive subjects.

4.2. Methods

4.2.1. HLA genotyping

HLA genotyping was performed with a PCR-based lanthanide-labeled hybridization method using time-resolved fluorometry for detection as described before (Mikk et al., 2014). The newborn infants were initially analyzed for the presence of HLA *DQB1*02*, *DQB1*0301*, *DQB1*0302* and *DQB1*0602/3* alleles using a homogeneous screening assay (Kiviniemi et al., 2007). Depending on the initial result, additional analyses included low resolution DQB1 full-house typing, DQA1 typing with three allele-specific probes and DR4 subtyping (Mikk et al., 2014) Samples positive for the *DQA1*05-DQB1*02* combination (the *DR3-DQ2* haplotype) and/or those with *DQB1*0302/4* without the presence of *DRB1*0403/6* (*DR4-DQ8*) were selected as risk-conferring genotypes if no protective haplotype (i.e. *DQB1*0602/3*, *DQB1*0503* or the combinations of *DQA1*05-DQB1*0301* or *DQA1*0201-DQB1*0303*) was present. The original purpose of this screening was to identify children with increased risk for T1D for follow-up in the DIABIMMUNE study. Therefore, if the initial HLA analyses showed definitely protective HLA alleles, no further analysis of such alleles was performed. For example, after the identification of *HLA DQB1*0602/3*, further analysis to define whether the allele was DQB1*0602 or DQB1*0603 was not performed. Similarly, if the original analysis did not find a potential risk genotype no further analyses were performed.

4.2.2. Assigning study subjects to the risk-groups according to HLA genotypes.

In the first two studies, where also children with neutral and/or protective alleles were included, subjects were divided into four groups based on their HLA-associated risk for T1D. The subjects with the combination of two susceptibility

associated haplotypes, i.e. the *DR3-DQ2/DR4-DQ8* genotype were categorized as the group with very high risk for T1D, those carrying the *DR4-DQ8/X* genotype (X=non-protective allele or haplotype) as a group with high risk and those with the *DR3-DQ2/X* genotype as a group with moderate risk for T1D. All other allele combinations were considered as neutral or protective for T1D and subjects, carrying those alleles categorized as the group neutral/protective against T1D (Ilonen, 2004) (Table 1).

In the third study, where children with neutral and/or protective alleles were not included all subjects were divided into three groups according to Hekkala et al. (Hekkala et al., 2011). The subjects with the *DR3-DQ2/DR4-DQ8* genotype were categorized as the high-risk group, those carrying the *DR4-DQ8/X* genotype (X= a non-protective allele, but not *DR3-DQ2*) as a group with moderate risk, and those with the *DR3-DQ2/Y* genotype (Y= a non-protective allele, but not *DR4-DQ8*) as a group with low, though increased, risk for T1D.

4.2.3. Assessment of anthropometric parameters

For the analysis of the association between HLA DR-DQ risk alleles and birth weight, relative birth weight was calculated and expressed as a standard deviation score (SDS): SDS=subjects birth weight minus average birth weight [adjusted for sex, gestational age (in days) and country of origin] divided by the standard deviation (SD) that was calculated for every gestational week, sex and country of origin using the study population internal data.

Table 1. HLA risk classes for T1D according to their genotype

Diabetes risk	HLA DQB1		HLA DRB1	
	Haplo 1	Haplo 2	Haplo 1	Haplo 2
Very high: *DR3-DQ2/DR4-DQ8*	*0302/4	*02[a]	*0401/2/4/5/7/8	
High: *DR4-DQ8/X*	*0302/4	X	*0401/2/4/5/7/8	
Moderate: *DR3-DQ2/X*	*02[a]	Y		
Neutral or protective	all other	all other		

X is not *DQB1*02 [a],*0503, *0602,*0603*, and not a combination of *DQB1*0303/DQA1*0201* or *DQB1*0301/DQA1*05,* but homozygosity is possible
Y is not *DQB1*0302,*0503, *0602,*0603*, and not a combination of *DQB1*0303/DQA1*0201* or *DQB1*0301/DQA1*05,* but homozygosity is possible
[a]DQB1*02/DQA1*0501

For the analysis of early postnatal growth at each follow-up visit length was measured with a length board and weight with a regularly calibrated electronic

scale. For all subjects, the data of measurements at the ages of 3, 6, 12, 18, and 24 months were expressed as standard deviation scores (SDS), calculated based on the WHO growth reference data for healthy boys and girls up to the age of 19 years (WHO Multicentre Growth Reference Study Group, 2006). Since every follow-up visit was performed within ± 1 month from the predefined time points, the SDS was calculated using the subject's actual age in days based on the corresponding WHO reference data for that age. For each risk-group for T1D and each time point the mean length and weight SDS were calculated. During the 24-month follow-up period, incomplete data sets of growth (i.e. missed at least one growth measurement at some time point) were seen in 12 subjects in the moderate risk group (6% of all children in that group), in eight children in the high-risk group (3%), in one child in the very high risk group (2%), and in 51 children in the neutral/control group (27%). As there are no WHO reference data to calculate birth weight corrected for gestational age, birth weights were expressed only as mean for every risk group.

4.2.4. Diabetes-associated autoantibodies

Insulin autoantibodies (IAA), and antibodies to glutamic acid decarboxylase (GADA), insulinoma-associated-2 (IA-2A), and zinc transporter 8 antibody (ZnT8A) were analyzed with specific radiobinding assays as described by Knip et al. (Knip et al., 2010). The cut-off level was 2.80 relative units (RU) for IAA, 5.36 RU for GADA, 0.78 RU for IA-2A, and 0.61 RU for ZnT8A. All samples obtained from cord blood (0 month) and at the follow-up visits at the age of 3, 6, 12, 18, 24, and 36 months were analyzed for those four biochemical autoantibodies. Islet cell antibodies (ICA) were analyzed with indirect immunofluorescence on human blood group 0 donor pancreas from all samples from any child testing positive for one or more of the biochemical autoantibodies. The detection limit was 2.5 Juvenile Diabetes Foundation units (JDFU).

4.2.5. IGF-I and IGFBP-3 measurements

Plasma concentrations of IGF-I and IGFBP-3 were measured with a solid-phase, enzyme-labeled chemiluminescent immunometric assay with the IMMULITE 2000 analyzer (Diagnostic Products, Los Angeles, CA) according to the manufacturer's protocols. The detection limit for IGF-I was 25 μg/L. According to Elmlinger et al. (Elmlinger et al., 2004), the intra-assay and inter-assay coefficients of variability (CV) for that method are less than 4% and 8%, respectively, for IGF-I and less than 4.5% and 7%, respectively, for IGFBP-3. In autoantibody-positive subjects, plasma IGF-I and IGFBP-3 concentrations were measured from samples obtained at the age when seroconversion to positivity for at least one autoantibody was detected (T1), and at the closest time-points before (T0) and after seroconversion (T2). For children with multiple autoantibodies, the IGF-I and IFGBP-3 concentrations were measured from

all available time points. In controls (autoantibody-negative children) IGF-I and IFGBP-3 concentrations were analyzed from samples taken at the same time points as in the corresponding case children. The number of subjects studied at different time points is presented in Table 2.

Table 2. Numbers of IGF-I and IGFBP-3 measurements at various ages in subjects with different HLA-related risk for T1D.

Age in months	HLA risk-groups						Number of subjects with seroconversion
	Low risk (n=45)		Moderate risk (n=57)		High risk (n=18)		
	Aab+ (n=15)	Aab- (n=30)	Aab+ (n=19)	Aab- (n=38)	Aab+ (n=6)	Aab- (n=12)	
0 (n=5)	1	3	0	1	0	0	0
3 (n=19)	3	7	2	6	1	0	1
6 (n=39)	6	11	5	11	2	4	4
12 (n=67)	9	16	12	21	3	6	8
18 (n=98)	12	18	16	34	6	12	12
24 (n=79)	9	15	15	28	4	8	3
36 (n=54)	5	7	10	23	3	6	11

Aab-, Aab+ − Autoantibody negative and Autoantibody positive, respectively.

4.2.6. Statistical analysis

The data were analyzed with the R-program (R 2.15.2, GraphPad, San Diego, CA) and SAS (SAS 9.2, SAS Institute Inc., Cary, NC).

4.2.6.1. Analysis of the association between birth weight and HLA risk alleles for type 1 diabetes

For the review of general characteristics of the study group, the mean birth weight in grams, gestational age, distribution by sex and mean maternal age at birth were calculated for all HLA-typed newborn infants in the entire study cohort as well as in the individual national cohorts. Comparison between risk-groups for T1D and between countries were performed using the ANOVA test. A p value < 0.05 was considered to indicate a significant difference.

According to relative birthweights children were divided into quartiles and the distribution of various quartiles in each HLA-risk group was compared in the whole study group as well as for each country separately. The Chi-square test was used for statistical comparison between the groups and quartiles. In

order to find factors influencing the birth weight SDS scores, a multiple linear regression analysis was also performed. As the country of origin, gender and gestational age were already taken into account during SDS calculation, HLA-risk class (very high, high, moderate, neutral or protective), maternal age and route of delivery were included into the multiple linear regression analysis.

Because all the subjects studied were analyzed for the presence of *DQB1*02*, **0301*, **0302* and **0602/3*, we also analyzed the distribution of subjects between the four birth weight SDS quartiles for all genotypes formed by these alleles.

4.2.6.2. Analysis of the association between early postnatal growth parameters and HLA risk-groups

Homoscedasticity and homogeneity of mean weight or height SDS of each risk-group were tested using the Levene's test. The normality of the length and weight SDS distributions in different sub-groups was assessed using the Kolmogorov-Smirnov test. At all time points, there was a normal distribution of length SDS in all risk groups. The distribution of the weight SDS differed at most of the time points from a normal one, but the maximum difference between group mean and median values did not exceed 0.1 SDS. Therefore, the differences in mean length and weight SDSs were compared between the groups at different time points using the one-way ANOVA test with post hoc Tukey HSD analysis for pair-wise comparison.

