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Factors that influence age of type 1 diabetes onset and beta cell function in children and adults with newly diagnosed type 1 diabetes

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ABBREVIATIONS

DM	diabetes mellitus
T1D	type 1 diabetes
T2D	type 2 diabetes
HbA1c	glycated haemoglobin 1c
IAA	antibody against insulin
GADA	antibody against glutamate decarboxylase
IA-2A	antibody against tyrosine phosphatase related molecules IA-2a
ZnT8	zinc transporter 8
HLA	human leucocyte antigen
OR	odds ratio
SNP	single nucleotide polymorphism
GWAS	genome-wide association studies
INS	insulin gene
PTPN22	protein tyrosine phosphatase, non-receptor type 22
LYP	lymphoid protein tyrosine phosphatase
CTLA4	cytotoxic T-lymphocyte associated antigen 4
IL2RA	interleukin 2 receptor, alpha chain
IL-2	interleukin-2
FOXP3	forkhead box P3
IL-10	interleukin 10
IL7Rα	interleukin 7 receptor
IL18RAP	interleukin 18 receptor accessory protein
SH2B3	SH2B adaptor protein 3
IFIH1	interferon-induced helicase C domain-containing protein 1
ERBB3	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3
CLEC16A	c-type lectin domain family 16 gene A
PTPN2	phosphotyrosine-protein phosphatase, non-receptor 2
IFN-γ	interferon γ
MHC	major histocompatibility complex
DCCT	Diabetes Control and Complications Trial
HSC	hematopoietic stem cells
MSC	mesenchymal stem cell
hESC	human embryonic stem cell
NOD	non-obese diabetic
Treg	T regulatory cells
CDKAL	CDK5 regulatory subunit associated protein 1-like 1
CDKN2A	cyclin-dependent kinase inhibitor 2A
FTO	fat mass and obesity associated
GCKR	glucokinase (hexokinase 4) regulator

HHEX/IDE	hematopoietically expressed homeobox/ insulin-degrading enzyme
HMGA2	high mobility group AT-hook 2
IGF2BP2	insulin-like growth factor 2 mRNA binding protein 2
KCNJ11	potassium inwardly-rectifying channel, subfamily J, member 11
KCNQ1	potassium voltage-gated channel, KQT-like subfamily, member 1
MTNR1B	melatonin receptor 1B
PPARG	peroxisome proliferator-activated receptor gamma
SLC30A8	solute carrier family 30 (zinc transporter), member 8
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)
THADA	thyroid adenoma associated
WFS1	Wolfram syndrome 1 (wolframin)
WBC	white blood cells
SD	standard deviation
DMSO	Dimethyl sulfoxide
NaCl	Sodium Chloride
TNC	total nucleated cells

1. INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease, characterized by the progressive destruction of the β cells of the pancreas, leading to severe insulin deficiency (Cnop et al 2005). The rising incidence of T1D over the last decade (Patterson et al 2009) has led to a growing interest in investigating the specific causes, genetic or environmental, that are responsible for T1D manifestation and disease progression (Todd 2010). While specific HLA genotypes are found to provide high susceptibility to T1D mainly by acting on the initiation of islet autoimmunity (Erlich et al 2008, Todd 2010), little is known whether non-HLA genes affect age at diagnosis and whether they continue to influence disease progression after diagnosis, by accelerating the loss of β cell function.

Both type 1 and type 2 diabetes (T2D) result from the metabolic consequences of inadequate insulin secretion or impaired β cell function, regardless of their differences in the basic pathogenic mechanism (American Diabetes Association 2012). Numerous T2D associated gene variants are reported to contribute to the development of T2D, by decreasing insulin secretion and impairing β cell function (Grarup et al 2010, van de Bunt et al 2010). It still remains unclear whether such T2D associated gene polymorphisms are associated with age at onset or β cell function in T1D. Aim of this thesis was to investigate for the first time the cumulative effect of various T1D and T2D associated SNPs on age of disease onset and β cell function.

Despite significant advances in diabetes management over the past years, controlling blood glucose levels with exogenous insulin administration remains still burdensome for patients and families (Gallagher et al 2011). As a vast source of

primitive hematopoietic stem cells and endothelial progenitor cells, cord blood is considered nowadays a safe treatment option for pediatric patients with various malignant and nonmalignant diseases (Smythe et al 2007, Rocha et al 2009, Wagner et al 2002). The introduction of cord blood in the regulation of immune imbalance in various autoimmune diseases such as T1DM has been suggested and gained great interest during the past years (Dejaco et al 2006). In the second part of this thesis, a non-randomized, open-label, controlled trial was performed in order to investigate the effect of a single autologous cord blood infusion on the natural course of β cell and immune function in newly diagnosed children.

1.1 Definition and Diagnosis of Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic disorders of multiple aetiology characterized by chronic hyperglycemia, resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association 2012). Several pathogenic processes are involved in the development of diabetes; these range from autoimmune destruction of the pancreatic β cells with consequent insulin deficiency to abnormalities that result in resistance to insulin action (American Diabetes Association 2012). The vast majority of cases of diabetes fall into two broad etiopathogenic categories; in the first category, T1D, the cause is an absolute deficiency of insulin secretion. In the second, T2D, the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (American Diabetes Association 2012).

The current diagnostic criteria of DM include the following: a) fasting plasma glucose ≥ 126mg/dl (7.0mmol/l), where fasting is defined as no caloric intake for at least 8 hours, or b) symptoms of diabetes -polyuria, polydipsia and unexplained weight loss- plus casual plasma glucose concentration ≥ 200mg/dl (11.1 mmol/l), where casual is defined as any time without regard to time since last meal, or c) 2-hour-plasma glucose ≥ 200mg/dl (11.1mmol/l) during an oral glucose tolerance test (Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 1997). The test should be performed according to WHO guidelines (Report of a WHO Study Group 1985), using a glucose load containing the equivalent of 1.75 g anhydrous glucose dissolved in water/kg body weight (max.75g). A new diagnostic criterion was added recently and concerns the level of glycated

hemoglobin (HbA1c), with a threshold of 6.5% (American Diabetes Association 2012).

Diabetes mellitus is one of the most rapidly growing chronic diseases worldwide; in 2010 the world prevalence of diabetes among adults (aged 20-79 years) was estimated to be 6.4% and late predictions indicate that it will rise to 7.7% by 2030. Between 2010 and 2030, there will be a 69% increase regarding adults with diabetes in developing countries and a 20% increase in developed countries (Shaw et al 2010).

1.2 Type 1 Diabetes

T1D is a chronic autoimmune disease, which involves the progressive autoimmune destruction of the β cells of the pancreas via effector T cells and macrophages, leading to severe insulin deficiency (Cnop et al 2005). Immune mediated β cell destruction has been found to begin months or sometimes years before the clinical manifestation of the disease, which usually appears after the majority of β cells have been destroyed (Kloppel et al 1985). Therefore, the first clinical symptoms are associated with lack of insulin and include polyuria, polydipsia, polyphagia and weight loss.

The symptomless, silent phase can be easily identified through specific serological markers of autoimmunity, which include the autoantibodies against insulin (IAA), the 65-kDa isoform of glutamate decarboxylase (GADA), protein tyrosine phosphatase related molecules IA-2a (IA-2A) as well as zinc transporter 8 (ZnT8) (Atkinson et al 2001, Achenbach et al 2009). Screening for autoantibodies displays nowadays a useful tool in identifying individuals at increased risk for T1D. Prospective studies following genetically at-risk children from birth have contributed to the understanding of the natural course of T1D with regard to autoantibody appearance (Ziegler et al 1993, Rewers et al 1996, Honeyman et al 2000, Kupila et al 2001). According to results of the German BABYDIAB trial, a longitudinal study examining the natural history of islet autoimmunity and T1D in 1650 offspring of diabetic parents, seroconversion to antibody positivity occurs mostly in the period between 9 months to 2 years, is found to be rare at the age of 6 months or younger and IAAs are usually the first to appear (Ziegler et al 2012).

The relative annual increase of T1D varies between countries and has been recently reported to be on average 3.9% in Europe. According to late predictions, if present trends continue, doubling of new cases of T1D in European children younger than 5 years is predicted between 2005 and 2020, and the prevalence of cases of individuals younger than 15 years will rise by 70% (Karvonen et al 2000, Patterson et al 2009).

1.3 Etiology of T1D

Several timelines have been proposed over the years that represent the outcome of the interplay between genetic and environmental factors in T1D. The most popular model proposed by Eisenbarth in 1986 (Figure 1), suggests that in the context of genetic predisposition, an environmental trigger induces islet autoimmunity and progressive β cell destruction, which lead to a sequence of prediabetic stages and finally clinical manifestation (Eisenbarth 1986).

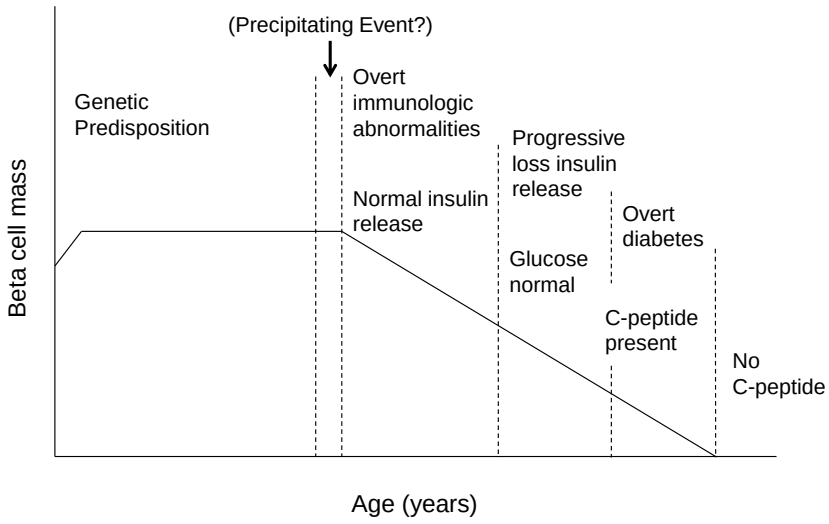

Figure 1. Stages in the development of T1D (Eisenbarth 1986).

1.3.1 Genetics

Children with a first degree relative suffering from T1D have a 5% risk for T1D by the age of 20 in contrast to a 0.3% risk for children without affected family members (Bonifacio et al 2010). Furthermore, it has been shown that by age of 60, the cumulative incidence of diabetes among monozygotic twins is 65% (Redondo et al 2008); both observations support the strong genetic contribution to T1D.

Human Leucocyte Antigen Complex (HLA)

Early studies have indicated that the HLA region on chromosome 6p21.31 (IDDM1) is a critical susceptibility locus for T1D, providing about 45% genetic susceptibility (Bertrams et al 1976, Buzzetti et al 1998). Specific HLA region genes, in particular the genes that encode the classical HLA class I (A, B, and C) and class II (DR, DQ and DP) antigens, are involved in the immune response to both environmental pathogens as well as in various autoimmune diseases (Noble et al 2000, Blackwell et al 2009). HLA class I molecules present endogenous antigens and class II molecules present exogenous antigens to T cells (Horton et al 2004). However, determining HLA associations with T1D is complicating, not only because of the large number of reported alleles at the HLA genetic loci, the interactions of HLA with other susceptibility factors but also because of the different allele frequencies and haplotypic combinations among populations (Noble et al 2000).

Regarding HLA class II locus, several genes are pivotal as their alleles are found to determine a susceptibility hierarchy ranging from protection to strong

susceptibility (Table1), with reported odds ratios (ORs) ranging from 0.02 to >11 (Erlich et al 2008).

Table 1. T1D risk associated with HLA-DR and HLA-DQ haplotypes (Erlich et al 2008).

Risk	DRB1	DQA1	DQB1
High risk	0401, 0402, 0405	0301	0302
	0301	0501	0201
Moderate risk	0801	0401	0402
	0101	0101	0501
	0901	0301	0303
Weak or moderate protection	0401	0301	0301
	0403	0301	0302
	0701	0201	0201
	1101	0501	0301
Strong protection	1501	0102	0602
	1401	0101	0503
	0701	0201	0303

DR3/4-DQ8 heterozygous haplotypes (DR3 is DRB1*03-DQB1*0201, DR4 is DRB1*04-DQB1*0302, DQ8 is DQA1*0301/DQB1*0302) represent the main predisposing molecules (Erlich et al 2008). It is important to note that circa 30-50% of patients with T1D carry the high risk genotype DR3/4-DQ2/8 (van Belle et al 2011). Other susceptible haplotypes include the DRB1*0402-DQA1*0301-DQB1*0302 and DRB1* 0404-DQA1*0301-DQB1*0302 (Atkinson et al 2001, Erlich et al 2008). On the other hand, the DRB1*1501-DQA1*0102-DQB1*0602 haplotype, found in circa 20% of the population but in only 1% of patients, is negatively associated with the disease in all populations and confers dominant protection to T1D (Erlich et al 2008). Further haplotypes, that were also found to play a protective role include DRB1*1401-DQA1*0101-DQB10503 and DRB10701-DQA1*0201-DQB1*0303 (Erlich et al 2008). Although not as strong as the effect of the known susceptible DR and DQ haplotypes, variations at class II DP molecule are also responsible for T1D risk (Noble et al 2000, Cruz et al 2004).

The exact mechanism of action of DR and DQ molecules in T1D remains elusive, however it has been associated with peptide-binding activity of the HLA class II molecules in antigen presenting cells for T lymphocyte peptide recognition. These peptides derive from the autoantigens: preproinsulin, insulinoma-associated antigen 2, glutamic acid decarboxylase and zinc transporter 8 and are presented to the CD4+ T cell antigen receptors in the thymus and in the periphery. CD4+ T cells trigger then the activation of CD8+ cytotoxic T cells (Todd 2010).

Regarding HLA class I, alleles at loci A and B have been found to provide susceptibility independently from class II; in particular, class I alleles have been found to associate with age at diagnosis of T1D (e.g. B*39) (Noble et al 2010), as well as with rapid C-peptide decline after manifestation (e.g. A*24) (Nakanishi et al 1995).

Non-HLA T1D loci

While HLA genotypes mainly act on the initiation of islet autoimmunity in T1D, it has been shown that other genes such as *IFIH1, INS* and *PTPN22* (Winkler et al 2011, Lempainen et al 2012) can influence progression to clinical overt T1D. With regard to advances in genetic screening technologies in the past 5 years as well as the application of single nucleotide polymorphisms (SNPs), genome-wide association studies (GWAs) have revealed more than 50 distinct genomic loci associated to T1D (Burren et al 2011). GWAs give information on thousands of SNPs across the entire genome by comparing SNP allele frequency differences between cases and controls (Visscher et al 2012). Many of these loci provide modest susceptibility or protection

to T1D, with ORs ranging from 0.6 to 2, and they serve to better define disease pathogenesis and potentially identify patients at risk. A few of these regions contain genes of unknown function (Burren et al 2011). Little is known whether these genes also affect other important factors regarding T1D manifestation and course, such as age at diagnosis or progression of β cell function loss.

One of the first discovered non-HLA regions is the insulin gene (*INS*), which is located on chromosome 11p15.5 and encodes the pre-proinsulin peptide. This locus has the second highest effect magnitude that attributes about 10% of genetic susceptibility to T1D (Bell et al 1984, Barratt et al 2004). It maps to a variable number of tandem mini-satellite repeats, where shorter forms of class I VNTR alleles (26 to 63 alleles, OR >2) predispose to the disease, while longer forms of class III VNTR alleles (140 to 210 repeats) have a dominant protective role (Vafiadis et al 1997). The mechanism for the protective effect of class III alleles can be explained, as class III VNTR alleles have been associated with 2 to 3 fold higher levels of insulin mRNA expression in the thymus and therefore with immune tolerance induction in contrast to class I VNTR alleles (Vafiadis et al 1997). Intermediate (class II) alleles are rare. Two SNPs of the *INS* gene have been associated with T1D, *rs689* (T minor allele, OR 0.42) (Smyth et al 2008) and *rs7111341* (T minor allele, no given OR) (Barrett et al 2009). *Rs689* has been associated with age at diagnosis of T1D as homozygosity for the protective T allele was found to be responsible for delaying the onset of the disease for approximately 2 years (Howson et al 2009).