Power calculation, based on the results derived from the entire population analysis, was performed for consecutive separate analysis of country-specific data. Given the country-specific numbers of study subjects, an effect size of 0.5 SDS and an SD equal of 1 SDS for mean length and an SD of 0.9 SDS for mean weight the power was 42% for length and 86% for weight in the Estonian cohort and 29% and 27%, respectively, in the Finnish cohort.

Since mid-parental height has only a modest effect on linear growth in infancy (Smith et al., 1976), the length SDS data were not corrected for that variable. However, in order to confirm the absence of any mid-parental height effect, this parameter was calculated for each subject using the formula (father's height + mother's height+13cm) / 2 for boys and (father's height + mother's height – 13cm) / 2 for girls. Subsequently the mean mid-parental height was calculated for all risk groups and compared between the groups using the one-way ANOVA test. A p value < 0.05 was considered to indicate a significant difference.

4.2.6.3. Analysis of the association between IGF-I/IGFBP-3 and the development of early signs of β-cell autoimmunity

The molar ratio of IGF-I/IGFBP3, the possible marker of free bioactive IGF-I in plasma (Juul et al., 1995), was calculated on the basis of molecular weights of

7.6 kDa for IGF-I (Rinderknecht and Humbel, 1978) and 42 kDa for IGFBP-3 (Matsumoto et al., 1996)

The normality of data distribution was assessed based on the Kolmogorov-Smirnov test. When the concentration of IGF-I was below the detection limit, a level of 25 μg/L was used for the statistical analysis. To compare the possible differences in plasma IGF-I and IGFBP-3 concentrations and IGFI/IGFBP-3 ratios between subjects with different HLA-risk groups for T1D, the ANOVA-test with post hoc Tukey HSD analysis was used. If the data were not normally distributed, the Kruskal-Wallis test with the post hoc Bonferroni (Dunn) t-test was applied. For comparison of IGF-I, IGFBP-3, and IGFI/IGFBP-3 molar ratio between autoantibody-positive and negative subjects, an unpaired two-tailed Student t-test or, in the case of data with a skewed distribution, the Mann-Whitney U-test were applied.

The changes in plasma IGF-I and IGFBP-3 concentrations and in IGF-I/IGFBP-3 ratio were calculated between the time point of seroconverion (T1) and the closest time points before seroconversion (T0) to positivity for at least one autoantibody (T1 – T0), and also the closest time points after seroconversion (T2 – T1). In matched control subjects the changes in IGF-I and IGFBP-3 concentrations as well as in the IGF-I/IGFBP-3 ratio were calculated between the same time points corresponding to those used in the cases. The paired t-test was applied to compare the changes in IGF-I, IGFBP-3 and IGF-I/IGFBP-3 ratio between cases and controls.

For the subgroup of autoantibody-positive children with multiple autoantibodies (n=12), a paired t-test analysis of possible changes in IGF-I, IGFBP-3 and IFG-I/IGFBP-3 ratio between the time points before and at the time of seroconversion to positivity for multiple antibodies, was performed as well. In addition the four subjects who progressed to clinical T1D were compared to their controls applying the paired t-test. A p value <0.05 was considered statistically significant.

5. RESULTS

5.1. Birth weights in subjects with HLA risk alleles for type 1 diabetes (Paper I)

5.1.1. General characteristics of the subjects

The general characteristics of the subjects are presented in Table 3. The mean birth weight in Russian Karelia was significantly lower than in Estonia ($p<0.001$) and Finland ($p<0.001$). The mean age of the mothers at delivery and mean duration of pregnancy was also significantly lower in Russian Karelia than in Finland and Estonia (both $p<0.001$). Estonian mothers were younger than the Finnish ones, and the duration of pregnancy was shorter in Estonia than in Finland ($p<0.001$).

Table 3. General clinical characteristics (mean and 95% CI) of the infants included in the study.

	Finland N =2931; 1402 girls	Estonia N= 2521; 1231 girls	Russian Karelia N= 2479; 1191 girls	Total N= 7931; 3824 girls
Mean BW (g)	3599 (3582–3616)*	3627 (3608–3646)*	3452 (3434–3469)	3562 (3552–3572)
Girls	3542 (3519–3565)	3547 (3521–3573)	3373 (3349–3397)	3491 (3477–3505)
Boys	3651 (3627–3675)	3704 (3677–3730)	3525 (3500–3549)	3628 (3613–3643)
Mean duration of pregnancy (days)	281.2 (280.9–281.6)**	280.2 (279.8–280.5)**	277.3 (277.0–277.6)**	279.7 (279.5–279.9)
Mean age of mother at delivery (years)	31.4 (31.3–31.6)**	28.8 (28.5–29.0)**	27.6 (27.4–27.8)**	29.4 (29.3–29.5)

* $p<0.001$ – compared to Russian Karelia
** $p<0.001$ – compared to the other two countries

Significant differences in the prevalence of HLA risk genotypes were seen between the countries, similar to a previous study (Nejentsev et al., 1998). In general, very high and high risk genotypes were more prevalent in Finland compared to the other two neighbouring countries. At the same time, the moderate risk and neutral or protective genotypes were more frequent in Estonia and

Russian Karelia compared to Finland. The detailed description of genotype distribution between the study populations is presented in Table 4.

5.1.2. The distribution of birth weight SDS between quartiles in subjects belonging to different HLA risk groups for type 1 diabetes

The distribution of newborn infants belonging to various HLA risk genotype classes into the lowest and highest quartile of relative birth weight (SDS) is shown in Table 5. There were no significant differences between any risk groups toward low or high relative birth weight when analyzed either in the total study population or separately in the Finnish, Estonian or Russian Karelian cohorts. Multiple linear regression analysis showed that maternal age as well as delivery by caesarean section were both directly associated with birth weight SDS. At the same time, HLArisk class did not significantly influence birth weight SDS. Because all the subjects studied were analysed for the presence of *DQB1*02*, **0301*, **0302* and **0602/3*, we also analyzed the distribution of subjects between the four birth weight SDS quartiles for all genotypes formed by these alleles. In this analysis, we observed a statistically significant shift toward higher relative birth weight for the neutral/protective HLA *DQB1*0302/DQB1*0602/3* genotype. For this group, the protective alleles *DQB1*0602* and *DQB1*0603* in cross-combination with the risk allele *DQB1*0302* were associated with increased birth weight. In this group, the distribution of children between quartiles was as follows: 18% in the first quartile (the total number, of newborns n=66), 53% in second and third quartiles (n=199), and 29% (n=109) in the fourth quartile. The difference between first and fourth quartiles was 11% and it was statistically significant [p=0.003; OR=0.52 (CI=0.36–0.75)]. Infants with this genotype constitute 4.7% of all study subjects and 5.8% of the children with neutral/protective genotypes. The distribution of newborn infants with these genotypes over the quartiles was similar in all three national groups. The combination of *DQB1*0302* or *DQB1*0602* and **0603* with any other allele did not show significant associations with increased birth weight (data not shown).

Table 4. Distribution of T1D risk-genotypes in the three study populations (Finland, Estonia, and Russian Karelia) and differences in their distribution between the countries.

Diabetes HLA-risk group	Finland	Estonia	Russian Karelia	
Very High	2.2%*	1.4%	1.4%	*Finland vs. Estonia p=0.03 OR 1.6 (CI 1.5–2.0) *Finland vs. Russian Karelia p=0.02 OR 1.6 (1.1–2.5)
High	11.7%	4.8%*	8.7%**	*Finland vs. Estonia p=0.03 OR 1.6 (CI 1.5–2.0) *Finland vs. Russian Karelia p=0.02 OR 1.6 (1.1–2.5)
Moderate	9.9%	9.2%	6.8%*	*Russia Karelia vs. Estonia p=0.002 OR 1.4 (CI 1.1–1.7) *Russian Karelia vs. Finland p<0.001 OR 1.5 (CI 1.2–1.8)
Neutral and protective	76.2%*	84.6%	83.1%	*Finland vs. Estonia p<0.001 OR 1.7 (CI 1.5–2.0) *Finland vs. Russian Karelia p<0.001 OR 1.5 (CI 1.3–1.7)

Table 5. The proportional distribution of subjects (%) and numbers (n) in the 1st and 4th quartiles of BW SDS in different HLA risk classes for T1D

HLA-risk group	Estonia		Finland		Russian Karelia		Total	
	Quartile		Quartile		Quartile		Quartile	
	1st (<25ts)	4th (>75ts)	1st (<25ts)	4th (>75ts)	1st (<25ts)	4th (>75ts)	1st (<25ts)	4th (>75ts)
Very high (n=144)	25% (n=10)	13.9% (n=5)	26% (n=17)	32% (n=21)	29% (n=10)	32% (n=11)	27% (n=37)	27% (n=37)
High (n=675)	23% (n=28)	23% (n=28)	26% (n=90)	24% (n=81)	22% (n=47)	26% (n=56)	24% (n=165)	24% (n=165)
Moderate (n=686)	23% (n=53)	25% (n=58)	25.9% (n=75)	26% (n=76)	24% (n=41)	28% (n=47)	25% (n=169)	26% (n=181)
Neutral and pro-tective (n=6426)	25% (n=540)	25% (n=533)	25% (n=559)	26% (n=572)	25% (n=513)	24% (n=492)	25% (n=1612)	25% (n=1597)
Total number	2521		2931		2479		7931	

5.2. Early postnatal growth in subjects with different HLA risk alleles for type 1 diabetes (Paper II)

5.2.1. General characteristics of the subjects

The difference in mean BW and mean gestational age between the HLA-related risk groups for T1D was non-significant (Table 3). The number and proportion of subjects with different HLA risk genotypes in Estonia and Finland are also presented in Table 6. The mean duration of breast-feeding varied from 10.0 months in the moderate risk group to 10.7 months in the very high risk group, the difference being non-significant (p=0.16).

5.2.2. Comparison of mean length SDS in subjects with different HLA risk groups for type 1 diabetes

The mean length SDS was significantly lower in subjects with very high risk for T1D compared to that in subjects in the control group at the age of 12 months {0.36 SDS [95% confidence interval (CI) 0.03; 0.70] vs. 0.83 SDS (CI 0.67; 0.96), p = 0.01} and at the age of 24 months [–0.11 SDS (CI –0.45; 0.20) vs. 0.47 SDS (CI 0.28; 0.65); p=0.035]. There were no other significant differences between the groups (Figure 1). When Estonian and Finnish cohorts were analyzed separately, a similar pattern in the mean length SDS distribution persisted between the groups in both countries (data not shown), but the differences remained non-significant, most likely due to the smaller number of subjects. Separate analysis for boys and girls showed that the differences in mean length SDS were more significant between the HLA groups among boys (p values 0.01, 0.08 and 0.002 at the age of 3, 12, and 24 months respectively) whereas in girls only non-significant trends could be seen (all p values >0.05). The mean mid-parental height was not significantly different between the groups (all p values >0.05, data not shown).

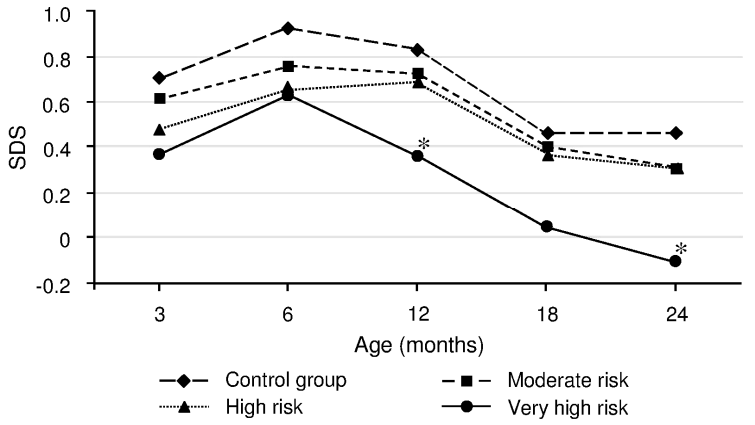
5.2.3. Comparison of mean weight SDS between subjects in different HLA risk groups for T1D

For the entire study cohort, the weight SDS was significantly lower in subjects with the very high-risk genotype for T1D compared to that in subjects in the control group at the age of 12 months {0.37 SDS (95% confidence interval (CI) 0.09; 0.66) vs. 0.92 SDS (CI 0.78; 1.1); p=0.001}, 18 months {0.32 SDS (CI 0.04; 0.61) vs. 0.89 SDS (CI 0.7; 1.1); p=0.001}, and 24 months {0.34 SDS (CI 0.06; 0.62) vs. 0.8 SDS (CI 0.59; 1.0); p=0.005} (Figure 2). At the age of 18 months, the mean weight SDS in the very high-risk group was also lower compared to the moderate-risk group (p=0.03) and the high-risk group (p=0.02) (Figure 2). At some time points the mean weight SDS in the control/neutral group was also higher than in the moderate and high-risk groups (Figure 2).

Table 6. HLA genotypes conferring risk for or protection against T1D, the number of children in each risk-class, the proportion of children in each group out of all groups with increased risk for T1D, distribution by country of origin. Mean birth weight (g) and gestational age (weeks) with 95% CI in brackets are shown.

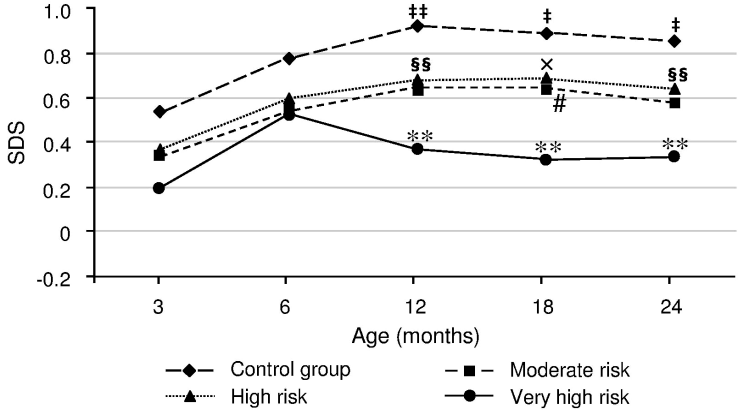
	Finland			Estonia			Combined		
HLA risk	N (%)	BW	GA	N (%)	BW	GA	N (%)	BW	GA
Very high DR3-DQ2[a]/DR4-DQ8[b]	24 (9)	3698 (3501–3896)	39.5 (39.0–40.1)	21 (9)	3487 (3273–3689)	39.4 (38.7–40.1)	45 (9)	3597 (3456–3738)	39.5 (39.0–39.9)
High DR4-DQ8/X[c]	152 (58)	3545 (3469–3622)	39.6 (39.4–39.9)	101 (43)	3487 (3273–3689)	39.2 (38.8–39.6)	253 (51)	3562 (3498–3627)	39.4 (39.2–39.7)
Moderate DR3-DQ2/Y[d]	85 (33)	3577 (3485–3668)	39.6 (39.3–39.9)	114 (48)	3487 (3273–3689)	39.6 (39.3–39.8)	199 (40)	3591 (3530–3653)	39.6 (39.4–39.8)
Neutral/ Protective All other genotypes	0			191	3631 (3562–3700)	39.5 (39.3–39.7)	191	3631 (3562–3700)	39.5 (39.3–39.7)

[a]DQA1*05-DQB1*02
[b]DRB1*0401/2/4/5/7/8-DQB1*0302/4
[c]Other than haplotypes including DQB1*0503, DQB1*0602, DQB1*0603, DQA1*05-DQB1*02, DQA1*0201-DQB1*0303 or DQA1*05-DQB1*0301
[d]Other than haplotypes including DQB1*0302/4, DQB1,*0503, DQB1*0602, DQB*0603, DQA1*0201-DQB1*0303 or DQA1*05-DQB1*0301



*p<0.05 – very high-risk group versus control group.

Figure 1. Changes in length SDS in the entire study cohort.



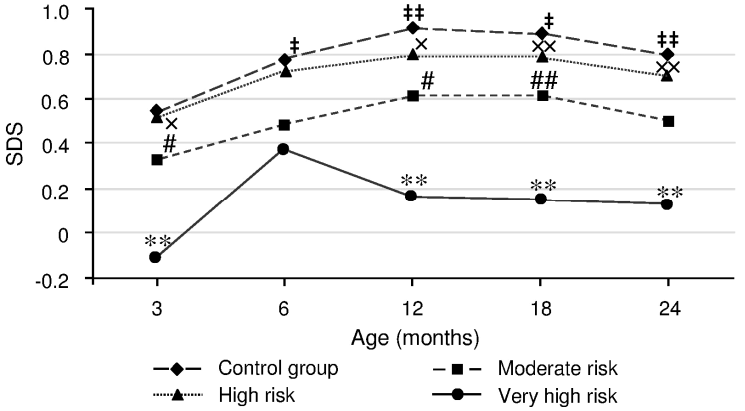
**p<0.01 – very high risk group vs. control group; #p<0.05– very high risk group vs. moderate risk group; ×p< 0.05 – very high risk group vs. high risk group; ‡p<0.05 – control group vs. moderate risk group; ‡‡p<0.01 – control group vs. moderate risk group; §§p<0.01 – control group vs. high risk group.

Figure 2. Changes in weight SDS in the entire study group.

When analyzed separately, the weight gain among the Estonian children showed a similar pattern between the groups as that seen in the entire study cohort: the mean weight SDS of the very high-risk group was significantly lower compared to the control group at most of the time-points (at 3 months p=0.002; at 12 months p=0.001; at 18 months p=0.002; and at 24 months p=0.0001), compared to the moderate-risk group at 3 months (p=0.04), at 12 months (p=0.04), 18 months (p=0.03) and compared to the high-risk group at 3, 12, 18, and 24 months (p=0.01, p=0.01, p=0.006 and p=0.003 respectively) (Figure 3). In addi-

tion, the mean weight SDS in the control group was higher compared to the moderate-risk group at 6 (p=0.01), 12 (p=0.01), 18 (p=0.04) and 24 months (p=0.004) (Figure 3). In the Finnish cohort, the differences between the groups were not statistically significant.

Separate analyses for boys and girls showed that the differences in mean weight SDS between the groups were significant among the boys whereas in the girls no significant differences were seen between the groups (all p values >0.05). Namely, in boys the mean weight SDS in the very high-risk group was lower compared to the control group at the age of 12 months (p=0.001), and at the age of 24 months (p=0.01). In addition, the mean weight SDS in the very high-risk group was significantly lower at the age of 12 and 24 months compared to the moderate-risk group (p=0.004 and 0.04, respectively), and high-risk group (p=0.01 and 0.04, respectively).



**p<0.01 – very high risk group vs. control group; #p<0.05 – very high risk group vs. moderate risk group; ##p<0.01–very high risk group vs. moderate risk group; ×p< 0.05 – very high risk group vs. high risk group; ××p< 0.01 – very high risk group vs. high risk group; ‡p<0.05 – control group vs. moderate risk group; ‡‡p<0.01 – control group vs. moderate risk group.

Figure 3. Changes in weight SDS in the Estonian participants**.**

5.3. Role of IGF-I and IGFBP-3 in HLA risk allele-related early postnatal growth and development of early signs of β-cell autoimmunity (Paper III)

5.3.1. Age-associated changes in IGF-I, IGFBP-3, and IFG-I/IGFBP-3 ratio in relation to HLA risk genotypes for T1D

In general, there is a rapid decline in IGF-I concentrations after birth up to the age of 6 months followed by a gradual recovery until the age of 18 months, when the levels are comparable with those at birth. After that the IGF-I

concentrations continue to increase slowly. Similar age-related changes were also seen in the IGF-I/IGBP-3 ratio. The IGFBP-3 concentrations are in contrast lowest in cord blood and increase continuously throughout the study period. At the age of 24 months, the mean IGF-I concentration was significantly lower in the children carrying the high-risk HLA genotype for T1D in comparison with the low-risk group (57.1 µg/L, 95% CI 45.1–69.1 µg/L vs 84.0 µg/L, 95% CI 68.1–99.8 µg/L, $p = 0.02$), (Fig. 4). Simultaneously also the mean IGFBP-3 concentration in the high-risk group (2.3 mg/L, 95% CI 2.1–2.6 mg/L; $p < 0.01$) was significantly lower when compared with both the moderate-risk (2.5 mg/L, 95% CI 2.4–2.7 mg/L; $p < 0.01$) and low-risk groups (2.9 mg/L, 95% CI 2.7–3.2 mg/L), (Fig. 5). There were no differences in the IGF-I/IGFBP-3 ratio at any time between the three HLA risk groups (Fig. 6).