PTPN22 (protein tyrosine phosphatase, non-receptor type 22) is the third major locus affecting risk for T1D after HLA-DR/DQ and *INS*. *PTPN22* is located on 1p13.2 and encodes a lymphoid protein tyrosine phosphatase (LYP) (Bottini et al

2004, Liu et al 2012), a down-regulator of T-cell activation, acting by dephosphorylating T-cell receptor-proximal signalling (Bottini et al 2004, Liu et al 2012). A single-nucleotide polymorphism (*rs2476601*) of *PTPN22* resulting in an amino acid substitution (R620W) of arginine by tryptophan, has been associated with increased inhibition of T-cell receptor signalling (Steck et al 2009b). T cells in patients carrying the risk allele for several autoimmune diseases were found to produce less interleukin-2 upon T-cell antigen receptor stimulation and the encoded enzyme was found to have a higher catalytic activity (Vang et al 2005). OR for T1D for *rs2476601* (A minor allele) was calculated to be 2.05 (Smyth et al 2008, Barrett et al 2009). *Rs2476601* has also been associated with other autoimmune diseases, such as Crohn's disease (Barrett et al 2009, Jostins et al 2012), rheumatoid arthritis (Begovich et al 2004, Stahl et al 2010) and systemic lupus erythematosus (Gateva et al 2009, Steck et al 2009a).

CTLA4 (cytotoxic T-lymphocyte associated antigen 4), located on the 2q33 locus, encodes a T-cell-specific transmembrane co-receptor. This gene is a good candidate for T1D as it is a negative regulator of T-cell activation (Walunas et al 1994). More specifically, it encodes a co-receptor on the cell surface of T-lymphocytes that binds the same B7 ligands that activate CD28, an important component of T-cell co-stimulation (Walunas et al 1994). *CTLA-4* transduces its inhibitory effect through cytoplasmic phosphatases (Chuang et al 2000). The first described polymorphisms, that proved an association between *CTLA4* and T1D, were found in the 5' flanking region of the gene (+49G >A in exon 1 and C318T in the promoter region) (Nistico et al 1996, Qu et al 2009a). Subsequent fine mapping of the *CTLA-4* gene region has rejected +49G >A as the only causal polymorphism

of T1D susceptibility (Qu et al 2009b) and stronger association has been found with *rs3087243* (A minor allele, OR 0.82), a polymorphism in the 3' flanking region (Ueda et al 2003, Smyth et al 2008). *CTLA4 p*olymorphisms have been associated with various autoimmune diseases, including celiac disease (Smyth et al 2008), Grave's disease, Addison's disease and rheumatoid arthritis (Stahl et al 2010).

One of the most recently associated via tag candidate gene is *IL2RA (interleukin 2 receptor, alpha chain)*, located on chromosome 10p15.1 (Vella et al 2005). *IL2RA* encodes the α chain of the interleukin-2 (IL2) receptor complex, also known as *CD25* (Leonard et al 1985). IL2 is a powerful growth factor for lymphocytes and *IL2RA* is highly expressed in forkhead box P3 $(FOXP3)^{+}$- regulatory $CD4^{+}$ T cells, which are known to play a key role in self-tolerance and therefore in inhibiting autoimmune diseases (Qu et al 2009a). In T1D, the noncoding variants in *IL2RA* are found to alter gene transcription, affecting expression of CD25 on the surface of naïve and memory T cells, and IL2 production by stimulated memory T-cells (Dendrou et al 2009). *IL2RA* expressed from the protective allele results in higher amounts of CD25 on memory CD4+ T cells and more CD25+ naïve CD4 T cells. Memory CD4+ T cells with higher amounts of CD25 on their surface lead to a greater IL2 secretion on stimulation (Dendrou et al 2009, Pociot et al 2010). The strongest T1D association occurs in SNPs at the 5' end of the gene and flanking region (Dendrou et al 2009) and include the following: *rs12722495* (G minor allele, OR 0.62), *rs11594656* (A minor allele, OR 0.87), *rs12722495* (G minor allele, OR 0.62) and *rs11594656* (A minor allele, OR 0.87) (Smyth et al 2008). *IL2RA* polymorphisms have been associated with other autoimmune diseases, such as multiple sclerosis (Maier et al 2009), vitiligo (Jin et al 2010) and rheumatoid arthritis (Plant et al 2010).

Several cytokine genes are included in the list of genetically mapped candidate genes. IL10, which is an anti-inflammatory cytokine, has been implicated in many autoimmune diseases, including T1D (Barrett et al 2009), ulcerative colitis (Franke et al 2008) and Crohn's disease (Franke et al 2010). The IL10 gene is located on the 1q32.1 chromosome region; the strongest association with T1D has been found with the *rs3024505* (C minor allele, OR 0.84) (Barrett et al 2009). The gene encoding the alpha chain of the IL7 cytokine receptor has been also implicated in the genetics of T1D and multiple sclerosis (Gregory et al 2007). Four different haplotypes have been described so far, however only *rs6897932* (T minor allele), which is associated with reduced splicing of the transmembrane domain, causing less production of soluble IL7Rα, displays protection against T1D (Todd et al 2007, Hoe et al 2010). At last but not least, a known celiac disease gene variant in the *IL18RAP* (interleukin 18 receptor accessory protein) gene, *rs917997* (A minor allele, OR 0.98), has been negatively associated with T1D (Smyth et al 2008, Barrett et al 2009).

The *SH2B3* (SH2B adaptor protein 3) gene is located on the 12q24.12 locus and encodes the lymphocyte adaptor protein, which plays a pivotal role as suppressor of T-cell receptor-mediated immune signaling and negative regulator of lymphopoiesis and early hematopoiesis (Rudd 2001, Takaki et al 2002). *SH2B3* was first associated with T1D (Todd et al 2007), in which a non-synonymous (ns) SNP, *rs3184504* Arg262Trp (A minor allele, odds ratio 1.28), was the best associated marker (Smyth et al 2008). Apart from T1D, *rs3184504* has been associated with celiac disease (Hunt et al 2008, Dubois et al 2010), rheumatoid arthritis (Stahl et al

2010) and multiple sclerosis (International Multiple Sclerosis Genetics Consortium (IMSGC) 2009).

The *IFIH1* gene (interferon-induced helicase C domain-containing protein 1, also known as MDA-5, melanoma differentiation-associated 5) is located on chromosome 2q24.2 and encodes an RNA helicase which plays a crucial role in the innate immune response to viral antigens (Kato et al 2006). It has been described, that *IFIH1* actually protects the host from viral infection by sensing viral nucleic acid in the cytoplasm and triggering a cellular antiviral and apoptotic response (Gitlin et al 2006). Among the viruses that *IFIH1* recognizes are viruses belonging to the picornavirus family, of which enterovirus is a member (Kato et al 2006). *IFIH1* is regarded as a good functional candidate for T1D because of the numerous associations between T1D and viral infections, especially enteroviruses (Dotta et al 2007). Four rare polymorphisms (*rs35744605*, *rs35337543*, *rs35732034*, *rs35667974*) of *IFIH1*, leading to inhibited function, have been found to offer protection against T1D. These rare *IFIH1* variants display stronger protective effects on T1D (OR 0.51-0.74) in contrast to the common nsSNP *rs1990760* (G minor allele, OR: 0.86) (Smyth et al 2008).

The *ERBB3* (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3) gene, located on the 12q13 locus, encodes the human epidermal growth factor receptor 3 of the type I receptor tyrosine kinase family (Nikitin et al 2010). The tyrosine kinase family also includes EGFR, HER2/neu and HER4 (Linggi et al 2006). All ErbB receptors are involved in a variety of cellular responses that range from migration to adhesion and from growth to apoptosis (Nikitin et al 2010). The membrane-bound protein HER3 forms heterodimers with other epidermal growth

factor receptor family member which leads to the activation of specific pathways like cell proliferation or differentiation (Singer et al 2001). Amplification of the *ERBB3* gene and/or over expression of HER3 has been associated with numerous types of cancer, including breast, prostate and bladder and gastric cancer (Wang et al 2010). *Rs2292239* is found to be associated with T1D and its heterozygotic OR (A risk allele) is reported to be 1.28 (CI 1.21-1.35) (Todd et al 2007).

The *CLEC16A* (C-type lectin domain family 16 gene A, formerly *KIAA0350*) gene, located on chromosome 16p13, is expressed almost exclusively in immune cells, including B lymphocytes, dendritic cells and natural killer cells, and encodes a predicted protein sequence with a C-type lectin domain (Hakonarson et al 2007). The function of *CLEC16A* involves antigen uptake and presentation by dendritic cells and β cells, as well as with activation, survival and differentiation of hematopoietic-derived cells (Bates et al 1999, Todd et al 2007). However, the functional mechanism of this locus remains unclear, as none of the associated SNPs change amino acids or show effects on mRNA levels (Hakonarson et al 2007). An association between *rs12708716* and T1D has been reported (G minor allele, OR 0.81) (Smyth et al 2008, Patterson et al 2009).

The *PTPN2* (phosphotyrosine-protein phosphatase, non-receptor 2) gene is located on 18p11.21 chromosome and is expressed in immune cells. Phosphatase *PTPN2* acts as a negative regulator of several signaling pathways, including Janus kinases, signal transducer and activator of transcription, p42/44 MAPK, epidermal growth factor receptor and insulin receptor β, and modulates β-cell apoptosis induced by interferon (IFN)-γ (Brown-Shimer et al 1990). The contribution of this gene in T1D pathogenesis is probably based on its role to protect β cells against

apoptosis due to negative feedback on IFN signaling (Colli et al 2010). Several SNPs have been studied and associated with T1D, including *rs1893217* (G minor allele), *rs45450789* (G minor allele, OR: 1.28) and *rs478582* (G minor allele, OR: 0.83) (Smyth et al 2008, Barrett et al 2009).

The majority of the best-established genes that contribute to T1D susceptibility are described above; for sure, additional genetic associations may result from whole-genome sequencing in the coming years.

Type 2 diabetes associated loci

Both T1D and T2D result from metabolic consequences of inadequate insulin effect and secretion, they have similar complications but they differ in their pathogenetic mechanisms (American Diabetes Association 2012). However, it has been suggested that T1D and T2D may share a common genetic aetiology (Wilkin 2001). Over the past years many loci showing genome-wide significant association with type 2 diabetes and hyperglycemia have been detected and evidence exists that most of these variants, for example *CDKAL1 rs10946398, CDKN2A rs2383208, IGF2BP2 rs4402960, KCNJ11 rs5215, MTNR1B rs1387153, SLC30A8 rs3802177* and *TCF7L2 rs7901695* act by impairing pancreatic β-cell function (Table 2) (Grarup et al 2010).

Of the known T2D associated loci, several have been tested in previous reports, (such as *TCF7L2, PPARG, IGF2BP2, WFS1, CDKAL1, SLC308A, CDKN2A/B, IDE/HHEX, KCNJ11, FTO and HNF1B)* but failed to prove any significant contribution to pediatric T1D risk (Field et al 2006, Qu et al 2008, Raj et al

2009). However, it remains unclear whether these loci may promote the early onset of T1D manifestation or whether they affect an individual's ability to preserve their residual β-cell function after T1D onset.

Table 2. Overview of loci associated with T2D at genome-wide significance (Grarup et al 2010, p.487-489)

Nearest gene(s)	Lead SNP	Proposed mechanism
Potassium Inwardly-Rectifying Channel, Subfamily J, Member 11 *(KCNJ11)*	*rs5219*	Impaired β-cell function and impaired glucagon suppression
Transcription Factor 7-Like *(TCF7L2)*	*rs7901696* *rs7903146*	Impaired incretin-stimulated insulin release
Wolfram Syndrome 1 *(WFS1)*	*rs10010131*	Impaired incretin-stimulated insulin release
Hematopoietically expressed homeobox *(HHEX)*	*rs1111875*	β-cell dysfunction
Insulin-degrading enzyme *(IDE)*	*rs5015480*	β-cell dysfunction
Solute carrier family 30 (zinc transporter), member 8 *(SLC30A8)*	*rs13266634*	Impaired formation of insulin granules impairing insulin release
CDK5 regulatory subunit associated protein 1-like 1 *(CDKAL1)*	*rs10946398*	β-cell dysfunction
Cyclin-dependent kinase inhibitor 2A *(CDKN2A)*	*rs10811661*	β-cell dysfunction
insulin-like growth factor 2 mRNA binding protein 2 *(IGF2BP2)*	*rs4402960*	β-cell dysfunction
Potassium voltage-gated channel, KQT-like subfamily, member 1 *(KCNQ1)*	*rs2237895*	Decreased incretin secretion
Thyroid adenoma associated *(THADA)*	*rs7578597*	Reduced β-cell mass due to increased apoptosis
Melatonin receptor 1B *(MTNR1B)*	*rs10830963*	Impaired melatonin-stimulated insulin release
Peroxisome proliferator-activated receptor gamma *(PPARG)*	*rs1801282*	Whole-body insulin resistance
Glucokinase (hexokinase 4) regulator *(GCKR)*	*rs780094*	Hepatic insulin resistance
Fat mass and obesity associated *(FTO)*	*rs8050136* *rs9939609*	Increased BMI-dependent insulin resistance

1.3.2 Environmental factors

Although genetic contribution to T1D is strong, it is widely supported that the rising incidence of the disease should be attributed to environmental factors (Gillespie et al 2004, Nikitin et al 2010). Several studies with twins support this opinion, as concordance rates between monozygotic twins amount to circa 30% while between

dizygotic twins to 10% (Barnett et al 1981, Kaprio et al 1992, Kyvik et al 1995). The majority of discordant identical twins of parents with T1D expresses diabetes specific autoantibodies and develops diabetes, but islet autoantibodies in the second twin may appear many years after the first twin develops diabetes (Redondo et al 2008). The most popular disease modifiers/environmental factors studied so far include: viral infections, early infant diet, toxins, vaccine administration, climatic influences and psychological stress (Knip et al 2010, van Belle et al 2011, Coppieters et al 2012).

An initial observation of the seasonal variation in the incidence of T1D has led to the conclusion that there might be an association with viral infections (Christy et al 1979). Since then, a significant number of viral strains have been associated with T1D, positively or negatively, including enteroviruses, with the best representative Coxsackie virus B, rotaviruses, mumps virus, rubella virus and cytomegalovirus; however, no direct relation has been proven so far (Menser et al 1978, Hyoty et al 1988, Pak et al 1988, Honeyman et al 1998, Honeyman et al 2000, Hyoty et al 2002, van Belle et al 2011).

The role of bacteria, and especially the bacterial composition of the intestine, has been and continues to be under examination, as autoimmunity could be strongly connected to a disturbance in the intricate microbial balance in the intestine (van Belle et al 2011). It appears that factors that could alter the balance of gut microbiota such as antibiotics and probiotics, may influence the immune response, but still a lot of investigation remains in terms of understanding the role of intestinal flora in T1D development (Vaarala et al 2008, Boerner et al 2011, Atkinson et al 2012).

Among the environmental factors that have been studied are vaccinations, as their introduction during childhood and the growing prevalence of T1D in developed countries appeared to happen concurrently. However most studies conducted so far have not established a clear association between increased risk for T1D and the routinely recommended childhood vaccines (Blom et al 1991, Jefferson et al 1998, DeStefano et al 2001).

Further environmental triggers that have been under investigation include the introduction of cow's milk and gluten in a child's diet during the first months of life and vitamin D deficiency (Knip et al 2010). Cow's milk has been implicated because of the cross-reactivity found between serum antibodies to albumin and β-cell surface protein ICA-1 (Karjalainen et al 1992). Gluten has been implicated too, because of the fact that T1D patients as well as their first-degree relatives present an increased prevalence of celiac disease (Cronin et al 1997). Recent studies have shown that removal of gluten in islet autoantibody positive children does not prevent from progression to T1D (Hummel et al 2002) and that early introduction (<3 months of age) of gluten-containing foods is a risk factor for the development of T1D associated autoimmunity in genetically at-risk offspring of parents with T1D (Norris et al 2003, Ziegler et al 2003). At last, the protective role of Vitamin D in T1D still remains unclear (Pani et al 2000, Nejentsev et al 2004).

In conclusion, although there is sufficient indirect evidence proving an association with T1D, the identification of specific environmental factors has been shown to be difficult. Aiming at this direction, an international consortium, the Environmental Determinants of Diabetes in the Young (TEDDY), has been initiated in order to follow from birth children with high genetic risk (TEDDY Study Group

2007). Results from this trial could provide insight into specific infectious agents, dietary factors or other environmental factors that trigger islet autoimmunity in genetically susceptible people.

1.4 Pathophysiology of T1D

The precise molecular and cellular pathways that lead to β cell destruction in human T1D are still not known. Animal studies (non-obese diabetic/NOD mice) have shown that a breakdown in immune regulation, which leads to the expansion of autoreactive CD4+ and CD8+ T cells, B cells and the activation of the innate immune system, is responsible for β cell destruction (DiLorenzo et al 2005). Findings from post-mortem studies of new onset patients are in agreement with these results and “insulitis” described as mononuclear cell infiltration into the pancreatic islets is a common finding (Foulis et al 1991, Moriwaki et al 1999). B-cell death in the course of insulitis is probably caused by direct contact with activated macrophages and T-cells (with a predominance of CD8+ T cells), and/or exposure to soluble mediators secreted by these cells, including cytokines, nitric oxide, and oxygen free radicals (Eizirik et al 2001). CD8+ T cells, found in insulitic lesions in mouse models and in human, destroy β cells upon activation via MHC class I expressed on β cells (Skowera et al 2008). Class II molecules in antigen presenting cells bind peptides from the known four T1D specific autoantigens and present these to CD4 T cell antigen receptors in the thymous and the periphery (e.g. pancreatic lymph nodes and islets of Langerhans) (von Herrath 2009). Another mechanism implicates Fas, which induces

apoptosis, found in β cells and Fas ligand, which is expressed by islet-infiltrating mononuclear cells (Moriwaki et al 1999).

1.5 Treatment of T1D - Clinical significance of residual endogenous insulin secretion

The major strategy for controlling blood glucose levels includes the exogenous administration of insulin, either by daily insulin subcutaneous injections or by insulin pumps. Despite the progress in insulin treatment achieved over the last years, exogenous insulin therapy remains expensive, is associated with a high rate of hypoglycaemias and brings lifestyle restrictions (Gallagher et al 2011). In addition, T1D is still associated with tremendous morbidity, premature mortality as well as a series of chronic complications, such as nephropathy, cardiovascular disease and retinopathy (Franco et al 2007).

C-peptide is considered to be a reliable index of residual β cell function in T1D patients because of its equimoral secretion with insulin and negligible hepatic extraction (Bratusch-Marrain et al 1984). The rate of C-peptide loss is variable among individuals, being especially rapid in infants and young children, and slower in adolescents and adults, some of whom may retain the ability to secrete insulin for several years after disease onset and therefore have a more stable glycemic control (Komulainen et al 1999).

The Diabetes Control and Complications Trial (DCCT) has clearly demonstrated that a good glycaemic control as well as intensive diabetes treatment, either through an insulin pump or an insulin schema of at least 3 insulin injections

per day, delays the onset and slows the progression of diabetic complications in subjects with T1D (Writing Team for the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group 2002). Retention of insulin secretion is associated with improved and more stable glucose control and data from the DCCT indicate that individuals with a low but clearly measurable stimulated C-peptide response (>0.2 pmol/ml) have a better metabolic control compared to individuals with stimulated responses below this level (The Diabetes Control and Complications Trial Research Group 1998). In addition, DCCT data demonstrate that patients with any C-peptide secretion, but especially those with the highest, have a reduced incidence of retinopathy and nephropathy (Diabetes Control and Complications Trial Research Group 1994, Writing Team for the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group 2002, Nathan et al 2005). Therefore, C-peptide is currently the appropriate end point for clinical trials performed in T1D individuals (Ludvigsson 2009).

1.6 New therapeutic approaches in T1D

Successful surgical approaches performed during the past years include whole pancreas transplantation as well as transplantation of purified pancreatic islets, currently the only techniques that can achieve normoglycemia with absolute prevention of severe hypoglycaemia and offer the potential for independence from daily insulin injections (Karvonen et al 2000, Robertson et al 2000). Both procedures are limited to only a few patients, who meet specific criteria. Pancreas transplantation is mostly performed in combination with kidney transplantation or in

patients who have already undergone kidney transplantation (Robertson et al 2003). Nevertheless, this therapeutic option is still associated with postoperative complications such as pancreatitis, thrombosis, anastomotic leak and gastrointestinal bleeding and the chronic use of immunosuppressive drugs has been associated with non-effective β cell survival (Robertson et al 2000, Naftanel et al 2004). At last but not least, although short-term results have been reported to be positive, recurrence of autoimmunity has been reported in several cases (Burke et al 2011).

Regarding islet transplantation, which is a non-invasive procedure, several protocols have been suggested in order to improve isolation, transplantation and preservation of the islets (Linetsky et al 1997, Lakey et al 1999). The introduction of Edmonton's protocol, which is mainly based on the use of a steroid free immunosuppressive formula, has shown that two-thirds of the recipients have remained insulin free one year after procedure; however most of the patients returned to insulin dependency 5 years later (Shapiro et al 2000, Ryan et al 2005). Like in pancreas transplantation, there is a lack in cadaveric donors, while in most cases recurrence of autoimmunity could not be avoided (Bosi et al 2001, Monti et al 2008). Nowadays, new techniques are focusing on microencapsulating human islets, in order to prevent rejection of the grafted tissue without the use of immunosuppressive drugs (Garfinkel et al 1998) as well as on xenogeneic islets, mainly deriving from pigs, in order to fill the gap between supply and demand in islet transplantation (Groth et al 1994, Koo et al 2008, Elliott 2011).

Currently numerous clinical intervention trials are being performed primarily in order to assess safety and efficacy of agents in preserving β cell mass and therefore

in proving long-term good metabolic control in T1D patients. These trials are either antigen specific, substitutive, palliative or combinations thereof (van Belle et al 2011).

1.6.1 The use of stem cells in regenerative medicine

Given their unique regenerative abilities, recent research into stem cells provides an exciting and potentially new approach towards finding a cure for T1D and other diseases. Stem cells are a class of undifferentiated cells found in all multicellular organisms that can divide under certain physiologic or experimental conditions through mitosis and differentiate into specialized cell types (Morrison et al 1997). In different tissues they have the ability to serve as an internal repair system, dividing essentially without limit to replenish other cells (Morrison et al 1997). Commonly, there are two types of stem cells: embryonic stem cells that are isolated from the inner cell mass of blastocysts, and non-embryonic somatic or adult stem cells that are found in various tissues. Stem cells can be categorized by their potential to differentiate into other cell types (Hall et al 1989), such as totipotent, pluripotent (e.g. embryonic stem cells and cells that derive from the mesoderm, endoderm, and ectoderm), multipotent (e.g. hematopoietic stem cells/HSCs), oligopotent (e.g. lymphoid or myeloid stem cells) and unipotent (e.g. adult muscle stem cells) (Forraz et al 2011). There are three possible sources of stem cells: bone marrow, peripheral blood and umbilical cord blood from newborns. Although bone marrow was the first source used in stem cell transplant, peripheral blood is used most often today (Champlin et al 2000).

The multilineage potential of embryonic stem cells and adult stem cells deriving from bone marrow has been examined extensively (Jiang et al 2002). Adult stem cells have been proposed as a practical alternative source, because of the ethical and political issues that accompany the use of embryonic stem cells (Borge et al 2003). In addition to ethical considerations, the use of embryonic stem cells can be limited due to the high risk of teratoma formation resulting from potential contamination with undifferentiated embryonic stem cells (Hentze et al 2009). On the other hand, adult stem cells have shown their positive effects and their therapeutic potential in the field of haematological/immune disorders, cardiovascular diseases, wound healing etc (Conrad et al 2005, Segers et al 2008).

Recently, attention has focused on controlled differentiation of stem cells to obtain specialized cells useful in treating different diseases. Several studies have demonstrated that stem cells are capable to differentiate into adipocytes, chondrocytes, osteoblasts, and myoblasts in vitro (Hauner et al 1987, Grigoriadis et al 1988, Wakitani et al 1995, Ferrari et al 1998, Johnstone et al 1998) and in vivo (Benayahu et al 1989, Bruder et al 1998a, Bruder et al 1998b). Furthermore, their immunoregulatory properties encouraged a possible use of modulating autoimmune responses and treat of autoimmune diseases (Zappia et al 2005, Sun et al 2009).

To date allogeneic and autologous hematopoetic stem cell transplantation is a treatment option for various haematological diseases such as acute myeloid leukemia, acute lymphoblastic leukemia, chronic myelogenous leukemia, myelodysplastic syndrome, T-cell lymphomas, myeloma etc (Ljungman et al 2006). Regarding autoimmune disorders, autologous haematopoietic stem cell transplantation, after appropriate conditioning to maximize immunosuppression, is

being proposed in several clinical protocols for selected patients with multiple sclerosis (Mancardi et al 2008), rheumatoid arthritis (Snowden et al 2008), systemic lupus erythromatosus (Marmont et al 2008), systemic sclerosis (Farge et al 2008) and Crohn´s disease (Cassinotti et al 2008). Transplantation as treatment for some of the above disorders is not only indicative for adults, but also for the pediatric population (Ljungman et al 2010).

Despite the reported clinical success of HSC transplantation in autoimmune diseases, the exact underlying mechanism still remains unclear. It is suggested that the subsequent generation of naïve T-lymphocytes lead to restoration of immunological tolerance by increasing the number of regulatory, FoxP3-positive T cells as well as by reactivating the thymic function (Hugle et al 2010). Unfortunately, persistence of auto-reactive cells or de novo reappearance of the autoimmune disease in a highly predisposed host sometimes leads to relapses (Hugle et al 2010).

1.6.2 Cell therapy in T1D

Stem cell therapy has emerged recently as a potential therapy for a variety of genetic, acquired and degenerative diseases (Conrad et al 2005, Segers et al 2008), (Ljungman et al 2006). Accumulating evidence suggests that embryonic stem cells are able to differentiate in vitro into insulin producing cells under specific conditions (Lumelsky et al 2001). Studies propose that after intravenous (iv) infusion of mesenchymal stem cells (MSCs), stem cells migrate and home to tissues and organs, including gut, kidney, lung liver, thymus, cardiac muscle, spleen, but also to injured sites such as pancreatic islets (Lumelsky et al 2001, Devine et al 2003, Sordi

et al 2005). To enhance the maturation process of human embryonic stem cells (hESCs)-derived insulin producing cells, a further modification of culture conditions as well as genetic manipulation methodologies has been performed (Hori et al 2002, Kahan et al 2003, Kania et al 2003). Not only embryonic but also adult stem cells have been examined regarding their abilities to differentiate to β cells and preserve β cell function (Ianus et al 2003). Transplantation of adult human bone marrow-derived MSCs could be a promising option in order to enhance insulin-producing cells due to their ability to suppress T cell responses, as mentioned above (Hugle et al 2010).

So far, a large number of experiments in diabetic mice investigated the effect of stem cells deriving from bone marrow and cord blood and their ability to facilitate the endogenous regeneration of β cells (Sordi et al 2008). The possibility of infusing stem cell transplantation to initiate tissue regeneration has also been explored as an alternative therapy in vascular diseases using both endothelial progenitor cells as well as hematopoietic progenitor cells in an effort to restore blood supply to skeletal muscle (Szmitko et al 2003).

In vitro, stem cells, and particularly MSCs, deriving from the bone marrow have been found to differentiate into insulin producing cells and in some cases to reverse diabetes in animal models (Ikehara et al 1985, Li et al 1996, Chen et al 2004, Tang et al 2004). Transplantation of adult bone marrow derived stem cells into mice with streptozotocin-induced pancreatic damage initiated endogenous pancreas regeneration and reduced hyperglycaemia 7 days after transplantation (Hess et al 2003). Furthermore, this experiment showed that the majority of the transplanted stem cells migrated to ductal and islet structures in pancreas, which resulted in the proliferation of recipient pancreatic cells and, thus, insulin production (Hess et al

2003). Additionaly, there is evidence for preventing T1D in non-obese diabetic (NOD) mice; according to a study, transplantation of purified hematopoietic stem cells into NOD mice could block the development of autoimmune diabetes (Beilhack et al 2003).

Regarding clinical trials in humans, Voltarelli et al reported in 2007 the first encouraging results of autologous hematopoietic stem cell infusion after nonmyeloablative immunosuppression with cyclophosphamide andantithymocyte globulin in a group of patients with recently diagnosed T1D. Two years later, the main results remained promising; preservation of β-cell mass as well as insulin independence was observed in the majority of the patients (Voltarelli et al 2007, Couri et al 2009). Further randomized controlled clinical trials using intrapancreatic bone marrow derived stem cell infusion in patients suffering from T2D have proven its safety and efficacy concerning endogenous insulin secretion, preservation of C-peptide levels and stabilization of blood glucose levels (Estrada et al 2008, Bhansali et al 2009). To date, clinical trials are performed to investigate the use of intra-arterial pancreatic infusion of autologous bone marrow and/or bone marrow-derived stem cells for the treatment of both T1D and T2D (Ciceri et al 2010).

1.6.3 Umbilical cord blood

Cord blood from neonates, although formerly considered biological waste, is a potential vast source of primitive HSCs, MSCs and endothelial progenitor cells available for clinical application to reconstitute the hematopoietic system and restore immunological function in affected individuals (Waller-Wise 2011). In addition, it is a

rich source of T-regulatory cells, dendritic cells and natural killer cells (Forraz et al 2011).

Cord blood can be harvested at birth by the obstetrician, who places a clamp across the umbilical cord, cannulises the umbilical vein and drains about 50-100ml of blood. Samples can be collected *in utero*, after the birth of the child and before delivery of the placenta or *ex utero*, after delivery of the placenta (Forraz et al 2011). A variety of collection methods have subsequently been proposed to optimize the collection volume and minimize the risk of bacterial contamination (Bertolini et al 1995). It is afterwards HLA typed and examined for infectious diseases. It can be safely cryopreserved for either public or private use and then transplanted at any time to patients. Cord blood stem cells compared to other adult stem cells are characterized by their high proliferative potential, as they are only at 9 months of gestational age and they have longer telomere length (van de Ven et al 2007, Waller-Wise 2011).

Regarding its haematopoietic potential, cord blood has shown many advantages, when compared to bone marrow or peripheral blood stem cells, as HSCs derived from cord blood produce larger hematopoietic colonies in vitro, are able to expand in long-term culture in vitro and have longer telomeres (Gluckman 2000, Liao et al 2011). Notably, cord blood derived CD34+ progenitors display a ~15-fold higher multilineage proliferative capacity compared to those from peripheral blood (Leung et al 1999). Except for HSCs, non-HSCs have, also, been isolated in cord blood and include characteristic mesenchymal stem cells, which are capable of in vitro differentiation to connect tissues, such as fat, cartilage and bone (Liao et al 2011). Recent studies have shown that cord blood contains specific cells capable of

more extensive differentiation; these multipotent cord blood-derived primitive stem cells can differentiate into cells from all three embryonic layers, endo-, ecto- and mesoderm (Liao et al 2011). Animal studies have indicated that these primitive cells do not have the risk to form teratomas, in contrast to embryonic stem cells. For example, the unrestricted somatic stem cells (USSCs), is a CD45neg/CD34neg population that can be differentiated in vitro into bone, adipocytes, cartilage, hematopoietic cells and neural cells and in vivo into myocardial cells, Purkinje fibers and hepatic cells (Liao et al 2011). Many studies have demonstrated their potential therapeutic applications regarding bone healing and improvement of recovery from myocardial infarction. Other isolated stem cell populations include the multilineage progenitor cells and a population of very small embryonic-like stem cells (CXCR4+lin-CD45-) (Kucia et al 2007). However, it should be noted that the number of some of these stem cell populations is influenced negatively by the thawing procedure in cases of cryopreserved umbilical cord blood (Bieback et al 2004, Kogler et al 2004, Liao et al 2011).