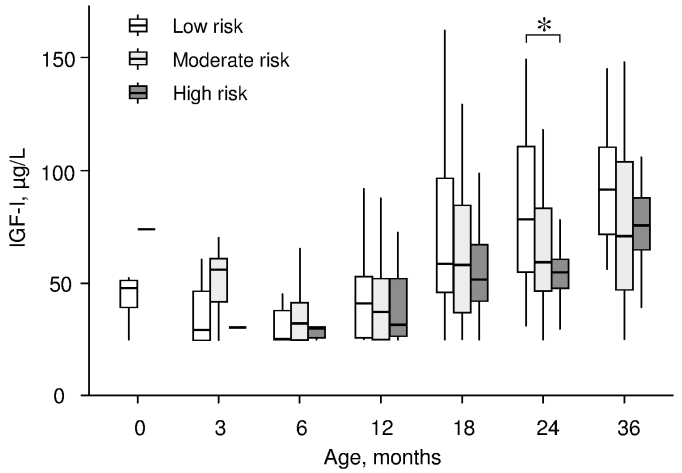


Figure 4. Box plot of plasma IGF-I concentrations over the first 3 years of life in the study participants in relation to their HLA risk genotype for type 1 diabetes. The ends of the whiskers represent the lower and the upper quartile (Q1 and Q4, respectively). The horizontal bar at 0 months represents the value measured for a single subject from the moderate-risk group, and that at the age of 3 months is for a single subject from the high-risk group. The asterisk marks $p = 0.02$ for the high risk vs. the low-risk group.

5.3.2. Comparison of IGF-I, IGFBP-3, and IFG-I/IGFBP-3 ratio between autoantibody-positive and negative subjects

There were no significant differences in plasma concentrations of IGF-I and IGFBP-3 concentrations or IFG-I/IGFBP-3 ratio between the autoantibody-positive and negative children at any time point from birth up to the age of 36 months, except a slightly lower mean IGF-I concentration in the autoantibody-positive subjects (39 µg/L, 95% CI 30–48 µg/L) compared to the autoantibody-negative infants (48 µg/L, 95% CI 42–54 µg/L) at the age of 12 months (*p*

<0.05), and a slightly lower IGFI/ IGFBP-3 ratio level in autoantibody-positive children at the age of 24 months (0.136, 95% CI 0.117– 0.156 *vs.* 0.151, 95% CI 0.140-0.161; $p < 0.05$). In the four cases who progressed to clinical T1D the concentrations of IGF-I and IGFBP-3 as well as the IGF-I/IGFBP-3 ratio were not different from other cases or controls.

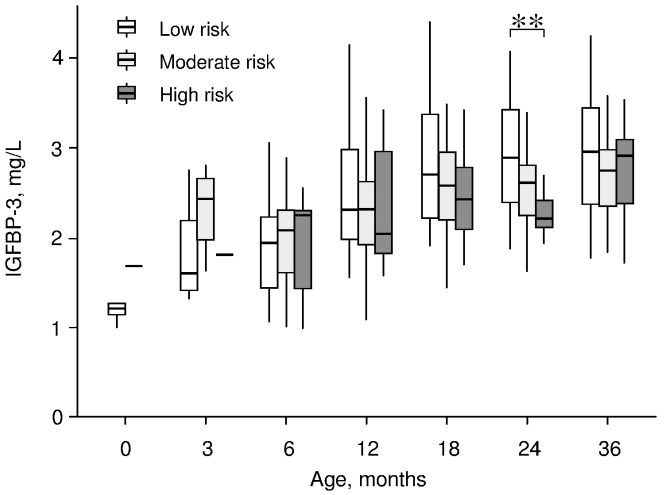


Figure 5. Box plot of plasma IGFBP-3 concentrations over the first 3 years of life in the study participants in relation to their HLA risk genotype for type 1 diabetes. The ends of the whiskers represent the lower and the upper quartile (Q1 and Q4, respectively). The horizontal bar at 0 months represents the value measured for a single subject from the moderate-risk group, and that at the age of 3 months is for a single subject from the high-risk group. The two asterisks mark $p < 0.01$ for the high risk vs. both the moderate-risk and low risk groups.

5.3.3. Changes in IGF-I, IGFBP-3, and IFG-I/IGFBP-3 ratio before and after seroconversion

When comparing the changes in IGF-I and IGFBP-3 concentrations as well as in the IFG-I/IGFBP-3 molar ratio between different time points, the mean increment in IGFBP-3 concentration preceding the seroconverison (T1 – T0) was significantly higher in autoantibody-positive children compared to the increase during the corresponding time interval in the autoantibody-negative subjects (0.43 mg/L 95% CI 0.29–0.56 mg/L *vs.* 0.22 mg/L, 95% CI 0.1–0.34 mg/L; $p = 0.005$; Fig. 7). In addition there was a trend towards a decrease in mean IGF-I and IGFBP-3 concentrations after seroconversion among the autoantibody-positive children (T2 – T1, $p = 0.06$ for both) compared to the autoantibody negative subjects. A subcohort analysis of the autoantibody-positive children with multiple autoantibodies, and their autoantibody-negative controls did not show

any significant differences in the increase in mean IGF-I and IGFBP-3 concentrations or in the IGF-I/IGFBP-3 molar ratio during the interval before and after seroconversion to multiple antibodies. The children who developed T1D showed similar trends to the other cases.

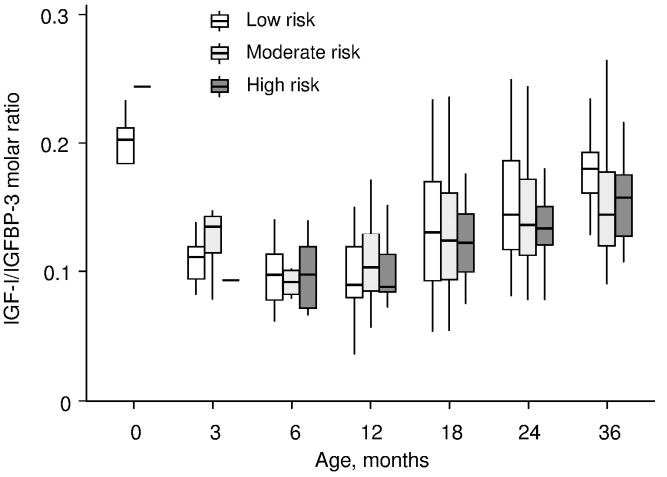


Figure 6. Box plot of plasma IGF-I/IGFBP-3 molar ratio over the first 3 years of life in the study participants in relation to their HLA risk genotype. The ends of the whiskers represent the lower and the upper quartile (Q1 and Q4, respectively). The horizontal bar at 0 months represents the value measured for a single subject from the moderate-risk group, and that at the age of 3 months is for a single subject from the high-risk group.

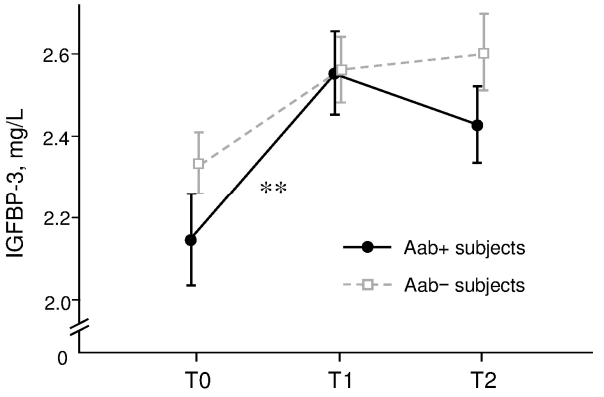


Figure 7. Mean plasma IGFBP-3 concentrations in autoantibody-positive (Aab+) children compared to the controls (Aab-) before (T0), at (T1) and after (T2) seroconversion for at least one diabetes-associated autoantibody. The statistical standard error of the mean (SE) is shown by error bars. The increase preceding the seroconversion was

significantly greater (** $p = 0.005$) in the Aab+ children than during the corresponding time interval in the Aab- children.

6. DISCUSSION

6.1. Birth weights in subjects with HLA risk alleles for type 1 diabetes (Paper I)

In our study, we did not find any direct association between HLA-risk genotypes (*DR3-DQ2/DR4-DQ8*, *DR3-DQ2/X*, *DR4-DQ8/X*) and birth weight. However, we observed that a relatively high birth weight was associated with the protective alleles HLA *DQB1*0602* and *DQB1*0603*, when combined with the strong risk allele *DQB1*0302*. This finding is partially consistent with previous studies, where an association of all these alleles with high birth weight has been reported (Aroviita et al., 2004; Järvinen et al., 2008; Stene et al., 2001; Larsson et al., 2005). The principal difference from previous reports was that in our study, only the cross-combinations of these alleles with each other were seen to be associated with a significantly increased birth weight, whereas no association with increased birth weight was observed if these alleles were combined with other alleles.

Most previous studies aimed at finding possible associations between HLA risk haplotypes for T1D and birth weight have been conducted in one particular country. In our study, we set out to investigate whether such an association exists in three adjacent countries with similar climate and geophysical conditions but multi-fold differences in the incidence of T1D and conspicuous differences in socio-economic level and living environment. Because environmental factors are most likely the main factors (Onkamo et al., 1999) responsible for the increase seen in T1D incidence over the last decades in developed countries (DIAMOND Project Group, 2006), we hypothesized that the impact of the HLA haplotype on birth weight would be strongest in the country with the highest incidence of T1D, that is, in Finland (Harjutsalo et al., 2013). Some observations speak in favor of this hypothesis. It has for example, been reported that some environmental factors such as maternal smoking and intrauterine infections may influence fetal growth modulated by HLA genotypes (Taylor et al., 2006; Larsson et al., 2007). Various HLA genotypes may influence for example, the microbial colonization of the newborn infant (De Palma et al., 2010). Therefore, differences in such factors between the countries studied here could potentially influence the association between the HLA genotype and birth weight. However, the distribution of birth weight over quartiles in different HLA risk classes or their combinations was not significantly different in any of the countries studied or in the whole study cohort. Even in groups with a non-homogeneous distribution of birth weight over quartiles, such as in the case of HLA *DQB1*0302/DQB*0602/3*, no significant differences was seen between Finland, Estonia and Russian Karelia.