Cord blood is a rich source of immune cells, including naturally arising regulatory T cells (CD4+CD25+ Treg), natural killer cells and dendritic cells (McKenna et al 2011). Actually, cord blood contains significantly higher absolute numbers of T, natural killer cells and B lymphocytes compared to adult peripheral blood and even bone marrow (Szabolcs 2010). Cord blood lymphocytes are naïve and immature, they are enriched in double-negative CD3+ cells and produce fewer cytokines. Moreover, cord blood cells express mRNA transcripts for interferon-γ, interleukin-4 (IL-4), IL-10 and IL-2, and they have a fully constituted polyclonal T-cell repertoire (Garderet et al 1998).

Tregs are an immunosuppressive subpopulation of CD4+ T helper cells that play a crucial role in the establishment and maintenance of immunological unresponsiveness to self-constituents, such as immunological self-tolerance and negative control of various immune responses to non-self-antigens (Sakaguchi 2004). They are capable of suppressing a wide variety of immune cells, encompassing those of both the innate and the adaptive immune systems (Godfrey et al 2005, Dejaco et al 2006). They are characterized by the production of regulatory cytokines such as IL-4, IL-10 and transforming growth factor β. Cord blood contains Tregs, which are different from adult Tregs as they predominantly express markers of naïve T cells, such as CD45RA and CD38, being able to suppress antigen-specific T-cell responses (Wing et al 2005). Recently, it has been shown that they are responsible for preventing transplant rejection. Taking all these into consideration Tregs could be suggested for the treatment of autoimmune diseases (Godfrey et al 2005, Dejaco et al 2006).

Furthermore, cord blood is a rich source of CD16-CD56+ natural killer cells, a group of circulating lymphocytes, which participate in innate immunity. They play an anti-infectious and antitumor cytolytic role by secreting various cytokines, such as interferon γ and other cytokines that regulate the haematopoiesis (McKenna et al 2011). Earlier studies concerning cord blood-derived NK cells have presented encouraging results with potential therapeutic advantages (McKenna et al 2011).

Dendritic cells are also found in cord blood (McKenna et al 2011) and apart from their antigen presenting ability, they are able to activate several types of immune effector cells, proving their important role in the immune response (Nestle et al 2005). Dendritic cells have been isolated from cord blood and expanded and there

is great evidence that their use may be effective in the clinical field (McKenna et al 2011).

Overall, the above data demonstrate the plasticity and immunomodulatory abilities of cord blood and provide strong evidence that cord blood has potential beyond haematopoietic differentiation and could be considered for further regenerative medicine research (Dejaco et al 2006). Cord blood is rapidly available, which can be of great importance concerning high-risk patients requiring urgent transplantation. However, the major advantage of cord blood over bone marrow transplantation is the higher immune tolerance of the former and the lower risk of graft versus host disease, which allows more liberal use of HLA-mismatched grafts with rapid availability when compared with conventional matched unrelated donor transplants (Gluckman 2000). The exact reasons for this reduced incidence remain unclear, however it is widely accepted that the immaturity of cord blood T cells play a major role (Gluckman 2000). In addition, there is a very low risk of transmissible infectious diseases, such as cytomegalovirus and Epstein-Barr virus. Further advantages include the non-invasive collection of umbilical cord MSCs, the lack of risk to the donor and the lack of donor attrition (Gluckman et al 1997, Gluckman 2000, Rocha et al 2000).

Several studies have shown that strong predictors of clinical outcomes after cord blood infusion are the number of total nucleated cells as well as the CD34+ cell dose (Bittencourt et al 2002). Recent findings on graft engineering and robust ex vivo expansion protocols have increased the potential of cord blood for immune and haematopoietic reconstitution (Escalon et al 2010). However, although cord blood transfusion displays a life-saving form of stem cell transplantation, it is limited by the

high incidence of opportunistic infections during the first 6 months, most of which are of viral origin (Szabolcs 2010).

The idea of using cord blood for disease therapy was developed in the late 1970s, but H.E.Broxmeyer transferred this idea to a clinical level in 1989, when he evaluated the haematopoietic potential of human cord blood in vitro and established methods for its collection and storage (Broxmeyer et al 1989). The first successful cord blood transplantation was performed in 1989 in France in a 5-year-old boy suffering from severe Fanconi´s anemia, using his newborn sister's HLA-matched umbilical cord blood (Gluckman et al 1989). The first cord blood bank was established later, in 1991, at the New-York Blood Center and the first unrelated transplantation was performed in 1993 in a 4-year-old-child with leukaemia (Gluckman et al 2011). Later, cord blood banks established the criteria regarding the collection, banking, processing, cryopreservation and distribution of cord blood for unrelated donor transplants in patients with various haematological malignant and non-malignant diseases and knowledge about the biological characteristics of cord blood cells has increased (Gluckman et al 2011).

Since the first transplantation in 1989, more than 25.000 allogeneic, related and unrelated, cord blood transplantations have been performed worldwide and cord blood is now considered a treatment option in adult and pediatric patients with hematologic malignancies and disorders, such as leukaemia and thalassemia, bone marrow failure syndromes, inherited metabolic disorders, immunological defects and other genetic diseases (Wagner et al 2002, Laughlin et al 2004, Liao et al 2011). Recent studies have examined the use of cord blood in children with sickle cell disease, β-thalassaemia (Locatelli et al 2003), Krabbe´s disease (Escolar et al 2005)

and Hurler´s syndrome (Escolar et al 2005). Promising results have been reported for the treatment of systemic lupus erythematosus (Sun et al 2010), epidermolysis bullosa (Tolar et al 2009), inherited metabolic diseases, neonatal asphyxia and cerebral palsy (Forraz et al 2011). In 2005 the first transplantations were performed using double cord blood transplants, in order to increase the cell dose pro kg body weight for patients who could not find a single unit with enough cells (McKenna et al 2011). However, reports published later revealed controversial results regarding the unit engraftment proving that these observations still need to be confirmed (Verneris et al 2009). Chimerism data showed that only one unit can be engrafted; in some studies that was the first infused, in others it was the one with the higher CD34+ cell dose (Barker et al 2005, Ballen et al 2007, Gluckman et al 2011).

Regarding T1D, in 2004 it was initially shown that cord blood cells contain a panel of markers considered essential in β cell differentiation (Pessina et al 2004). These markers include nestin, CK-8 and CK-18, as well as the transcription factors Is1-1, Pdx-1, Pax-4 and Ngn-3 (Pessina et al 2004). Cord blood contains nestin positive islet derived progenitors, which are able to proliferate and differentiate into the main neural phenotype, neuron, astroglia and oligodendroglia, but, most importantly, they express functional receptors for glucagon-like peptide 1, which is known to induce NIPs to express insulin promoter factor 1 and to differentiate them into insulin producing cells (Abraham et al 2002, Suzuki et al 2003). Recently, Gao et al reported that human cord blood derived MSCs differentiate into islet-like cells in vitro (Gao et al 2008). In animal models, the transplantation of human umbilical cord blood cells had positive effects on glycemia and glomerular hypertrophy in mice with T2D (Ende et al 2004b). In mouse models with T1D, the same intervention resulted

in decreased blood glucose levels and provided a great protection from insulitis (Ende et al 2004a). Reversal of autoimmune diabetes has also been reported in NOD mice through stem cell infusion (Ryu et al 2001, Kodama et al 2003, Zorina et al 2003). While these results are promising, there are a number of safety factors to consider when applying these results to clinical trials.

Cord blood has become nowadays a very popular stem cell alternative source and its use in clinical research is still evolving. Several new methods in order to enhance engraftment such as the addition of mesenchymal stem cells, Notch mediated ex vivo cord blood expansion (Delaney et al 2010) and the infusion of expanded Tregs (Godfrey et al 2005) are under investigation.

2. OBJECTIVES

The rising incidence of T1D has led to a continuously growing interest in investigating the specific causes which are responsible for T1D manifestation and disease progression (Patterson et al 2009). While HLA genotypes mainly act on the initiation of islet autoimmunity in T1D, it has been shown that non-HLA genes such as *IFIH1, INS* and *PTPN22* (Winkler et al 2011, Lempainen et al 2012) can influence progression to clinical overt T1D. Little is known whether these genes or gene combinations can affect age at clinical onset and whether they continue to play an essential role in disease progression after manifestation, by influencing the rate of β cell function loss. In addition, there are various T2D associated gene variants, which are found to have a predominant effect on insulin secretion and β cell function and contribute therefore to the development of T2D (Grarup et al 2010, van de Bunt et al 2010). It remains unclear whether T2D associated SNPs can affect age at onset or β cell function loss in T1D individuals. Identification of the specific genetic parameters, that influence disease progression could provide great insight into disease pathogenesis and would therefore be rational in improving risk stratification and designing new, individualized intervention strategies. In this sense, the objectives of the first part of this thesis are:

1. to evaluate the effect of T1D and T2D associated loci on the age of disease manifestation in a cohort of children and young adults,
2. to study the association between T1D and T2D associated gene variants and fasting C-peptide values at disease onset,

3. to identify the genetic parameters, which predict C-peptide decline after T1D manifestation and

4. to identify novel risk allele scores from known T1D and T2D associated loci that accelerate disease progression, by affecting age at diagnosis or rate of C-peptide decline.

The introduction of cord blood in the treatment of various autoimmune diseases such as T1D has gained great interest during the past years, because it displays a safe alternative in regulating the immune imbalance present in such diseases (Dejaco et al 2006, Sun et al 2010). So far, Haller et al. have shown that the application of cord blood in children with T1D is safe, but fails to preserve β cell function (Haller et al 2011). It remains unclear, however, whether infused children demonstrated a better course of C-peptide preservation in contrast to natural controls. It is well described that the number of infused total nucleated cells (TNC) and CD34+ cells associate with rate of engraftment and clinical outcome in hematopoietic disorders (Bittencourt et al 2002, Rocha et al 2002, Wagner et al 2002), but unclear whether these parameters also determine the outcome in autoimmune diseases.

A non-randomized, open-label, controlled intervention trial was performed, with the starting hypothesis that autologous umbilical cord blood administered to young children with newly diagnosed T1D could preserve C-peptide levels and improve glycemic control. The objectives of the second part of this thesis are:

1. to compare the courses of metabolic (stimulated C-peptide levels, HbA1c and daily insulin use/kg) and immune function parameters (islet autoantibody titer

and T cell repertoire) over 1 year of follow-up after a single autologous cord blood infusion with the natural courses of a recruited control group and

2. to investigate the effect of the dose of TNC and CD34+ cells on metabolic outcome 6 months post-infusion.

3. PATIENTS AND METHODS

3.1. Study Population and Study Design

3.1.1 Genetic associations on new onset patients

The first analysis including genetic associations with age at diagnosis has been performed on a young population of T1D subjects (DiMelli cohort), while for the second an additional population of older patients taking part in the following intervention trials: Altered Peptide Ligand NBI-6024 (Walter et al 2009), Calcitriol (Walter et al 2010) and ChAglyCD3 trials (Keymeulen et al 2005).

3.1.1.1 DiMelli Cohort

The DiMelli study is a population-based incidence cohort study of children and adolescents with recent onset of any type of diabetes mellitus, diagnosed within Bavaria, Germany. The cohort consists of patients younger than 20 years of age at diabetes manifestation, who are registered within six months after diagnosis. The objectives of the trial are to determine incidence trends and phenotype changes of T1D and T2D and to assess the contribution of genetic predisposition and environmental risk factors to the different types of diabetes (Thumer et al 2010). For the present analysis only autoimmune T1D cases, defined by presence of at least one disease associated autoantibodies and clinical symptoms, were included. Children with negative diabetes associated autoantibodies, T2D, Maturity Onset Diabetes of the young (MODY) or neonatal diabetes were excluded. Blood samples for the determination of fasting C-peptide, HbA1c%, T1D-associated autoantibodies were obtained within 6 months after confirmation of disease diagnosis or after start

of insulin therapy. The DiMelli trial is approved by the ethics committee of Bavaria, Germany (Bayerische Landesaerztekammer, #08043) and informed consents have been obtained from children and parents/guardians. The trial was performed using the Guidelines of Good Clinical Practice.

3.1.1.2. Altered Peptide Ligand NBI-6024

A multicenter, randomized, four-arm, placebo-controlled phase 2 trial was performed with the primary objective to assess the effect of repeated administrations of NBI-6024 on endogenous insulin production as measured by C-peptide concentration in adult and adolescent patients with recent-onset type 1 diabetes (ClinicalTrials.gov Identifier: NCT00873561). Insulin usage, glycemic control, and immune function were also assessed. NBI-6024 is a soluble altered peptide ligand, which contains two natural L-amino acid substitutions in the (9-23) sequence of the B-chain of insulin; alanine is substituted for tyrosine at position 16 and at position 19 for cysteine (Giannoukakis 2002, Walter et al 2009). A total of 21 patients with a median age of 20.0 years (IQR: 15.0, 24.5 years) with recent-onset T1D were recruited between January 2003-February 2004 in the Forschergruppe Diabetes, Technische Universität München, Germany and were randomly assigned to subcutaneous administration of placebo or 1.0, 0.5 or 0.1 mg NBI-6024. All participants fulfilled the following criteria: symptom duration for no longer than 6 months, treatment with insulin for less than 3 months, positive islet autoantibodies, stimulated C-peptide peak concentration between 0.4 and 3.0 pmol/ml, Body Mass Index (BMI) <28 kg/m ^{2} and laboratory and electrocardiography screening results within normal ranges. Pregnant and lactating women were excluded. Written informed consents were

obtained from all participants, as well as from parents/guardians from subjects younger than 18 years. The study drug was administered at baseline visit, weeks 2 and 4, and then monthly until month 24. Participants were examined every 3 months in order to assess fasting and peak C-peptide concentrations during a 2-hour mixed meal tolerance test (MMTT). To avoid possible confounding through differences in glycemic control among the groups, diabetes management and glycemic targets were standardized as much as possible in all patients. All patients were treated with intensive insulin therapy (Giannoukakis 2002). The whole study design (Figure 2) as well as the results have been published; NBI-6024 did not demonstrate statistically significant efficacy compared with placebo (Giannoukakis 2002, Walter et al 2009). The Altered Peptide Ligand NBI-6024 trial has been approved by the ethics committee of Bavaria, Germany (Bayerische Landesaerztekammer, #02038) and informed consents have been obtained from all participants. The trial was performed using the Guidelines of Good Clinical Practice.

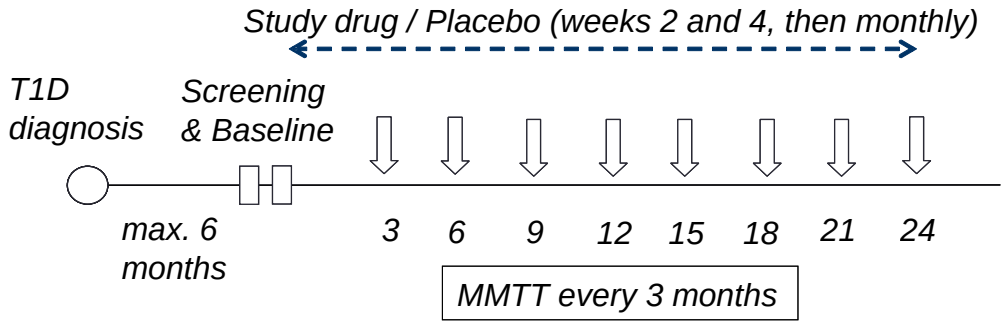

Figure 2. Flowchart presenting the study design of the NBI-6024 trial.

3.1.1.3. Calcitriol Trial

A two-phase clinical trial was performed by the Forschergruppe Diabetes, Germany starting from November 2000 (ClinicalTrials.gov Identifier: NCT00960635), in order to assess safety of daily oral $1.25(OH)_2D_3$ (Calcitriol) intake and efficacy in reducing β cell loss in recent-onset T1D patients. According to the inclusion criteria, all patients had been treated with insulin for less than 2 months, had at least one positive result for T1D autoantibodies (GADA or IA-2A) and presented screening safety parameters within normal ranges (calcium, alkaline phosphatase and creatinine). Exclusion criteria included malignancies, arterial hypertension, kidney diseases as well as calcium metabolism disorders. Pregnant or lactating female subjects were not allowed to take part in the trial (Walter et al 2010). The phase 1 trial was an open study and was designed to assess safety at a dose of 0.25μg $1.25(OH)_2D_3$ as Rocaltrol (F.Hoffmann-La Roche, Basel, Switzerland) taken daily at breakfast for 9 months. 36 new onset patients with a median age of 32.0 years (IQR: 24.1, 37.0 years) were enrolled in the trial. The phase 2 trial was a randomized, double-blind, placebo-controlled study. 39 new onset patients with a median age of 28.0 years (IQR: 22.0, 33.0 years) were recruited, randomized and assigned to oral 0.25μg $1.25(OH)_2D_3$ or placebo daily at breakfast over a 9 month period. All patients were followed for 18 months. A MMTT was performed at baseline, month 9 and 18 in order to assess β cell function (Figure 3). In both phases of the study, patients continued their normal insulin regimen as intensive insulin therapy, unless changes were clinically indicated. To avoid possible confounding through differences in glycemic control among the groups, diabetes management and glycemic targets were standardized as much as possible in all patients. Both trials were approved by the ethics committee of the Ludwig-Maximilians University, Munich, Germany

(#336/00) and written informed consent was obtained from all participants. The trial was performed using the Guidelines of Good Clinical Practice. Published results presented failure of Vitamin D supplementation to preserve β cell function in new onset patients with T1D (Walter et al 2010).

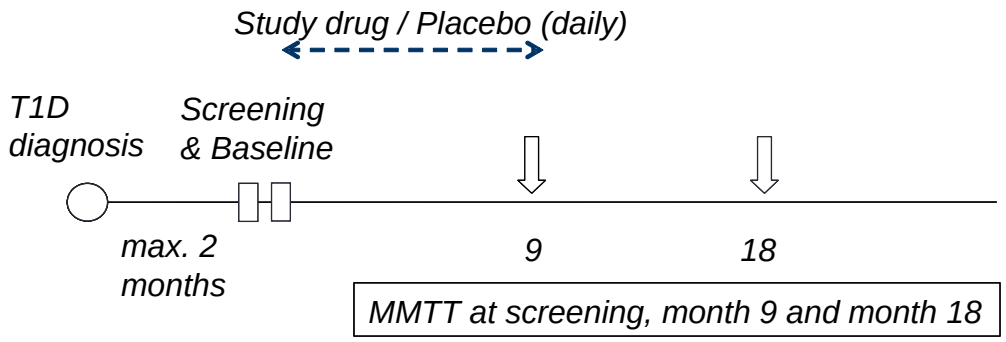

Figure 3. Flowchart presenting the study design of the Calcitriol trials.

3.1.1.4. ChAglyCD3 Study

The ChAglyCD3 study, a phase 2 multicenter, randomized, double blind, placebo-controlled trial, was performed for new onset T1D patients in order to assess efficacy of study drug in preventing β cell function decline. 26 new onset patients with a median age of 29.6 years (IQR: 23.6, 34.6 years) were recruited by the Forschergruppe Diabetes and were assigned to 8mg of a humanized antibody, an aglycosylated human IgG1 antibody directed against CD3 or placebo for 6 consecutive days. According to the inclusion criteria all participants presented the following characteristics by screening: had been treated with insulin for less than 4 weeks, had positive results for islet-cell and/or GADA, had a random plasma C-peptide >0.20nmol/l and a plasma glucose level of 180 to 250 mg/dl, had had polyuria for less than 6 months and had lost less than 10% of their body weight in the

previous 6 months (Keymeulen et al 2005). The trial was approved by the Belgian Diabetes Registry as well as the ethics committee of Bavaria, Germany (Bayerische Landesaerztekammer, #01060) and written informed consents were obtained from all participants. β cell residual function was determined by a two-phase glucose-clamp test. Patients were followed every 6 months for max.48 months (Figure 4).

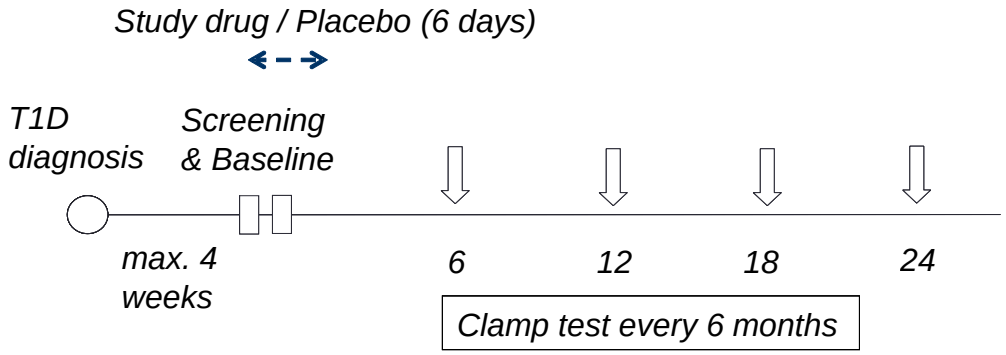

Figure 4. Flowchart presenting the study design of the ChAglyCD3 trial.

3.1.2. Autologous cord blood infusion in newly diagnosed children: The cord blood trial

A phase 2, pilot, non-randomized, open-label, controlled trial for young children with newly diagnosed type 1 diabetes was conducted in the Forschergruppe Diabetes München (ClinicalTrials.gov Identifier: NCT00989547) in order to assess safety and clinical efficacy of a single autologous cord blood infusion. Between September 2008 and March 2012, children with newly diagnosed type 1 diabetes were recruited across Germany according to the following inclusion criteria: age >1 year, disease duration <1 year, possession of a suitable autologous cord blood sample stored in the accredited, private cord blood bank Vita34 (Leipzig, Germany), laboratory results (complete blood count and basic metabolic profile) within normal ranges and compliance with intensive insulin treatment. Cord blood samples met the following

selection and testing criteria: cord and mother's blood at time of collection were free of infectious disease markers and viability of cord blood cells was >50%. The following exclusion criteria were applied: positive test for hepatitis B virus (HBV), hepatitis C virus (HCV), Treponema pallidum, human immunodeficiency virus (HIV), parvovirus B19, cytomegalovirus (CMV), human T-lymphotropic virus types 1 and 2 (HTLV-1/2), or other active infections and chronic disease requiring immunosuppressive therapy. In parallel, children who were also older than 1 year and with recent onset type 1 diabetes (duration <1 year) and normal laboratory baseline results were recruited across Germany as natural controls. As of February 2013, 7 children of Caucasian origin, with a median age of 3.02 years (range 1.82-5.38 years) with new onset T1D underwent an autologous cord blood infusion and an additional group of 10 new onset individuals (median age 6.60 years, range 3.59-10.85 years) were recruited as natural controls. Children who received cord blood infusion were followed every 3 months during the first and every 6 months during the second year post-infusion (Figure 5), while controls were examined at months 0, 6, 12 and 24 (Figure 6).

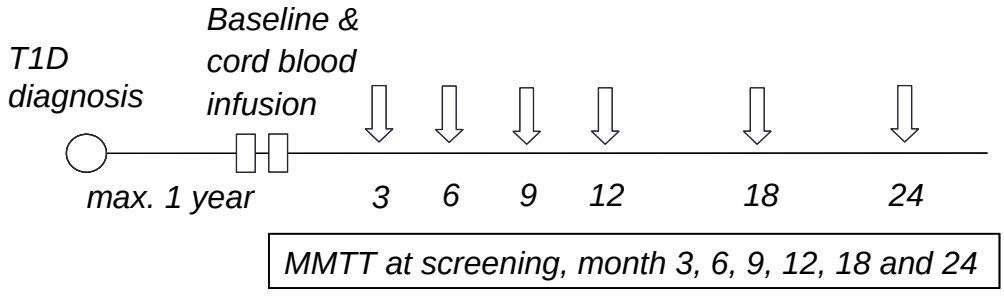

Figure 5. Flowchart presenting the study design of the cord blood trial. Umbilical cord blood recipients are followed every 3 months during the first year post-infusion and every 6 months during the second year post-infusion.

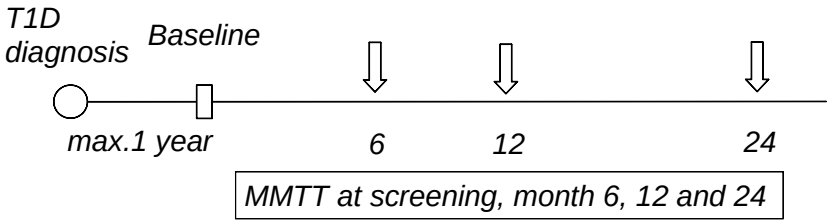

Figure 6. Flowchart presenting the examination plan of the participants of the control group. An MMTT is performed at screening and at months 6, 12 and 24.

A mixed meal tolerance test (MMTT), complete blood count, basic metabolic panel, HbA1c, T cell repertoire (peripheral T regulatory cells, memory T regulatory cells, recently activated T cells and CD4+ T helper cells to CD8+ cytotoxic T cell ratio) and autoantibody titer were performed at each visit. Throughout the trial subjects were advised to use intensive insulin regimes to achieve the best glycemic control (HbA1c ≤7.5% was intended). Use of hypoglycaemic agents other than insulin was not permitted. Regarding cases, safety parameters (complete blood count, electrolytes, serum creatinine and urea) were examined regularly at each visit.

Furthermore, all children were examined physically, adverse events were documented, glucose monitoring logs as well as insulin doses for the 7 days prior to each visit have been reported. Based on the personal insulin diaries, insulin requirements per kg per day were calculated for each subject.

All parents/guardians were informed about the purpose, eligibility criteria, procedures, risks and benefits of the study and written informed consent for the enrolment into the intervention trial as well as for the genetic screening was obtained. The trial was approved by the ethics committee of the Technische Universität München, Germany (# 2022/08) as well as by the Paul-Ehrlich-Institute,

German Federal Ministry of Health. The trial was performed using the Guidelines of Good Clinical Practice.

3.2 Methods

3.2.1 Genotyping

HLA class II alleles HLA-DRB1, HLA-DQA1 and HLA-DQB1 were determined using PCR-amplified DNA and non-radioactive sequence-specific oligonucleotide probes by Conexio Genomics, Australia. Individuals were defined as having a high risk HLA genotype on the basis of whether they had one of the TEDDY Study inclusion high risk genotypes: DR4-DQA1*030X-DQB1*0302@/DR3-DQA1*0501-DQB1*0201; DR4-DQA1*030X-DQB1*0302@/DR4-DQA1*030X-DQB1*0302@; DR4-DQA1*030X-DQB1*0302@/DR8-DQA1*0401-DQB1*0402, DR3-DQA1*0501 DQB1*0201/DR3-DQA1*0501-DQB1*0201; DR4-DQA1*030X-DQB1*0302@/DR4-DQA1*030X-DQB1*020X; DR4-DQA1*030X-DQB1*0302@/DR1-DQA1*0101-DQB1*0501; DR4-DQA1*030X-DQB1*0302@/DR13-DQA1*0102-DQB1*0604, DR4-DQA1*030X-DQB1*0302/DR4-DQA1*030X-DQB1*0304, DR4-DQA1*030X-DQB1*0302@/DR9-DQA1*030X-DQB1*0303; DR3-DQA1*0501-DQB1*0201/DR9-DQA1*030X-DQB1*0303; where @ includes DQB1*0302 and *0304 (TEDDY Study Group 2007).

Genetic typing for specific gene variants known to be associated with T1D and T2D (Table 3) has been performed with the MassARRAY system using the iPLEX^{TM} chemistry (Sequenom, San Diego, CA, USA) by Research Unit Molecular Epidemiology, Helmholtz Zentrum München, Germany. DNA from EDTA blood was transferred into a 96 well microtiter plates (ABgene Thermo-Fast 96 PCR Plate Skirted Kat.-Nr.AB-0800). The allele-dependent primer extension products were

loaded onto one 384-element chip using a nanoliter pipetting system (SpectroCHIP, SpectroPOINT Spotter; Sequenom), and the samples were analysed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (Bruker Daltonik, Leipzig, Germany). The resulting mass spectra were analysed for peak identification via the SpectroTYPER RT 3.4 software (Sequenom). To control for reproducibility, 16.3% of samples were genotyped in duplicate with a discordance rate less than 0.5%. All SNPs were tested for deviation from Hardy-Weinberg equilibrium by means of chi-square or Fischer's exact test (Winkler et al 2011).

Table 3. Overview of the examined T1D and T2D associated SNPs.

T1D associated SNPs	T2D associated SNPs
CD25 rs11594656	*CDKAL1 rs10946398*
IL7R rs6897932	*CDKN2A rs2383208*
COBL rs4948088	*FTO rs8050136*
CTLA4 rs3087243	*GCKR rs780094*
ERBB3 rs2292239	*HHEX/IDE rs10882102*
IFIH1 rs1990760	*HMGA2 rs1531343*
IL2 rs4505848	*IGF2BP2 rs4402960*
IL10 rs3024505	*KCNJ11 rs5215*
IL18RAP rs917997	*KCNQ1 rs2237892*
KIAA0350 rs12708716	*MTNR1B rs1387153*
PTPN2 rs1893217	*PPARG rs1801282*
PTPN22 rs2476601	*SLC30A8 rs3802177*
SH2B3 rs3184504	*TCF7L2 rs7901695*
INS rs689	*THADA rs11899863*
	WFS1 rs1801214

Of the examined T2D associated polymorphisms, the following were previously reported to be associated with reduced insulin secretion and impaired β cell function: *CDKAL1 rs10946398, CDKN2A rs2383208, HHEX/IDE rs10882102, IGF2BP2 rs4402960, KCNJ11 rs5215, MTNR1B rs1387153, SLC30A8 rs13266634 and TCF7L2 rs7901695* (Grarup et al 2010, van de Bunt et al 2010).

According to the risk allele known to be associated with T1D or T2D, the participants were coded as 2 if they were homozygous for the risk allele, 1 if they were heterozygous and 0 if they were homozygous for the non-risk allele. Different risk allele scores including T1D or T2D associated gene variants were calculated for each patient by adding the number of risk alleles for each SNP (0, 1 or 2). Only one variant at each locus was chosen and only children with complete genotype data from the examined variants were included in risk score analyses. Successful typing for T1D associated gene variants was obtained for 450 patients and for T2D genes for 256 patients (Table 4).

Table 4. Genotyping for T1D and T2D loci in the whole study population.

	DiMelli	ChAglyCD3	Calcitriol	Altered Peptide Ligand NBI-6024
Total number	357	26	75	21
Genotyping (n)				
• *T1D genes*	357	16	56	21
• *T2D genes*	200	9	33	14

3.2.2 Mixed Meal Tolerance Test (MMTT)

MMTTs were performed in order to determine residual β cell function assessed by fasting, peak and Area Under the Curve (AUC) C-peptide (Altered Peptide Ligand NBI-6024, Calcitriol trials and cord blood trial). Through a peripheral vein catheter, blood samples were drawn at 0, 15, 30, 60, 90 and 120 minutes after ingesting 6ml per kg body weight of a standard oral mixed formula liquid meal (Boost Nutritional Energy drink, Nestle Healthcare Nutrition Inc., USA). The maximal dose was determined to be 360ml and the subjects were advised to remain sitting throughout the test. It was recommended that the participants had fasted at least 10 hours before performing the test; abstinence from coffee, tea, caffeine containing drinks,

sodas or chewing gums and vigorous exercise was also recommended. Furthermore, the subjects were advised to consume high (>150g) carbohydrate meals for 3 days prior to the test. As for insulin therapy, subjects were instructed to withhold long-acting insulin on the morning of the test and those wearing an insulin pump continued to use the usual basal rate; however, it was important that no rapid-acting insulin was administered before or during the stimulation test. In case of infectious illness or other physical stress the test was postponed. Each MMTT was initiated before 10:00 a.m., after measuring fasting blood glucose using a glucometer (Contour meter and test strips, Bayer). In case of a fasting glucose value less than 70mg/dl or greater than 200mg/dl, the test was rescheduled. Blood glucose was evaluated again at the end of the test and, when necessary, appropriate insulin correction doses were administered.