One reason why we did not detect any association between common HLA-risk genotypes and birth weight might be the relatively small number of subjects in each country for the detection of such an association as that one seen in the Swedish study comprising 16 709 newborn infants (Larsson et al., 2005). The

second reason might be slightly different classifications applied for HLA risk categories. For example, in our study, DR4 in DQB1*0302 positive subjects was additionally subtyped to find the protective allele DRB1*0403 in order to exclude those subjects from the high-risk group. However, after applying exactly the same classification for HLA risk groups as that one used by Larsson et al. (Larsson et al., 2005), the distribution of children over quartiles remained the same as in our initial analysis (data not shown). The third reason might be the difference in how birth weight SDS was calculated. In the Swedish study (Larsson et al., 2005), the birth weight SDS was based on the mean birth weight derived from national standardized intrauterine growth curve. In our study, such a standardized curve was available only for the Finnish cohort (Pihkala et al., 1989). The comparison of Finnish birth weight data (mean birth weight by gestational week and sex and their standard deviation) with the Finnish national reference growth curve showed that at least this national cohort represented the general population very well (data not shown).

One might also expect an effect opposite to our hypothesis. Namely, in Finland, where the incidence of T1D is the highest, the effect of HLA on birth weight may remain unrecognized because of other powerful risk factors affecting birth weight. Under such circumstances, an effect of HLA on birth weight could be expected to be seen in countries with lower prevalence of T1D. However, both in Estonia and Russian Karelia, no such differences in birth weight distribution were observed. There have also been other studies looking for possible associations between HLA risk group and birth weight (Stene et al., 2001; Locatelli et al., 2007; Järvinen et al., 2008; Hummel et al., 2007). Järvinen with colleges demonstrated in 2008 that only one population-specific relatively rare *DQB1*0302-DR4* haplotype with the *B56* allele conferring increased risk for T1D was associated with higher birth weight, whereas other HLA haplotypes conferring risk for T1D were associated with only a modest and non-significant trend toward higher birth weight. In a German study (Hummel et al., 2007), the association of *HLA DQB1*0302-DR4* with high birth weight was demonstrated only in offspring of diabetic mothers. Our results are more similar to those reported by Stene et al. and Locatelli et al. who did not find any significant associations between a high birth weight and HLA haplotypes conferring increased risk for T1D. (Stene et al., 2001; Locatelli et al., 2007) Our results also agree with the outcome of the recent study by Sterner et al., where the populations of four countries with similar socioeconomic level but with different climatic conditions and genetic background were compared, and no difference between groups with different HLA-risk genotypes was seen (Sterner et al., 2011).

We observed a positive association between the HLA genotype and increased birth weight for the HLA *DQB1*0302/DQB1*0602/3* combination. Stene et al. demonstrated a positive association between high birth weight and the protective HLA *DQB1*0602* allele. (Stene et al., 2001). Other authors have noticed some associations between an increased birth weight and the protective HLA *DQB1*0603* allele (Larsson et al., 2005) and the predisposing allele HLA

*DQB1*0302* allele (Larsson et al., 2005; Järvinen et al., 2008; Hummel et al., 2007). We suggest that these alleles have an additive effect on birth weight and when combined, the effect on birth weight is most obvious. If those alleles are present separately, the additive effect vanishes and in our study, we did not see any impact on birth weight.

Our study has a couple of limitations. First is the selection bias. Because of technical difficulties in emergency situations, the cord blood samples were not obtained from a few children born by caesarean section. We were not able to find out the exact number of such missed subjects. Therefore, the data of these children who are often extremely large or because of their prematurity very small are missing in the study. The second limitation is a possible information bias. Namely, the gestational age of some infants might be misclassified. One sign of such a possible misclassification is the fact that the duration of pregnancy was statistically different between the countries. This might be caused by the differences in the methodology of estimation of pregnancy duration between the countries. In our SDS calculation, however, we have taken into consideration the country of origin and therefore the influence of possible misclassification of gestational age should be eliminated. The third limitation is that we were not able to identify exactly the number of previous deliveries by the mother. We asked about the number of siblings but not about the number of previous deliveries. Therefore, we could not adjust the birth weight data for birth order. It is known that the birth weight is increasing along with birth order in singleton pregnancies (Wilcox et al., 1996). However, many studies on HLA associations with birth weight have shown that adjustment for birth order does not influence the results (Larsson et al., 2005; Locatelli et al., 2007; Hummel et al., 2007); and therefore, we may assume that the same is true for the current study. In addition, birth weight is influenced by many biological, medical and socioeconomic factors, information of which was not available and thus could not be adjusted for neither in previous studies nor in the current study.

To conclude, in this study, we were not able to confirm the previously reported observation of an association between common HLA risk haplotypes for T1D and increased birth weight. However, we found a significant association between increased relative birth weight and two strongly protective alleles in cross-combination with the DQB1*0302 risk allele. Further comprehensive epidemiological studies are needed to assess to which extent HLA haplotypes conferring susceptibility to T1D are contributing to the increased birth weight associated with enhanced disease risk and the role of different environmental factors in this process. Our observations suggest that the mechanisms behind the association between high birth weight and risk for T1D could be related to other factors than the HLA genotypes.

6.2. The role of HLA risk alleles for type 1 diabetes in early postnatal growth (Paper II)

In this study we showed that the early growth pattern in children carrying high-risk HLA genotypes for T1D differs significantly from that seen in other young children. In particular, up to the age of 24 months the children in the very high-risk group had gained significantly less weight compared to the children in the other groups. The same pattern was also seen in length, but it was less conspicuous. Similarly to the very high-risk group, at some time points children in the other risk groups gained less weight than those with neutral genotypes.

The current results were surprising because the majority of previously published studies have reported a positive correlation between increased linear growth, weight gain, and development of T1D (Blom et al., 1992; Bognetti et al., 1998; Ljungkrantz et al., 2008; Larsson et al., 2008; Hyppönen et al., 2000; EURODIAB Substudy 2 Study Group, 2002; Knerr et al., 2005). Therefore, we also expected a similar positive correlation between growth and HLA-conferred risk for T1D. However, there are also reports, in which inverse association (Brown et al., 1994; DiLiberti et al., 2002; Leslie et al., 1991), or positive associations only in some specific subgroups (Brown et al., 1994; Japan and Pittsburgh Childhood Diabetes Research Groups, 1989; DiLiberti et al., 2002; Songer et al., 1986; Leslie et al., 1991; Kharagjitsingh et al., 2010) have been observed between risk for T1D and accelerated growth. The principal difference between our study and the previous ones is that we monitored prospectively a cohort of children carrying HLA genotypes conferring genetic susceptibility to T1D, while earlier studies have analyzed growth data retrospectively in children with established T1D. Moreover, the role of HLA genotypes conferring susceptibility to T1D is not well defined earlier in relation to linear growth and weight gain (Court et al., 1982; Larsson et al., 2008; Carlsson et al., 2012). The recent Swedish study by Carlsson et al., which investigated the association between BMI and HLA genotypes conferring risk for T1D in 2403 children with recent-onset T1D, showed that disease development was associated with increased BMI in subjects with low or moderate risk HLA genotypes, i.e. the association was positive, whereas in the group with the highest genetic risk, the development of T1D was associated with low BMI, i.e. there was an inverse association (Carlsson et al., 2012). Very similar results were also recently shown in another large study (Yang et al., 2014). Those findings are in line with our results.

The current study confirms that the association between different HLA genotypes and weight and length gain is directly related to the HLA genotypes. For example, an inverse association between linear growth and presentation of T1D has been observed in children who developed T1D early in life (Brown et al., 1994; DiLiberti et al., 2002). It has also been shown that the HLA genotype conferring very high risk for T1D (HLA *DR3-DQ2/DR4-DQ8*) is overrepresented among those who present with overt disease before the age of 5 years (Gillespie et al., 2002). Accordingly, when combined, those observations are

consistent with the current finding of a retarded growth among children who carry the HLA genotype conferring the highest risk for T1D.

It should be noted that any direct linking of our results with the development of T1D should be avoided, since the vast majority of children with HLA-conferred risk for T1D will never develop diabetes (Erlich et al., 2008; Knip et al., 2005). Information on the progression of the autoimmune process towards clinical T1D would provide additional light on the possible link between the growth pattern and the development of T1D, but only a few subjects seroconverted to positivity for T1D-associated autoantibodies by the age of 24 months.

We found some differences in the growth pattern between the two countries represented in this study. While the mean length SDS changes in all risk groups showed similar patterns in both countries, the weight SDS of the very high and moderate-risk groups was significantly lower at several time points over the study period compared to the control group only in Estonia, but not in Finland. Increased growth and weight gain are known to be risk factors for the development of T1D (Blom et al., 1992; Bognetti et al., 1998; Ljungkrantz et al., 2008; EURODIAB Substudy 2 Study Group, 2002; Hyppönen et al., 2000; Kibirige et al., 2003; Knerr et al., 2005). Our hypothesis at the beginning of the study was that some unknown environmental exposures, associated with the development of T1D, also promote a more rapid weight and length gain and this phenomena is more obvious in children from Finland, were the incidence of T1D is approximately three times higher than in Estonia (Teeäär et al., 2010; Harjutsalo et al., 2013). This hypothesis is supported by studies showing that in countries with low T1D rates (such as India or Japan), no positive correlation has been observed between rapid linear growth and further development of T1D (Japan and Pittsburgh Childhood Diabetes Research Groups, 1989; Ramachandran et al., 1994).