3.2.3 Determination of C-peptide levels

DiMelli trial, Calcitriol trials: C-peptide concentrations were measured in 250 µl EDTA samples using a two-site immunoenzymometric assay (AIA-360, Tosoh Bioscience, Inc, San Francisco, CA). The interassay coefficient of variation of the C-peptide assay is 4.2% at a concentration of 0.24 ng/ml and the lower limit of detection is 0.02 ng/ml.

Altered Peptide Ligand NBI-6024 trial: C-peptide concentrations were measured at a central laboratory (ICON Central Laboratories, Farmingdale, New York) with a radioimmunoassay (Diagnostic Systems Laboratories). The reported interassay

coefficient of variation of the C-peptide assay is 5.3% at a concentration of 0.55 nmol/l and the lower limit of detection is 0.03 nmol/l.

ChAglyCD3 study: C-peptide levels were measured in EDTA tubes with the use of a time-resolved fluorescence immunoassay (PerkinElmer) in Vrije Universiteit Brussel Central United, Brussels, Belgium.

Cord blood trial: C-peptide determination was performed in the Northwest Lipid Research Laboratories, Washington using a two-site immunoenzymometric assay (Tosoh, USA). The reference values of C-peptide determined in apparently healthy donors was found to be 1.1-3.3 ng/ml. The highest measurable concentration without dilution was circa 15.0 ng/ml and the lowest measurable concentration was 0.05 ng/ml. Samples with values in the range between 9.99 – 14.99 ng/ml were repeated without dilution, whereas all results ≥15ng/ml were repeated with dilution. Samples whose results were less than 0.05ng/ml were not given a value but were reported as 0 ng/ml.

3.2.4 Glycosylated haemoglobin (HbA1c)

Cord blood trial: HbA1c was assessed centrally in EDTA blood through turbidimetry at the Institute for Clinical Chemistry of the Klinikum Schwabing, Munich, Germany.

3.2.5 Islet autoantibody measurements

IAA, GADA, IA-2A, and ZnT8A were determined centrally by the Institute of Diabetes Research Munich, Helmholtz Zentrum Munich using radiobinding assays as

previously described (Ziegler et al 1999, Achenbach et al 2009). Briefly, IAA were measured by Protein A/G radiobinding assays using ^{125}I-recombinant human insulin labelled at tyrosine aa14. GADA, IA-2A, and ZnT8A were measured separately by Protein A radiobinding assays using ^{35}S-methionine-labelled *in vitro* transcribed/translated recombinant human GAD65, IA-2ic, and the carboxy-terminal portion of ZnT8 for the two major variants at amino acid 325, respectively. The upper limit of normal for each assay was determined using Q–Q plots and corresponded to the 99th percentile of control children. Values above 1.5 units are considered as IAA positive, above or equal 35 units are considered as GADA positive and values above or equal 5 units are considered as IA-2A positive in this assay. As for Zn antibodies values ≥16 units are considered positive for ZnT8A-C-Arg and values ≥ 30 units are considered positive for ZnT8A-C-Tryp antibodies. Offspring were considered islet autoantibody-positive when two consecutive samples collected after birth were positive. All islet autoantibody assays were evaluated by the Diabetes Autoantibody Standardization Program (Torn et al 2008, Schlosser et al 2010, Lampasona et al 2011).

3.2.6 Autologous cord blood infusion

All autologous umbilical cord blood samples were stored in the private, accredited, German cord blood bank Vita34 (Leipzig, Germany). Before storage, cord blood samples were examined in order to prove that all selection and testing criteria are met; cord blood cell viability should be >50% and both the unit and a maternal blood sample drawn right after delivery should be free of infectious disease markers. Cord blood samples were mixed with a Citrate-Phosphate-Dextrose solution and NaCl

0.9% and then stored in cryopreservation in liquid nitrogen, where dimethyl sulfoxide (DMSO) was used as cryoprotectant. As the circumstances require, 2-3 weeks before cord blood infusion various tests, including HLA and blood group confirmation, estimation of viability, number of CD34+ cells and total nucleated cells, were performed in an aliquot of cord blood. Upon arrival at the Department of Pediatrics (Technische Universität München, Germany) all children were examined physically and in case of illness the infusion was rescheduled. Subjects' weight was obtained and body temperature was measured in the beginning as well as at the end of the procedure. There were no restrictions regarding consumption of breakfast or use of insulin before or during infusion. An emergency schedule was calculated separately for each individual. Blood samples were obtained for the assessment of the following parameters: complete blood count, basic metabolic panel, liver enzymes, infection markers, disease associated autoantibodies and HbA1c%. Meanwhile cord blood samples were sent from Vita34 to the Department of Pediatrics using standard shipping methods for frozen cord blood cells (temperature <-130^{o}C). Cord blood was then prepared for infusion (thawed [Plasmatherm, Fa.Barkey GmBH&Co KG Germany] to max. 37^{o}C and washed with NaCl 0.9% twice [Sepax Biosafe Kit CS 600.1]). This procedure lasted circa 1.5 hour and the final product had a total volume of ca. 50ml. During the preparation of the final product, subjects received intravenous pre-treatment with mannitol (2ml/kg body weight infused in 30 min) and pethidin (Dolantin 1mg/kg body weight infused in 30 min) in order to eliminate possible reactions to the cord blood sample. Additionally the subjects received a 20cc/kg normal saline by intravenous infusion bolus in order to perfuse the kidneys and assist in excretion of cellular debris from the thaw procedure. No chemotherapy or other immunosuppressive drug was administered.

Cord blood was infused through the peripheral intravenous line for 20 to 30 minutes. During the infusion a pulse oximetry was applied and vital signs including blood pressure, heart and breathing rate were monitored every 15 minutes during and 1 hour after transfusion. After a monitoring period of circa 6 hours the subjects were dismissed but returned the next morning to the hospital for a physical examination and a blood control of safety parameters (complete blood count, electrolytes, serum creatinine and urea). Safety parameters were determined centrally at the Institute for Clinical Chemistry of the Klinikum Schwabing, Munich, Germany using accredited methods.

3.2.7 T cell assay

Cord blood trial: Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation over Lymphoprep (Axis-Shield) and freshly stained for the markers CD3, CD4, CD8, CD25, CD45RO, CD127, and FOXP3 using standard techniques. Cells were acquired on a Becton Dickinson LSR-II flow cytometer with FACS Diva software. At least 50000 gated CD4+ events were acquired for each sample and analyzed using FlowJo software version 7.6.3 (TreeStar Inc.). T cell measurements including peripheral Tregs, memory Tregs and recently activated T cells, which had not been performed immediately after collection in fresh blood, were excluded from the present analysis, because samples which were stored in the meantime were found to have significantly lower T cell numbers.

3.3 Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 20.0;SPSS Inc., Chicago, IL, USA) with $p < 0.05$ indicating significance.

3.3.1 Genetic associations on new onset patients

Linear regression analysis was performed in order to investigate the associations between examined gene variants and age at diagnosis. For the comparison of allelic frequencies in different age groups, binary logistic regression was performed. Binary outcome was either “early age at diagnosis” (age at diagnosis ≤ 5 coded as 1) or “late age at diagnosis” (age at diagnosis ≥ 15 coded as 1).

For the investigation of possible genetic effects on fasting C-peptide, adjusted linear regression analyses for age, gender and disease duration were executed. Because of the fact that fasting C-peptide was not normally distributed in the studied populations, the logarithmic form *ln*(fasting C-peptide+1) was used for the execution of statistical tests. This form has been introduced by several authors in similar cases in the literature (Palmer et al 2004, Lachin et al 2011). Correction for multiple testing was performed for the two tested variables, age at diagnosis and fasting C-peptide, by dividing the level of significance by the number of tested loci (Bonferroni corrections).

With regard to the analysis performed in the second patient group, who participated in Altered Peptide Ligand NBI-6024, Calcitriol and ChAglyCD3 interventional trials, data evaluation showed that there were only two common time

points for all patients. These were the screening visit, which took place close to disease manifestation, and month 18. To assess the course of fasting C-peptide over these 18 months after disease onset, the difference of fasting C-peptide: C-peptide$_{month18}$ minus C-peptide$_{screening}$, was calculated. According to the result, two different patient groups were created; one, in which a decline in C-peptide was observed (negative result) and a second, in which the patients preserved or gained C-peptide over time (zero or positive result). For all analyses, adjusted comparisons for age, gender, diabetes duration, baseline values for C-peptide and intervention group were performed by linear regression model. All data are presented as median (interquartile range [IQR]). Correction for multiple testing was performed for the tested variables, by dividing the level of significance by the number of tested loci (Bonferroni corrections).

3.3.2 Cord blood trial

Area Under the Curve (AUC$_{0\text{-}120min}$) C-peptide (AUC C-peptide) was calculated using the formula introduced by Tai (Tai 1994). Unadjusted comparisons between cases and controls were performed using the non-parametric Mann Whitney U test. Linear regression was used to adjust for age, gender, type 1 diabetes duration and baseline levels. Absolute changes from baseline to month 6 of AUC C-peptide, peak C-peptide, daily insulin use and HbA1c were compared with the total number of infused nucleated cells and the number of infused CD34+ cells using Pearson's correlation coefficient. All data are presented as median (range). To account for potential

attrition bias, sensitivity analyses for all examined parameters including only those participants with complete measurements at each time point was performed.

4. RESULTS

4.1 Genetic determinants of T1D diagnosis age and β cell function

4.1.1 Characteristics of the study cohort

The present analysis was performed in a population of 357 children (149 female, 208 male) with newly diagnosed T1D participating in the DiMelli study. Median age at diagnosis was 10.42 years (IQR: 6.66, 13.17 years); 15.7% of the cohort was diagnosed before the age of 5 years and 12.0% had an age at diagnosis of >15 years. Median fasting C-peptide was 0.4 ng/ml (IQR: 0.0, 0.7 ng/ml) and median diabetes duration at time of blood examination was 9 days (IQR: 6, 13 days). A significant negative correlation between age and fasting C-peptide levels was observed (Pearson's r=0.323, $p < 10^{-6}$). Female participants had significantly higher C-peptide levels (median C-peptide in girls 0.5 vs C-peptide in boys 0.3 ng/ml, p=0.006 adjusted for age and diabetes duration) and also tended to have younger age at diagnosis in comparison to males (median age at diagnosis in girls 9.94 vs age at diagnosis in boys 10.81 years, p=0.076).

4.1.2 T1D associated loci and their association with age at diagnosis

Linear regression analysis was initially performed for all T1D associated SNPs in order to investigate their effect on age at diagnosis (Table 5). Since 14 genes were analyzed, the required level for statistical significance was defined as p<0.004.

There were no significant associations between the number of risk alleles for each SNP and age at diagnosis.

As stated in the introduction, GWAS have shown that the contribution of each SNP separately to T1D is very small, with ORs <2 (Barrett et al 2009). According to the hypothesis, their effect would become stronger, if a combination of more than two genes in the form of a risk score was used. The first risk score included all 14 examined SNPs (*CD25 rs11594656, IL7R rs6897932, COBL rs4948088, CTLA4 rs3087243, ERBB3 rs2292239, IFIH1 rs1990760, IL2 rs4505848, IL10 rs3024505, IL18RAP rs917997, KIAA0350 rs12708716, PTPN2 rs1893217, PTPN22 rs2476601, PTPN22 rs6679677, SH2B3 rs3184504, INS rs689*). This risk allele score was tested in the whole cohort (Figure 7A, n=165 children, Pearson's r=-0.065, p=0.408), as well as in patients carrying the HLA risk genotypes (Figure 7B, n=78 children, Pearson's r=-0.131, p=0.253) in order to trace possible correlations with age at diagnosis, but no significant correlations were found.

All SNPs which were found to have a negative unstandardized coefficient b in the linear regression model were added to form a second risk score (Table 5). This score consisted of the variants of the following 10 genes: *SH2B3, PTPN2, COBL, IL18RAP, CD25, IL2, IL7R, CTLA4, KIAA0350* and *IFIH1.* A significantly inverse correlation was found between the number of risk alleles for this score and age at diagnosis (p=0.022, r=-0.168, n=187, Figure 8A); patients carrying a higher number of risk alleles were diagnosed at a significantly younger age compared with those who carried less risk alleles. The same significant correlation remained inside the HLA high risk group (p=0.028, r=-0.235, n=88, Figure 8B).

To determine the effect of each SNP between different age groups, binary logistic regression analyses using two cut-offs (5 and 15 years) were performed. Both cut-offs, 5 and 15 years, were defined based on the ages at which differences in rates of complications and mortality have been observed (Finne 2005, Harjutsalo 2011). For the first binary logistic analysis, “early age of onset” was defined as the outcome variable, and patients were coded as “1” if age at diagnosis was ≤ 5 years or “0” if age at diagnosis was >5 years. None of the 14 examined SNPs was associated with early age at onset (Table 6), both in the whole cohort and inside the HLA high risk group (Table 7).

In order to investigate whether a high risk allele load associates with early age at onset ≤5 years, logistic regression analysis was performed for the previously described risk scores (Table 8). There was a significant association between the second risk allele combination (*SH2B3, PTPN2, COBL, IL18RAP, CD25, IL2, IL7R, CTLA4, KIAA0350* and *IFIH1*) and age at diagnosis ≤5 years, inside the whole cohort (p=0.014) and inside the HLA high risk group (p=0.023).

To determine the effect of each T1D associated SNP on late age at onset, binary logistic regression analyses with “late age of onset” (age at diagnosis ≥15 years) as outcome were performed. HLA high risk allele carriers were found to be diagnosed at younger ages (onset < 15 years) in contrast to the rest patients (OR: 0.35, 95% CI: 0.17, 0.73, p=0.005). None of the 14 examined T1D associated SNPs associated with late onset of T1D significantly, not only inside the whole cohort (Table 9) but also inside the HLA high risk group (Table 10).

Neither the first score containing all 14 examined SNPs nor the second one, was found to associate with late onset ≥15 years, when the analysis was performed

inside the whole cohort (Table 11) and in patients carrying the HLA high risk genotypes (Table 11).

4.1.3 T2D associated loci and their association with age at diagnosis

In order to explore whether T2D associated genes can promote the early manifestation of T1D by impairing insulin secretion or insulin sensitivity, linear regression model analysis was performed for all 15 examined T2D gene variants (Table 12). Since 15 genes were analyzed, the required level for statistical significance was defined as $p<0.003$. *HHEX/IDE rs10882102* risk allele associated with younger age at diagnosis significantly ($p=0.001$). Homozygotes for the risk allele had a significantly younger age at diagnosis compared with children without any risk allele (median age 8.02 vs. 12.53 years, $p=0.002$).

In order to investigate the cumulative effect of multiple T2D associated risk alleles on age at diagnosis, all 15 examined T2D SNPs were added to form a risk score. No significant correlation between the number of risk alleles and age at diagnosis was observed (Figure 10A, n=113 children, Pearson's $r=-0.172$, $p=0.068$). A second T2D risk score was created by adding all SNPs whose risk alleles were found to have a negative effect on age at diagnosis (negative b unstandardized coefficient, Table 12). The number of risk alleles for this score, including the SNPs *HHEX/IDE rs10882102*, *GCKR rs780094*, *FTO rs8050136*, *CDKN2A rs2383208*, *CDKAL1 rs10946398*, *KCNQ1 rs2237892*, *MTNR1B rs1387153*, *PPARG rs1801282*, *TCF7L2 rs4506565*, *THADA rs11899863* and *WFS1 rs1801214,* associated with lower age at diagnosis significantly (Figure 10B, n=173 children, Pearson's $r=-0.256$, $p=0.001$).

The study cohort was stratified into two groups according to the number of risk alleles for this score, using as cut-off the median number of risk alleles (=14 risk alleles): first group <14 risk alleles (n=75 children) and second group: ≥14 risk alleles (n=89 children). Children from the first group were found to be diagnosed at significantly younger age at diagnosis in comparison with children from the second group (median age at diagnosis 8.65 years vs. 11.62 years, p<0.001, Figure 11).

To investigate the effects of the examined T2D SNPs inside different age groups ("early age of onset" and "late age of onset"), binary logistic regression analyses were performed. When "early age of onset" was defined as binary outcome, no significant associations were found regarding each SNP separately (Table 13) and risk scores (Table 14).

To determine the effect of T2D associated SNPs on late age at onset, binary logistic analysis was performed, in which "late age of onset" (age at diagnosis ≥15 years) was defined as outcome. There were no significant associations between each SNP separately and late age at diagnosis (Table 15). The risk score containing the genes *HHEX/IDE, GCKR, FTO, CDKN2A, CDKAL1, KCNQ1, MTNR1B, PPARG, TCF7L2, THADA and WFS1* associated with diagnosis age younger than 15 years (Table 16).

4.1.4 Associations of genetic loci with C-peptide as a measure of β cell function in newly diagnosed T1D patients

β cell function, assessed by fasting C-peptide, differs at the time of disease manifestation among individuals (Karjalainen et al 1989). This parameter is greatly affected by age as well as by disease duration; compared with older patients,

younger appear to have lower or even undetectable values of C-peptide at disease onset, which disappear rapidly within a few months (Karjalainen et al 1989, Palmer 2009, Greenbaum et al 2012). For this analysis, fasting C-peptide levels were tested at first in the DiMelli cohort and secondly in the cohort consisting of adolescents and young adults (16-45 years).

Effect of T1D and T2D associated loci on residual β cell function in newly diagnosed individuals ≤ 20 years

The effect of each T1D and T2D associated SNP separately on fasting C-peptide, measured at disease onset, was examined in patients from the DiMelli study (Tables 17, 18). There were no significant associations between each SNP and fasting C-peptide levels.

The T1D risk score, containing all 14 examined T1D associated SNPs (*ERBB3, PTPN22, KIAA0350, IL10, SH2B3, PTPN2, COBL, IL18RAP, CD25, IL2, IFIH1* and *CTLA4)* did not correlate with fasting C-peptide levels significantly (p=0.983 adjusted for age, gender and diabetes duration, unstandardized b coefficient=0). Similarly, the T2D risk score containing all 15 examined SNPs did not correlate with fasting C-peptide levels significantly (p=0.842 adjusted for age, gender and diabetes duration, unstandardized b coefficient=-0.002). A third risk score was created by adding all T2D SNPs, previously reported to be associated with impaired β cell function and insulin secretion (*CDKAL1, CDKN2A, IGF2BP2, KCNJ11, MTNR1B, SLC30A8* and *TCF7L2).* The number of risk alleles for this score did not correlate with fasting C-peptide levels (p=0.638 adjusted for age, gender and diabetes duration, unstandardized b coefficient=0.006).

Effect of T1D and T2D associated loci on residual β cell function in newly diagnosed adolescents and young adults (16-45 years)

All participants were examined for fasting C-peptide at screening, performed close to disease onset (median diabetes duration 39.50 days, IQR: 26, 47 days) and at month 18. Fasting C-peptide levels were analyzed for possible associations with all T1D and T2D SNPs (Tables 19, 20). There were no significant associations between the examined SNPs and fasting C-peptide levels.

Identification of risk allele scores that predict loss of β cell function

86 patients have been identified with a C-peptide decline during the first 18 months after disease manifestation. Their median age at screening was 30.81 years (IQR: 23.50, 35.71 years). The second group included 36 patients, who retained or gained C-peptide within the same period of time. The median age at screening for the second group of patients was 26.95 years (IQR: 21.21, 33.67 years). Both groups were compared in order to determine whether these two C-peptide groups differed significantly in the number of risk alleles for T1D and T2D associated risk scores. In comparison to patients, who had a C-peptide gain, patients who had a decline in C-peptide, had more risk alleles in the T1D score which contained all 14 examined T1D associated SNPs (Figure 12, p=0.004, adjusted for age, gender, diabetes duration, screening C-peptide and intervention group, unstandardized b coefficient=-1.636).

With regard to T2D loci and their effect on fasting C-peptide decline, a first score consisting of all examined T2D SNPs (*SLC30A8 rs13266634* was not included because of incomplete genotyping) and a second consisting of SNPs previously

found to be associated with impaired β cell function -*CDKAL1, CDKN2A, IGF2BP2, KCNJ11, MTNR1B, SLC30A8* and *TCF7L2*- (Grarup et al 2010, van de Bunt et al 2010), did not associate significantly with C-peptide decline in the first 18 months after disease manifestation (adjusted for age, gender, diabetes duration, screening C-peptide and intervention group p=0.818 and p=0.973, respectively, Figure 13A and 13B).

TABLES

Table 5. Analysis of the effect of T1D associated SNPs on age at diagnosis, considering the different genotypes.

Polymorphism	Risk allele	N	Age at diagnosis (years) Median (IQR)	Unstandardized Coefficient Beta	p value
1. INS					
AA	A	198	10.14 (6.37, 13.25)		
AT		86	10.25 (5.91, 12.92)		
TT		3	12.12 (11.88, -)	0.025	0.962
2. PTPN22 rs2476601					
AA	A	15	10.73 (6.40, 15.20)		
AG		95	10.80 (7.56, 12.62)		
GG		244	10.25 (6.37, 13.46)	0.178	0.669
3. IL2 rs4505848					
GG	G	50	10.40 (5.97, 12.63)		
AG		165	10.52 (7.06, 13.14)		
AA		136	10.39 (6.45, 13.38)	**-0.051**	0.881
4. SH2B3 rs3184504					
TT	T	101	10.01 (5.67, 13.16)		
TC		176	10.47 (7.44, 13.08)		
CC		75	11.16 (7.95, 13.42)	**-0.668**	0.043
5. ERBB3 rs2292239					
AA	A	42	10.44 (7.01, 13.48)		
AC		160	10.79 (7.41, 13.23)		
CC		152	10.13 (6.21, 13.15)	0.152	0.644
6. COBL rs4948088					
CC	C	231	10.46 (7.45, 13.38)		
CG		14	11.21 (8.21, 13.53)	**-0.525**	0.647
7. PTPN2 rs1893217					
CC	C	7	7.34 (5.66, 12.95)		
CT		110	10.39 (6.83, 13.39)		
TT		237	10.46 (6.68, 13.15)	**-0.134**	0.765
8. CTLA4 rs3087243					
AA	A	115	10.14 (6.79, 12.54)		
AG		89	10.80 (6.52, 13.62)		
GG		132	10.30 (6.36, 13.16)	**-0.053**	0.849
9. IL18RAP rs917997					
AA	A	15	10.90 (8.89, 15.38)		
AG		126	9.92 (5.77, 12.27)		
GG		210	10.52 (6.72, 13.41)	**-0.240**	0.552
10. IL10 rs3024505					
CC	C	246	10.75 (7.24, 13.44)		
CT		101	9.71 (6.13, 13.05)		
TT		6	9.77 (7.62, 11.96)	0.538	0.245
11. IFIH1 rs1990760					
TT	T	142	10.23 (5.68, 12.75)		
TC		166	10.70 (7.31, 13.66)		
CC		44	10.04 (5.48, 12.93)	**-0.250**	0.473
12. CD25 rs11594656					
TT	T	184	9.94 (5.98, 13.28)		
TA		116	10.58 (6.90, 12.98)		
AA		7	8.89 (5.55, 13.98)	**-0.368**	0.427

13. KIAA0350 rs12708716					
AA	A	156	10.20 (6.97, 13.15)		
AG		168	10.62 (6.68, 13.39)		
GG		33	10.46 (6.03, 12.97)	**-0.027**	0.941
14. IL7R rs6897932					
CC	C	157	10.45 (6.74, 13.59)		
CT		101	10.80 (8.44, 13.12)		
TT		14	9.55 (6.57, 13.71)	**-0.166**	0.705

Differences in age at diagnosis between genotypes were calculated with the linear regression model. P <0.004 indicates significance (Bonferroni correction). N is the number of participants.

Table 6. Comparison of the allelic frequencies of T1D associated loci and their association with early age at diagnosis.

Gene/Polymorphism	Risk allele	Age comparison			
		Onset ≤ 5 *years* *n*	***Onset > 5*** *years* *n*	**Odds Ratio (95%CI)** *for the risk allele*	***p*** **value**
1. INS	A	45	242	0.69 (0.37, 1.28)	0.237
2. PTPN22 rs2476601	A	55	299	0.60 (0.33, 1.10)	0.096
3. IL2 rs4505848	G	55	296	1.07 (0.70, 1.62)	0.752
4. SH2B3 rs3184504	T	53	299	1.26 (0.83, 1.92)	0.282
5. ERBB3 rs2292239	A	55	299	0.83 (0.53, 1.28)	0.394
6. COBL rs4948088	C	30	215	1.87 (0.24, 14.83)	0.555
7. PTPN2 rs1893217	C	55	299	0.83 (0.46, 1.48)	0.521
8. CTLA4 rs3087243	A	53	283	1.15 (0.82, 1.63)	0.414
9. IL18RAP rs917997	A	54	297	1.37 (0.85, 2.21)	0.201
10. IL10 rs3024505	C	55	298	1.15 (0.64, 2.08)	0.639
11. KIAA0350 rs12708716	A	56	301	0.94 (0.60, 1.46)	0.769
12. IL7R rs6897932	C	35	237	2.05 (1.01, 4.19)	0.048
13. IFIH1 rs1990760	T	56	296	1.18 (0.77, 1.82)	0.460
14. CD25 rs11594656	T	49	258	1.16 (0.65, 2.08)	0.613

ORs and p values derived from a logistic regression model; outcome tested is age at diagnosis ≤5 years vs all other patients. P <0.004 indicates significance (Bonferroni correction).

Table 7. Comparison of the allelic frequencies of T1D associated loci and T1D risk scores and their association with early age at diagnosis in HLA high risk children.

Gene/Polymorphism	Risk allele	Age comparison			
		Onset ≤ 5 *years* *n*	***Onset > 5*** *years* *n*	**Odds Ratio (95%CI)** *for the risk allele*	***p*** **value**
1. INS	A	25	107	1.18 (0.47, 3.02)	0.723
2. PTPN22 rs2476601	A	29	129	0.8 (0.36, 1.18)	0.599
3. IL2 rs4505848	G	30	128	1.75 (0.95, 3.24)	0.075
4. SH2B3 rs3184504	T	28	129	1.17 (0.65, 2.11)	0.602
5. ERBB3 rs2292239	A	29	129	0.61 (0.31, 1.18)	0.143
6. COBL rs4948088	C	14	94	1.22 (0.14, 10.60)	0.855
7. PTPN2 rs1893217	C	29	128	1.09 (0.48, 2.49)	0.830
8. CTLA4 rs3087243	A	28	122	1.00 (0.62, 1.62)	1.000
9. IL18RAP rs917997	A	28	129	1.14 (0.58, 2.24)	0.710
10. IL10 rs3024505	C	29	129	0.95 (0.45, 2.01)	0.895
11. KIAA0350 rs12708716	A	30	129	1.15 (0.59, 2.24)	0.688
12. IL7R rs6897932	C	17	106	1.34 (0.54, 3.36)	0.527
13. IFIH1 rs1990760	T	30	128	1.10 (0.61, 1.98)	0.759
14. CD25 rs11594656	T	27	115	1.39 (0.63, 3.04)	0.412

ORs and p values derived from a logistic regression model; outcome tested is age at diagnosis ≤5 years vs all other patients. P <0.004 indicates significance (Bonferroni correction).

Table 8. Comparison of the allelic frequencies of T1D risk allele scores and their association with early age at diagnosis in the whole cohort and only in HLA high risk patients.

Risk allele scores	**Onset ≤ 5** *years* *n*	**Onset > 5** *years* *n*	**Odds Ratio (95%CI)** *for the number of risk alleles*	***p*** **value**
● *WHOLE COHORT:*				
1. CD25, IL7R, COBL, CTLA4, ERBB3, IFIH1, IL2, IL10, IL18RAP, KIAA0350, PTPN2, PTPN22, PTPN22, SH2B3, INS	18	147	1.07 (0.88, 1.31)	0.483
2. SH2B3, PTPN2, COBL, IL18RAP, CD25, IL2, IL7R, CTLA4, KIAA0350 AND IFIH1.	23	164	1.30 (1.05, 1.60)	**0.014**
● *HLA HIGH RISK:*				
1. CD25, IL7R, COBL, CTLA4, ERBB3, IFIH1, IL2, IL10, IL18RAP, KIAA0350, PTPN2, PTPN22, PTPN22, SH2B3, INS	9	69	1.18 (0.89, 1.58)	0.253
2. SH2B3, PTPN2, COBL, IL18RAP, CD25, IL2, IL7R, CTLA4, KIAA0350 AND IFIH1.	11	75	1.44 (1.05, 1.97)	**0.023**

ORs and p values derived from a logistic regression model; outcome tested is age at diagnosis ≤5 years vs all other patients.

Table 9. Comparison of the allelic frequencies of T1D associated loci and their association with late age at diagnosis.

Gene/Polymorphism	Risk allele	Age comparison			*p* value
		Onset < 15 *years* *n*	***Onset ≥ 15*** *years* *n*	**Odds Ratio (95%CI)** *for the risk allele*	
1. INS	A	255	32	1.21 (0.55, 2.66)	0.630
2. PTPN22 rs2476601	A	311	43	1.16 (0.67, 2.01)	0.598
3. IL2 rs4505848	G	309	42	1.08 (0.68, 1.72)	0.757
4. SH2B3 rs3184504	T	309	43	0.84 (0.54, 1.33)	0.463
5. ERBB3 rs2292239	A	311	43	0.75 (0.46, 1.24)	0.263
6. COBL rs4948088	C	215	30	1.87 (0.24, 14.83)	0.555
7. PTPN2 rs1893217	C	311	43	0.90 (0.48, 1.69)	0.738
8. CTLA4 rs3087243	A	296	40	0.96 (0.65, 1.42)	0.848
9. IL18RAP rs917997	A	309	42	1.03 (0.59, 1.79)	0.924
10. IL10 rs3024505	C	310	43	1.55 (0.76, 3.16)	0.225
11. KIAA0350 rs12708716	A	314	43	1.23 (0.74, 2.06)	0.420
12. IL7R rs6897932	C	237	35	1.81 (0.91, 3.59)	0.092
13. IFIH1 rs1990760	T	310	42	1.02 (0.63, 1.65)	0.940
14. CD25 rs11594656	T	275	32	1.07 (0.54, 2.13)	0.849

ORs and p values derived from a logistic regression model; outcome tested is age at diagnosis ≥15 years vs all other patients. P <0.004 indicates significance (Bonferroni correction).

Table 10. Comparison of the allelic frequencies of T1D associated loci and their association with late age at diagnosis in HLA high risk children.

Gene/Polymorphism	Risk allele	Age comparison			
		Onset < 15 *years* *n*	***Onset ≥ 15*** *years* *n*	**Odds Ratio (95%CI)** *for the risk allele*	***p* value**
1. INS	A	122	10	1.05 (0.27, 4.10)	0.942
2. PTPN22 rs2476601	A	147	11	2.57 (0.99, 6.64)	0.052
3. IL2 rs4505848	G	148	10	0.81 (0.29, 2.28)	0.693
4. SH2B3 rs3184504	T	146	11	0.91 (0.38, 2.18)	0.826
5. ERBB3 rs2292239	A	147	11	0.44 (0.14, 1.35)	0.150
6. COBL rs4948088	C	102	5	NA	0.999
7. PTPN2 rs1893217	C	146	11	1.35 (0.42, 4.42)	0.616
8. CTLA4 rs3087243	A	139	11	0.66 (0.31, 1.40)	0.279
9. IL18RAP rs917997	A	146	11	1.40 (0.54, 3.68)	0.490
10. IL10 rs3024505	C	147	11	1.49 (0.41, 5.36)	0.544
11. KIAA0350 rs12708716	A	148	11	0.65 (0.24, 1.79)	0.407
12. IL7R rs6897932	C	115	8	2.55 (0.54, 11.99)	0.236
13. IFIH1 rs1990760	T	147	11	1.02 (0.41, 2.51)	0.972
14. CD25 rs11594656	T	133	9	0.52 (0.17, 1.56)	0.243

ORs and p values derived from a logistic regression model; outcome tested is age at diagnosis ≥15 years vs all other patients. P <0.004 indicates significance (Bonferroni correction).