The differences in growth pattern depending on T1D-related HLA genotypes could be explained by the role of insulin resistance which has been shown to contribute to the development of T1D in several studies (Fourlanos et al., 2004; Gardner et al., 2008; Xu P et al., 2007). Rapid weight gain has been observed to be associated with insulin resistance (Mericq et al., 2005). Children with neutral and protective HLA genotypes could have a higher degree of insulin resistance compared to those with high-risk HLA genotypes. This idea is supported by the recent study by Andersen et al. who reported a positive association between stronger HLA-conferred susceptibility to T1D and lower insulin secretion, better insulin sensitivity, and a reduced capacity to compensate for an increasing insulin demand in adults (Andersen et al., 2012). Unfortunately, we do not have any data on insulin resistance in our study subjects, and therefore this explanation is only hypothetical.

There are several major strengths of our study. It is one of the largest studies which has analyzed the association between different T1D risk-related HLA genotypes and early growth. Moreover, previous studies have focused mostly on the association between growth and further development of T1D. In such studies the HLA genotypes have been considered as potential confounders, and

their possible effect on growth has been analyzed accordingly (Court et al., 1982; Larsson et al., 2008). To our best knowledge, this study is the first one directly focusing on the effect of HLA genotypes conferring susceptibility to T1D on postnatal growth. Previous studies on the HLA impact on growth have been mostly case-control studies. There is only one earlier study, in which subjects with genetic risk for T1D were allocated according to their HLA genotypes and thereby providing the possibility to assess the potential effect of specific HLA genotypes on growth characteristics (Carlsson et al., 2012). Generally speaking, the results of that study were in line with our results. Recently our results have been reproduced by another large cohort study (Yang et al., 2014). Another strength of our study is the inclusion of subjects from two countries with similar genetic background and close geographic location, but with about a threefold difference in the incidence of T1D (Teeäär et al., 2010; Harjutsalo et al., 2013), suggesting a possible major impact of the environment and lifestyle on the development of the disease. The conspicuous difference in the T1D rate between Estonia and Finland must be explained mostly by environmental and lifestyle differences between these two countries.

The major limitations of our study are related to the control/neutral group. This group included only children from Estonia whereas the risk groups for T1D comprised subjects from both Estonia and Finland. When comparing growth charts of Estonian and Finnish children, the mean height and weight at the age of 2 years are very similar (88.5 cm/13400 g vs. 88cm/13000g in boys and 87.5 cm/12500 g vs. 86.6/12500 in girls, respectively). Accordingly we do not expect that the growth pattern in Finnish children with protective or neutral HLA genotype would have been significantly different from that seen in Estonian children. Moreover, the mean birth weight of newborn infants carrying neutral or protective HLA genotypes, as well as the mean birth weights of babies with other HLA genotypes, were not significantly different between these two countries (Peet et al., 2012). The second limitation, also related to the control group, is the fact that the subjects in this group were followed not within the framework of the DIABIMMUNE study but by their primary care physicians. However, the weight and length measurements are well standardized in young children, and therefore the growth data collected from the primary care case records can be expected to be reliable and comparable with the data collected within the DIABIMMUNE project. The third limitation, also related to the control group, was a relatively high proportion of incomplete growth data sets within this group as 27% of the subjects had one or more missing data point during the study period while this proportion was only 1 to 5% in the risk groups. Such a high number of missing data is due to the fact that the 18-month visit to a primary care physician is not obligatory in Estonia, and therefore many families do not attend. Another limitation of the current study is the low number of children in the very high risk group (n=45). It leads to significant lack of power in country-specific analysis and a high likelihood of false-negative results in comparison of Estonian and Finnish subgroups. In addition, there are some possible confounders, such as the children's diet, frequency of

intermittent diseases or medications, that could have an influence on the results, but the detailed analysis of their impact was beyond the scope of the current study.

In conclusion, we have observed that early growth was significantly slower in children with HLA genotypes conferring very high risk for T1D than in those with neutral or protective HLA genotypes. Contradictory to our initial hypothesis, the subjects with very high risk gained less weight and height during the first 24 months of life, and this was more pronounced in the Estonian cohort. From these observations we hypothesize that the development of T1D requires a higher degree of insulin resistance among children with neutral and protective HLA genotypes when compared to those with high-risk genotypes, and this seems to be the case more so in Estonia with a median disease incidence than in Finland with the highest rate of T1D in the world.

6.3. The role of IGF-I and IGFBP-3 in HLA risk allele-related early postnatal growth and development of early signs of β-cell autoimmunity (Paper III)

In this study we observed several significant associations between the IGF-I/IGFBP-3 system on one hand and the development of diabetes-associated autoantibodies and HLA genotypes conferring risk for T1D on the other. First of all, we found that at the age of 24 months the mean IGF-I and IGFBP-3 concentrations in subjects with the high-risk HLA genotype for T1D (*DR3-DQ2/DR2-DQ8*) were significantly lower compared to the subjects with low-risk HLA genotypes. The results are in line with our previous study in the entire DIABIMMUNE study cohort where we found that by the age of 18–24 months the subjects with high HLA risk-genotypes for T1D demonstrated a pronounced slowdown in linear growth as well as in weight (Peet et al., 2014). The current results suggest that the decreased circulating IGF-I and IGFBP-3 concentrations may be involved in the slower growth in children carrying the high-risk genotype. In the study by Beyerlein et al. the association of IGF-I and IGFBP-3 in relation to HLA status was not analyzed (Beyerlein et al., 2014).

The GH-IGF-I axis starts to play a major role in the regulation of human growth from the age of around 2 years (Karlberg, 1989). This may explain why the difference in IGF-I and IGFBP-3 concentrations between subjects with different HLA genotypes was seen only at the age of 24 months and not before. Accordingly, it would be interesting to follow these children to see whether a slower growth rate and lower IGF-I and IGFBP-3 concentrations will continue to persist or whether this is just a temporary finding followed by some catch-up both in growth as well as in hormone concentrations. Studies in slightly older children (Yang et al., 2014; Carlsson et al., 2012) and in adults (Fourolanos et al., 2014), in whom increased HLA-conferred risk for T1D was observed to be associated with lower BMI, indicate that the former scenario is more likely to occur. We suggest that the slower linear growth and weight gain in

DIABIMMUNE subjects (Peet al., 2014) and lower BMI in other children and adults with high HLA-conferred risk for T1D (Yang et al., 2014; Carlsson et al., 2012; Fourlanos et al., 2014) may be associated with lower circulating concentrations of IGF-I and IGFBP-3 concentrations. It is known that IGF-I has a protective effect on the process of autoimmune destruction of the β-cells (Castrillo et al., 2000; George et al., 2002; Anguela et al., 2013; Chen et al., 2004; Mabley et al., 1997; Bergerot et al., 1995). Since the mechanisms behind the HLA-conferred genetic susceptibility to T1D are poorly defined (Xie et al., 2014), we could speculate that the reduced IGF-I concentrations in subjects with the high-risk HLA genotype could explain, at least partly, the HLA-related risk for T1D given that high IGF-I concentrations are protective against the development of T1D (Castrillo et al., 2000; George et al., 2002; Anguela et al., 2013; Chen et al., 2004; Mabley et al., 1997; Bergerot et al., 1995). Fourlanos et al. proposed, however, recently that an increased BMI simply promotes the development of T1D in subjects carrying lower-risk HLA risk genotypes by increasing the penetrance of lower risk HLA genes, increasing thus the proportion of lower risk HLA genes among subjects with higher BMI (Fourlanos et al., 2014). The presence of HLA genotype-related differences in BMI in children without any sings of T1D, as was reported by Yang et al. (Yang et al., 2014) or, as shown in the present study, in children with early signs of β-cell autoimmunity speaks against that suggestion.

The most important result of our study is the demonstration of a significantly higher increment in IGFBP-3 before seroconversion to autoantibody positivity when compared to the corresponding time interval in autoantibody-negative subjects. In the only other relevant study (Beyerlein et al., 2014), the IGF-I and IGFBP-3 concentrations were compared at certain time points irrespectively of the time of seroconversion, whereas our study looked at the rate of IGF-I and IGFBP-3 increments before the time of seroconversion. This approach evaluates more directly the role of IGF-I and IGFBP-3 in the process of autoimmunity development.

Our initial hypothesis was that there should be a significant difference in the IGF-I/IGFBP-3 molar ratio as one of the proposed markers of bioavailable IGF-I (Juul et al., 1995). To a lesser degree, we expected to see differences in the changes in IGF-I or IGFBP-3 concentrations. In our study only the latter was observed. The interpretation of an isolated higher increment of IGFBP-3 concentrations in autoantibody-positive subjects compared to autoantibody-negative ones represents a challenge as the serum IGFBP-3 concentration is predominantly regulated post-translationally by stabilization through its GH-dependent binding partners IGF-I and ALS. Thus, at the serum level, IGFBP-3 is GH-dependent (Baxter and Martin, 1986) but at the transcriptional level, independent of GH (Olivecrona et al., 1999). In some diseases, such as T1D and obesity, the serum IGF-I concentration is often within the normal range whereas the GH level is high reflecting some degree of disintegration of the GH-IGF-I axis. (Olivecrona et al., 1999). We suggest that this type of disintegration of the GH-IGF-I axis may exist to some extent also in subjects seroconverting to posi-

tivity for diabetes-associated autoantibodies. This suggestion is supported by the absence of any significant difference in the increment in the IGF-I/IGFBP-3 molar ratio when autoantibody-positive and negative children were compared in the period preceding the seroconversion (see Fig. 7). Such a change in the IGF-I/IGFBP-3 molar ratio should be expected if the IGFBP-3 increase in the two groups is not accompanied by a similar increase in the IGF-I concentration. As a matter of fact, a small difference in the mean IGF-I/IGFBP-3 molar ratio was actually seen between autoantibody-positive and autoantibody-negative subjects when the entire groups were compared at the age of 24 months. However, the observations regarding the IGF-I/IGFBP-3 molar ratio should be interpreted cautiously in terms of its biological role. Actually it has recently been questioned whether the IGF-I/IGFBP-3 molar ratio is a reliable marker of free bioactive IGF-I as this has not been validated and proven experimentally (Baxter, 2014). The molar ratio changes in the same direction as bioavailable IGF-I in some, but not in all situations (Frystyk, 2004).

It is not clear whether IGFBP-3 protects β-cells against autoimmune damage or accelerates the death of β-cells. Increasing IGFBP-3 concentrations right before seroconversion to autoantibody positivity could be a compensatory reaction that increases the half-life of circulating IGF-I, thus providing β-cell protection. An independent role of IGFBP-3 in the development of autoimmune β-cell damage cannot be excluded. The IGF-I-independent apoptotic and growth-inhibitory effects of IGFBP-3 have been seen in many different cell types (Johnson and Firth, 2014). Furthermore, in several large studies an association between IGFBP-3 and the development of type 2 diabetes has been observed (Rajpathak et al., 2008; Frystyk et al., 1999; Rajpathak et al., 2012). Accordingly, the role of the fast increase in plasma IGFBP-3 concentration right before seroconversion will remain a subject for further studies.

In addition to the lower IGF-I/IGFBP-3 ratio at the age of 24 months in autoantibody-positive children compared to autoantibody-negative peers, we also have found a similar difference in the IGF-I concentrations at the age of 12 months. Both findings are different from those reported by Beyerlein et al. where serum IGF-I and IGFBP-3 concentrations at 9 and 24 months of age did not differ between seropositive and seronegative subjects (Beyerlein et al., 2014).

Our study has several limitations. First, the number of children who developed multiple autoantibodies or T1D was small. It is known that only a minority of subjects with one autoantibody will progress to clinical T1D (Knip and Siljander, 2008). A second limitation is the modest sensitivity of the IGF-I assay with a lower detection limit of 25 μg/L as quite many subjects (13 cases and 14 controls) had such results at the time point before seroconversion. The interpretation of the results provides also some challenges because of the relatively small number of IGF-I and IGBP-3 samples in the particular subgroups based on HLA risk genotypes, and the autoantibody status. This drawback was due to the limited availability of plasma samples from the study participants. We should also take into consideration possible confounders, such as the dura-

tion of breastfeeding, which is known to influence the serum IGF-I concentrations (Grunewald et al., 2014). However, the differences in IGF-I and IGFPB-3 plasma concentrations were seen around 24 months of age when the potential effect of breastfeeding should be marginal.

In conclusion, our study showed that circulating IGF-I and IGFBP-3 may play a role in the development of β-cell autoimmunity in young children. These results suggest a possible link between the previously reported association between HLA genotypes and linear growth rate and weight gain in children with HLA-conferred susceptibility for T1D.

7. CONCLUSIONS

1. Our study did not confirm the previously reported observation of an association between common HLA risk genotypes for T1D and increased birth weight. The distribution of birth weight over quartiles in different HLA-risk classes or their combinations was not significantly different in any of the countries studied as well as in the whole study cohort. However, we found a significant association between increased relative birth weight and protective alleles *DQB1*0602* or *DQB1*0603* in cross-combination with the *DQB1*0302* risk allele. Our observations suggest that the mechanisms behind the association between high birth weight and risk for T1D are hardly HLA related.

2. Early postnatal growth in children with HLA genotype conferring very high risk for T1D was significantly slower than in those with neutral or protective HLA genotypes. Contradictory to our initial hypothesis, the subjects with very high risk gained less weight and height during the first 24 months of life, and this was more pronounced in the Estonian cohort. We hypothesize that the development of T1D requires a higher degree of insulin resistance among children with neutral and protective HLA genotypes when compared to those with high-risk genotypes. It seems to be more pronounced in Estonia with a moderate disease incidence rather than in Finland with the highest rate of T1D in the world.

3. Circulating IGF-I and IGFBP-3 may play a role in the development of β-cell autoimmunity in young children. Our results suggest a possible link between the previously reported association between HLA genotypes conferring risk for T1D, early postnatal growth and the IGF-I/IGFBP-3 system.

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SUMMARY IN ESTONIAN

Pre- ja postnataalne kasv lastel 1. tüüpi diabeedi riski kandvate HLA genotüüpidega

Üle kogu arenenud maailma kasvab 1. tüüpi diabeedi (T1D) esinemissagedus keskmiselt 3–4% aastas (Patterson jt, 2009). T1D etioloogia on multifaktoriaalne, kus olulist rolli mängivad nii geneetilised kui ka keskkonnafaktorid (Knip jt, 2005; Onkamo jt, 1999). Geneetilistest teguritest on tähtsaim HLA 2. klassi geenide osakaal, mis moodustavad T1D tekkes kogu geneetilisest riskist umbes 50% (Noble jt, 1996; Concannon jt, 2005). HLA riskigenotüübid on levinud umbes 20% valgenahalistest inimestest, kuid ainult 5% nendest haigestub 1. tüüpi diabeeti (Knip jt., 2005). Isegi geograafiliselt suhteliselt väikeses Läänemere piirkonnas erineb T1D haigestumus riigiti kuni 6 korda (Tuomilehto J jt, 1992; Teeäär jt, 2010; Harjutsalo jt, 2013). Kõik need faktid viitavad sellele, et suhteliselt kiire ja püsiva T1D haigestumuse tõusu põhjuste taga on eeskätt muutused meie elukeskkonnas, mitte geneetilises taustas (Knip jt, 2005; Onkamo jt, 1999).

Paljud uuringud on näidanud, et enne haiguse avaldumist kasvavad 1. tüüpi diabeediga lapsed kiiremini nii pikkuses kui ka kaalus, võrreldes tervete lastega (Bloom jt, 1992; Larsson jt, 2008; Ljungkrantz jt, 2008; EURODIAB Substudy 2 Study Group, 2002; Hyppönen jt, 2000; Knerr jt, 2005). Nende tulemuste valguses püstitati nn. aktseleratsiooni või ülekoormuse hüpotees (Wilkin, 2001). Selle hüpoteesi kohaselt viib pidev toidu üleküllus, rasvumine ja kiirenenud kasv juba varases lapsepõlves insuliini produktsiooni vajaduse tõusuni, mille tulemusel kõhunäärme β-rakud on sunnitud intensiivsemalt töötama, mis muudab nad aga igasuguse stressi suhtes vastuvõtlikumaks. Viimane omakorda kiirendab apoptoosi (programmeeritud rakkude surm) ja rakkude destruktsiooni erinevate faktorite toimel (Wilkin, 2001). Olulist rolli selle hüpoteesi kohaselt mängib insuliini resistentsus, mille väljakujunemist soodustab liigne kaalutõus. Insuliini resistentsus stimuleerib β-rakkude metaboolset aktiivsust, suurendab nende haavatavust ja antigeenseid omadusi (Wilkin, 2001). Viimane soodustab omakorda autoimmuunset kõhunäärme Langerhansi saarekeste põletikku (Dahlquist, 2006; Gale, 2005) ja β-rakkude kadu (Wilkin, 2009). Suurema sünnikaalu ja T1D haigestumise riski vaheline positiivne korrelatsioon osutab sellele, et kiirenenud kasv koos teiste keskkonnafaktoritega võivad mängida olulist rolli T1D väljakujunemises juba intrauteriinselt (Cardwell jt, 2010; Harder jt, 2009).

1. tüüpi diabeedi eelsoodumust põhjustavate geneetiliste faktorite roll kiirenenud pre- ja postnataalses kasvus ei ole selge. Publitseeritud on rida uuringuid, mis on andnud vasturääkivaid tulemusi. On leitud, et teatud HLA genotüübid mõjutavad kasvu positiivselt, teised negatiivselt (Carlsson jt, 2012; Larsson jt, 2005; Larsson jt, 2008; Stene jt, 2001; Järvinen jt, 2008; Yang jt, 2014). Osa uuringuid on näidanud, et ka keskkonnategurid võivad mõjutada HLA genotüübi ja kasvamise vahelist seost (Taylor jt, 2006; Hummel jt, 2007; Larsson jt, 2007). Kuna ka keskkonnafaktorid mängivad olulist rolli T1D

esinemissageduse tõusus (Knip jt, 2005; Onkamo jt, 1999), võib oletada, et HLA genotüüpide mõju kasvule võiks olla rohkem väljendunud kõrge T1D esinemissagedusega riikides. Sellistes riikides peaks diabetogeense keskkonna surve kasvule ning HLA genotüübi ja kasvu vahelisele seosele olema suurem. Käesoleva ajani läbiviidud uuringud on piirdunud enamasti ühe riigiga (Järvinen jt, 2008; Larsson jt, 2005; Stene jt, 2006) või väga sarnase T1D esinemissagedusega riikidega (Yang jt, 2014; Sterner jt, 2011).

Kuna kasvuhormooni ja insuliini sarnase kasvufaktori I (IGF-I) telg on lapseeas kõige olulisem kasvu reguleeriv süsteem, siis diabeedi avaldumisele eelnev kiirenenud kasv võib osutada sellele, et IGF-I võib mängida olulist rolli ka diabeedi tekkes. Sellele viitavad uuringud, mis on näidanud IGF-I (Agudo jt, 2008; Anguela jt, 2013; Castrillo jt, 2000; George jt, 2002) ja seda peptiidi siduva peamise valgu (*binding protein*) (IGFBP-3) (Jones ja Clemmons, 1995; Butler jt, 1996; Chan jt, 2005; Kim jt, 2007) rolli T1D arengus. Mitmed loommudelid on näidanud, et IGF-I suurendab eksisteerivate β-rakkude replikatsiooni ja proliferatsiooni pärast kahjustust (Agudo jt, 2008), kaitseb β-rakke apoptoosi (Castrillo jt, 2000) ja Langerhansi saarekesi lümfotsütaarse infiltratsiooni eest (George jt, 2002) ning pidurdab autoimmuunse diabeedi progressiooni (Anguela jt, 2013). IGFBP-3 omab rida IGF-I sõltumatuid funktsioone (Kim, 2014; Johnson ja Firth, 2014), mille roll diabeedi patogeneesis on jäänud ebaselgeks. Mõned uuringud on näidanud positiivset seost seerumi IGFBP-3 taseme ja 2. tüüpi diabeedi vahel (Frystyk jt, 1999; Rajpathak jt, 2012). Tsirkuleeriva IGF-I ja IGFBP-3 rolli β-rakkude vastase autoimmuunsuse väljakujunemises on uuritud väga vähe, nagu ka IGF-I ja IGFBP-3 süsteemi ning HLA genotüübi kombineeritud mõju varajasele postnataasele kasvule.

Uurimuse eesmärgid

1. Uurida T1D riski kandvate HLA genotüüpide mõju sünnikaalule ja nende omavahelisi seoseid kolmes väga erineva T1D esinemissagedusega riigis.
2. Uurida T1D riski kandvate HLA genotüüpide mõju varajase postnataalse kasvu ja T1D riski vahelisele seosele kahes väga erineva T1D esinemissagedusega riigis.
3. Uurida veres tsirkuleeriva IGF-I ja IGFBP-3 rolli β-rakkude vastase autoimmuunsuse väljakujunemises ning nende võimalikku mõju T1D riski kandvate HLA genotüüpide ja varajase postnataalse kasvu vahelisele seosele.

Patsiendid ja meetodid

Käesolev töö toimus Euroopa Liidu 7. raamprojekti poolt finantseeritud uuringu DIABIMMUNE raames. Selle käigus genotüpiseeriti T1D riski kandvate HLA haplotüüpide suhtes ligi 12000 last Eestis, Soomes ja Venemaal Karjalas. Genotüpiseerimine toimus PCR-baasil, kasutades lantaniidiga markeeritud

hübridisatsiooni ja aeglahutatud fluoromeetria meetodit (Mikk jt, 2014). Esiteks toimus analüüs HLA *DQB1*02, DQB1*0301, DQB1*0302 ja DQB1*0602/3* alleelide esinemise suhtes (Kiviniemi jt, 2007). Kui see analüüs kinnitas, et tegemist on riski, neutraalse või protektiivse genotüübiga, siis edasist täpsemat genotüpiseerimist ei tehtud. Teistel juhtudel teostati rida täiendavaid analüüse, et välja selgitada lõplik riski suurus.

Esimeses uuringus kasutati esialgu 8369 vastsündinu andmeid. Diabeediga emade, enne 35 gestatsiooninädalat või mitmikrasedusest sündinud laste andmed eemaldati analüüsist. Seega jäi uuritavaid lapsi esimesse uuringusse 7931. Igale lapsele arvutati suhteline sünnikaal, mis väljendati standardhälve punktina (SDS), mille arvutamiseks kasutati vastava riigi soole ja gestatsioonivanusele vastavaid sünnikaalu aritmeetilise keskmise ja standardhälve (SD) väärtusi. Lähtudes HLA genotüüpidest, jaotati lapsed nelja rühma vastavalt T1D riskile: väga kõrge, kõrge ja mõõduka riskiga ning neutraalse/protektiivse HLA genotüübiga rühmadeks. Sünnikaalude jaotuvus kvartiilide vahel igas riskirühmas analüüsiti rühmasiseselt, rühmade vaheliselt kogu uuritavas kohordis ja ka riigiti eraldi.

Teises uuringus osales 496 last Eestist ja Soomest, kellel DIABIMMUNE uuringu raames oli vastsündinuperioodis avastatud T1D suurenenud riski kandvad HLA genotüübid ja 191 Eesti last neutraalse või protektiivse genotüübiga. DIABIMMUNE uuringus jälgiti T1D suurenenud riskiga laste kasvu 24 kuu vanuseni, neutraalse või protektiivse HLA genotüüpidega laste kasvu andmed saadi perearstide käest. Analoogselt esimese uuringuga, lähtudes HLA genotüüpidest, jaotati lapsed nelja riskirühma. Igale lapsele arvutati suhteline pikkuse SDS ja kaalu SDS vanuses 3, 6, 12, 18, ja 24 kuud, kasutades selleks soole ja vanusele vastavaid WHO kasvukõveraid (WHO Multicentre Growth Reference Study Group, 2006). Kuna Kolmogorov-Smirnovi testi tulemusena ei erinenud andmete jaotuvus erinevates rühmades normaalsest jaotuvusest, kasutati rühmade kaalude SDS ja pikkuste SDS võrdlemiseks ANOVA testi koos *post hoc* Tukey HSD analüüsiga.

Kolmandas uuringus osales 563 DIABIMMUNE uuringu last Eestist ja Soomest, kellel esinesid T1D riski kandvad HLA genotüübid. Vanuses 3, 6, 12, 18, 24 ja 36 kuud määrati neil vereseerumis autoantikehad IAA, GAD65, IA-2, ZnT8 ja ICA, kasutades selleks vastavaid spetsiifilisi teste ja piirväärtusi (Knip jt, 2010). Kui üks nendest autoantikehadest osutus positiivseks, nimetati seda serokonversiooniks. Kõigil 40 lapsel, kellel tekkis serokonversioon, määrati plasma IGF-I ja IGFBP-3 kontsentratsioon. IGF-I ja IGFBP-3 määrati ka 80 lapse kontrollil, kes sobitati vanuse, soo, päritoluriigi ja HLA genotüüpi järgi sama kohordi laste seast, kuid kellel serokonversooni ei esinenud. IGF-I ja IGFBP-3 määrati tahkefaasilise ensüümiga markeeritud kemiluminestsents-immunomeetrilise testiga kasutades IMMULITE 2000 analüsaatorit. Lisaks arvutati IGF-I ja IGFBP-3 molaarne suhe (IGF-I/IGFBP-3). Selles uuringus jagati lapsed, kellel kõigil oli suurenenud risk T1D tekkeks, HLA genotüüpide alusel kolme riskirühma: kõrge, mõõduka ja väikese (kuid suurenenud) riskiga haigestuda T1D. IGF-I ja IGFBP-3 taset võrreldi autoantikehade suhtes

negatiivsete ja positiivsete laste vahel ning erinevate HLA riskirühmadesse kuuluvate laste vahel. Andmete jaotuvust kontrolliti Kolmogorov-Smirnovi testiga. Autoantikehade suhtes negatiivsete ja positiivsete laste võrdluseks kasutati paaritut kahepoolset Student t-testi, mitte-normaalse andmete jaotuvuse korral Mann-Whitney U-testi. IGF-I ja IGFBP-3 taset erinevates riskirühmades võrreldi ANOVA testi abil koos *post hoc* Tukey HSD analüüsiga, mitte-normaalse jaotuvusega andmete puhul Kruskal-Wallis testi abil *post hoc* Bonferroni (Dunn) t-testiga. Samuti määrati IGF-I, IGFBP-3 ja IGF-I/IGFBP-3 juurdekasv ajamomentidel enne ja pärast serokonversiooni, kontroll-lastel analoogsetel ajamomentidel. Rühmadevaheline võrdlus tehti paaris t-testiga.

Tulemused

Esimeses uuringus otsest seost erinevate T1D riski kandvate HLA genotüüpide (*HLA DR3-DQ2/DR4-DQ8, DR3-DQ2/X ja DR4-DQ8/X*) ja sünnikaalu vahel kogu uuritavas kohordis, samuti ka eraldi vaadeldud riikides, ei leitud. Samas, protektiivsed alleelid HLA *DQB*0602* ja *603* kombinatsioonis riskialleeliga HLA *DQB1*0302* assotsieerusid suure sünnikaaluga: esimeses kvartiilis oli lapsi 18% *versus* 29% neljandas kvartiilis (P=0.003; OR=0.52 (CI=0.36–0.75).

Teises uuringus leidsime, et T1D suurimat riski kandva HLA genotüübiga (*DR3-DQ2/DR4-DQ8*) laste keskmise pikkuse SDS 24 kuu vanuses on oluliselt väiksem, võrreldes lastega, kel oli neutraalne/protektiivne genotüüp ($p<0.05$). Sama kehtis ka kaalu SDS suhtes vanuses 12, 18 ja 24 kuud ($p<0.05$). Kahe riigi kohordi eraldi analüüs näitas, et pikkuse SDS ja kaalu SDS osas säilisid mõlemas riigis sarnased trendid nagu kogu kohordis, kuid statistiliselt oluline erinevus oli vaid kaalu SDS osas ja ainult Eestis ($p<0.05$).

Kolmandas uuringus olid plasma IGF-I kontsentratsioon 12 kuu vanuses ja IGF-I/IGFBP-3 24 kuu vanuses oluliselt madalamad lastel, kellel olid tekkinud autoantikehad, võrreldes nendega, kellel neid polnud ($p<0.05$). Tsirkuleeriva IGFBP-3 juurdekasv enne serokonversiooni oli oluliselt suurem, võrreldes kontroll-lastega (0.43 mg/l; 95% CI 0.29–0.56 *versus* 0.22 mg/l; 95% CI 0.10–0.34 mg/l; $p<0.01$). Võrreldes väiksesse riskirühma kuuluvate lastega, oli kõrge T1D riski genotüübiga lastel plasma IGF-I ja IGFBP-3 tase 24 kuu vanuses oluliselt madalam (vastavalt $p<0.05$ ja $p<0.01$).

Järeldused

Meie uuring ei leidnud seost T1D riski kandvate HLA genotüüpide ja sünnikaalu vahel. Seos suurema sünnikaaluga oli ainult spetsiifilisel genotüübil, kus on kombineeritud protektiivsed alleelid *DQB1*0602* või *DQB1*0603* riski-alleeliga *DQB1*0302*.

Lapsed, kes on suurima geneetilise riskiga haigestuda T1D (HLA genotüüp *DR3-DQ2/DR4-DQ8*), kasvavad esimesel kahel eluaastal nii pikkuses kui ka kaalus aeglasemalt, võrreldes lastega, kel suurenenud risk puudub (neut-

raalse/protektiivse HLA genotüübiga). See seos on enam väljendunud Eestis, kus T1D esinemissagedus on oluliselt madalam kui Soomes.

Tsirkuleeriv IGF-I ja IGFBP-3 võivad osaleda β-rakkude vastase autoimmuunsuse väljakujunemises. Uuringu tulemused osutavad võimalikule seosele T1D riski kandvate HLA genotüüpide poolt modifitseeritud postnataalse kasvu ning IGF-I ja IGFBP-3 süsteemi vahel.

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List of publications

Talvik I, Peet A, Talvik T. Three-year follow-up of a girl with chronic paroxysmal hemicrania. Pediatr Neurol 2009; 40:68–69.

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