Table 11. Comparison of the allelic frequencies of T1D risk allele scores and their association with early age at diagnosis in the whole cohort and only in HLA high risk patients.

Risk allele scores	**Onset < 15** *years* *n*	**Onset ≥ 15** *years* *n*	**Odds Ratio (95%CI)** *for the number of risk alleles*	***p* value**
● *WHOLE COHORT:*				
1. CD25, IL7R, COBL, CTLA4, ERBB3, IFIH1, IL2, IL10, IL18RAP, KIAA0350, PTPN2, PTPN22, PTPN22, SH2B3, INS	149	16	1.11 (0.90, 1.37)	0.319
2. SH2B3, PTPN2, COBL, IL18RAP, CD25, IL2, IL7R, CTLA4, KIAA0350 AND IFIH1.	169	18	1.00 (0.80, 1.26)	0.991
● *HLA HIGH RISK:*				
1. CD25, IL7R, COBL, CTLA4, ERBB3, IFIH1, IL2, IL10, IL18RAP, KIAA0350, PTPN2, PTPN22, PTPN22, SH2B3, INS	76	2	1.03 (0.57, 1.84)	0.933
2. SH2B3, PTPN2, COBL, IL18RAP, CD25, IL2, IL7R, CTLA4, KIAA0350 AND IFIH1.	86	2	0.96 (0.47, 1.98)	0.915

ORs and p values derived from a logistic regression model; outcome tested is age at diagnosis ≥15 years vs all other patients.

Table 12. Analysis of the effect of T2D associated SNPs in T1D age at diagnosis, considering the different genotypes.

Polymorphism	Risk allele	N	Age at diagnosis (years) Median (IQR)	Unstandardized Coefficient Beta	p value
1. CDKAL1 rs10946398					
CC	C	13	11.69 (6.46, 14.24)		
CA		100	9.86 (5.88, 12.74)		
AA		86	10.12 (6.07, 13.38)	**-0.033**	0.950
2. CDKN2A rs2383208					
AA	A	147	9.94 (5.8, 13.15)		
AG		45	9.91 (6.12, 12.32)		
GG		7	13.12 (10.18, 16.68)	**-0.884**	0.137
3. FTO rs8050136					
AA	A	41	9.79 (6.13, 11.96)		
CA		141	10.42 (6.38, 13.46)		
CC		102	10.16 (6.3, 13.49)	**-0.124**	0.746
4. GCKR rs780094					
GG	G	62	9.6 (6.22, 13.13)		
AG		97	9.91 (5.87, 12.46)		
AA		38	11.22 (5.49, 14.03)	**-0.347**	0.444
5. HHEX / IDE rs10882102					
GG	G	71	8.02 (5.66, 11.38)		
CG		85	11.08 (6.52, 13.66)		
CC		28	12.53 (7.88, 14.68)	**-1.611**	**0.001***
6. HMGA2 rs1531343					
CC	C	1	14.67		
CG		9	11.29 (5.73, 12.95)		
GG		189	9.94 (6.12, 13.14)	0.932	0.460
7. IGF2BP2 rs4402960					
TT	T	16	9.14 (4.16, 13.56)		
TG		90	10.10 (6.03, 13.20)		
GG		93	10.22 (6.37, 12.55)	0.026	0.958
8. KCNJ11 rs5215					
CC	C	35	10.21 (7.45, 12.29)		
CT		86	9.58 (6.29, 13.49)		
TT		75	10.80 (5.52, 13.15)	0.091	0.837
9. KCNQ1 rs2237892					
CC	C	173	9.79 (5.74, 12.46)		
CT		25	13.52 (9.33, 14.93)		
TT		1	6.36	**-1.973**	0.025
10. MTNR1B rs1387153					
TT	T	17	11.36 (2.52, 13.66)		
CT		82	9.62 (5.62, 12.45)		
CC		96	10.33 (6.69, 13.46)	**-0.696**	0.160
11. PPARG rs1801282					
CC	C	152	10.03 (5.84, 13.13)		
CG		42	9.84 (6.33, 13.87)		
GG		3	13.12 (5.59, -)	**-0.241**	0.726
12. SLC30A8 rs13266634					
CC	C	116	10.34 (6.38, 13.15)		
CT		92	10.06 (5.60-13.12)		
TT		13	8.29 (4.38, 14.25)	0.466	0.350
13. TCF7L2 rs4506565					
TT	T	19	8.99 (4.92, 13.40)		
AT		79	9.61 (6.85, 12.49)		
AA		101	10.21 (5.80, 13.43)	**-0.294**	0.540
14. THADA rs11899863					
CC	C	164	9.60 (5.84, 12.63)		
CT		35	11.08 (6.95, 14.21)	**-1.221**	0.137

15. WFS1 rs1801214					
TT	T	77	10.14 (6.36, 13.15)		
CT		95	9.91 (5.50, 12.44)		
CC		28	10.32 (6.86, 14.54)	**-0.269**	0.559

Differences in age at diagnosis between genotypes were calculated with the linear regression model. P <0.003 indicates significance (Bonferroni correction). N is the number of participants.

Table 13. Comparison of the allelic frequencies of T2D associated SNPs and their association with early age at diagnosis.

Polymorphism	Risk allele	Age comparison			
		Onset ≤ 5 years N	*Onset > 5 years N*	**Odds Ratio (95%CI)** *for the risk allele*	***p* value**
1. CDKAL1 rs10946398	C	33	166	1.12 (0.60, 2.07)	0.727
2. CDKN2A rs2383208	A	33	166	1.51 (0.67, 3.38)	0.319
3. FTO rs8050136	A	44	240	0.81 (0.50, 1.31)	0.390
4. GCKR rs780094	G	33	164	0.74 (0.44, 1.27)	0.276
5. HHEX/IDE rs10882102	G	29	155	1.33 (0.74, 2.39)	0.350
6. HMGA2 rs1531343	C	33	166	0.54 (0.08, 3.88)	0.537
7. IGF2BP2 rs4402960	T	32	167	1.24 (0.69, 2.24)	0.467
8. KCNJ11 rs5215	C	32	164	0.97 (0.57, 7.64)	0.900
9. KCNQ1 rs2237892	C	33	166	2.60 (0.60, 11.29)	0.202
10. MTNR1B rs1387153	T	32	163	1.53 (0.87, 2.71)	0.140
11. PPARG rs1801282	C	33	164	1.20 (0.52 (2.81)	0.669
12. SLC30A8 rs13266634	C	39	182	0.77 (0.44, 1.34)	0.356
13. TCF7L2 rs4506565	T	33	166	1.14 (0.65, 1.99)	0.644
14. THADA rs11899863	C	32	167	1.60 ,(0.52, 4.88)	0.413
15. WFS1 rs1801214	T	33	167	1.16 (0.67, 2.03)	0.593

ORs derived from a logistic regression model; outcome tested is age at onset ≤5 years vs all other patients. P <0.003 indicates significance (Bonferroni correction). N is the number of participants.

Table 14. Comparison of the allelic frequencies of T2D risk allele scores and their association with early age at diagnosis.

T2D risk allele scores	Onset ≤ 5 *years n*	Onset > 5 *years n*	Odds Ratio (95%CI) *for the number of risk alleles*	*p* value
1. CDKAL1, CDKN2A, FTO, GCKR, HHEX/IDE, HMGA2, IGF2BP2, KCNJ11, KCNQ1, MTNR1B, PPARG, SLC30A8, TCF7L2, THADA AND WFS1	20	93	1.12 (0.91, 1.38)	0.293
2. HHEX/IDE, GCKR, FTO, CDKN2A, CDKAL1, KCNQ1, MTNR1B, PPARG, TCF7L2, THADA AND WFS1	26	147	1.11 (0.91, 1.35)	0.316

ORs derived from a logistic regression model; outcome tested is age at onset ≤5 years vs all other patients.

Table 15. Comparison of the allelic frequencies of T2D associated SNPs and their association with late age at diagnosis.

Polymorphism	Risk allele	Age comparison			
		Onset < 15 years N	*Onset ≥ 15 years N*	**Odds Ratio (95%CI)** *for the risk allele*	***p* value**
1. CDKAL1 rs10946398	C	178	21	1.28 (0.61, 2.67)	0.515
2. CDKN2A rs2383208	A	178	21	0.63 (0.30, 1.34)	0.231
3. FTO rs8050136	A	251	33	0.68 (0.39, 1.20)	0.181
4. GCKR rs780094	G	176	21	0.85 (0.45, 1.61)	0.609
5. HHEX/IDE rs10882102	G	163	21	0.42 (0.22, 0.82)	0.011
6. HMGA2 rs1531343	C	178	21	0.00 (0.00, -)	0.999
7. IGF2BP2 rs4402960	T	178	21	1.16 (0.57, 2.35)	0.681
8. KCNJ11 rs5215	C	175	21	1.03 (0.55, 1.93)	0.927
9. KCNQ1 rs2237892	C	178	21	0.49 (0.17, 1.37)	0.174
10. MTNR1B rs1387153	T	174	21	0.61 (0.28, 1.33)	0.215
11. PPARG rs1801282	C	176	21	0.81 (0.32, 2.04)	0.660
12. SLC30A8 rs13266634	C	195	21	0.88 (0.45, 1.70)	0.700
13. TCF7L2 rs4506565	T	178	21	0.74 (0.35, 1.53)	0.413
14. THADA rs11899863	C	178	21	0.90 (0.28, 2.85)	0.853
15. WFS1 rs1801214	T	179	21	0.63 (0.33, 1.21)	0.165

ORs derived from a logistic regression model; outcome tested is age at onset ≥15 years vs all other patients. P <0.003 indicates significance (Bonferroni correction). N is the number of participants.

Table 16. Comparison of the allelic frequencies of T2D risk allele scores and their association with late age at diagnosis.

T2D risk allele scores	**Onset < 15** *years n*	**Onset ≥ 15** *years n*	**Odds Ratio (95%CI)** *for the number of risk alleles*	***p* value**
1. CDKAL1, CDKN2A, FTO, GCKR, HHEX/IDE, HMGA2, IGF2BP2, KCNJ11, KCNQ1, MTNR1B, PPARG, SLC30A8, TCF7L2, THADA AND WFS1	100	13	0.79 (0.61, 1.02)	0.075
2. HHEX/IDE, GCKR, FTO, CDKN2A, CDKAL1, KCNQ1, MTNR1B, PPARG, TCF7L2, THADA AND WFS1	153	20	0.73 (0.58, 0.92)	**0.007**

ORs derived from a logistic regression model; outcome tested is age at onset ≥15 years vs all other patients.

Table 17. Analysis of the effect of all T1D associated SNPs on fasting C-peptide (ng/ml), considering the different genotypes.

Polymorphism	Risk allele	N	Fasting C-peptide (ng/ml) *Median (IQR)*	Unstandardized Coefficient Beta	p value Adjusted for age, gender and diabetes duration
1. INS					
AA	A	198	0.4 (0.2, 0.7)		
AT		86	0.4 (0.2, 0.7)		
TT		3	1.8 (0.0, -)	-0.005	0.890
2. PTPN22 rs2476601					
AA	A	15	0.4 (0.0, 0.7)		
AG		95	0.4 (0.2, 0.8)		
GG		244	0.4 (0.0, 0.7)	0.002	0.928
3. IL2 rs4505848					
GG	G	50	0.5 (0.3, 0.8)		
AG		165	0.4 (0.0, 0.7)		
AA		136	0.4 (0.0, 0.7)	0.020	0.342
4. SH2B3 rs3184504					
TT	T	101	0.3 (0.0, 0.7)		
TC		176	0.4 (0.1, 0.8)		
CC		75	0.4 (0.2, 0.6)	0.000	0.992
5. ERBB3 rs2292239					
AA	A	42	0.4 (0.0, 0.5)		
AC		160	0.4 (0.2, 0.7)		
CC		152	0.4 (0.0, 0.7)	-0.005	0.812
6. COBL rs4948088					
CC	C	231	0.4 (0.0, 0.7)		
CG		14	0.3 (0.0, 0.4)	0.093	0.214
7. PTPN2 rs1893217					
CC	C	7	0.3 (0.0, 0.6)		
CT		110	0.4 (0.2, 0.7)		
TT		237	0.4 (0.0, 0.8)	0.000	0.994
8. CTLA4 rs3087243					
AA	A	115	0.4 (0.0, 0.7)		
AG		89	0.4 (0.0, 0.6)		
GG		132	0.4 (0.2, 0.7)	-0.007	0.668
9. IL18RAP rs917997					
AA	A	15	0.5 (0.3, 0.7)		
AG		126	0.4 (0.0, 0.8)		
GG		210	0.4 (0.0, 0.7)	0.013	0.605
10. IL10 rs3024505					
CC	C	246	0.3 (0.0, 0.7)		
CT		101	0.5 (0.3, 0.8)		
TT		6	0.5 (0.4, 0.5)	-0.073	0.011
11. IFIH1 rs1990760					
TT	T	142	0.4 (0.0, 0.6)		
TC		166	0.4 (0.0, 0.8)		
CC		44	0.4 (0.0, 0.7)	0.006	0.774
12. KIAA0350 rs12708716					
AA	A	156	0.4 (0.0, 0.7)		
AG		168	0.4 (0.2, 0.7)		
GG		33	0.4 (0.0, 0.9)	-0.001	0.970
13. IL7R rs6897932					
CC	C	157	0.4 (0.0, 0.8)		
CT		101	0.4 (0.1, 0.6)		
TT		14	0.5 (0.0, 0.8)	0.027	0.330

14. CD25 rs12251307					
TT	T	4	0.5 (0.1, 1.9)		
CT		58	0.4 (0.2, 0.7)		
CC		287	0.4 (0.0, 0.7)	-0.011	0.703

Differences in fasting C-peptide between genotypes were calculated with the linear regression model and adjusting for age, gender and T1D duration. P <0.004 indicates significance (Bonferroni correction). N is the number of participants.

Table 18. Analysis of the effect of all T2D associated SNPs on fasting C-peptide (ng/ml), considering the different genotypes and homozygotes of the risk alleles.

Polymorphism	Risk allele	N	Fasting C-peptide (ng/ml) *Median (IQR)*	Unstandardized Coefficient Beta	p value Adjusted for age, gender and diabetes duration
1. CDKAL1 rs10946398					
CC	C	13	0.4 (0.1, 0.6)		
CA		100	0.4 (0.3, 0.7)		
AA		86	0.4 (0.2, 0.8)	-0.015	0.647
2. CDKN2A rs2383208					
AA	A	147	0.4 (0.2, 0.8)		
AG		45	0.4 (0.2, 0.7)		
GG		7	0.4 (0.2, 1.2)	0.025	0.507
3. FTO rs8050136					
AA	A	41	0.5 (0.3, 0.9)		
CA		141	0.4 (0.2, 0.7)		
CC		102	0.4 (0.0, 0.6)	0.021	0.372
4. HHEX/IDE rs10882102					
GG	G	71	0.4 (0.0, 0.6)		
CG		85	0.5 (0.3, 0.8)		
CC		28	0.5 (0.2, 0.9)	0.005	0.862
5. HMGA2 rs1531343					
CC	C	1	1		
CG		9	0.3 (0.0, 0.6)		
GG		189	0.4 (0.2, 0.8)	-0.121	0.136
6. IGF2BP2 rs4402960					
TT	T	16	0.4 (0.3, 0.8)		
TG		90	0.4 (0.2, 0.8)		
GG		93	0.4 (0.3, 0.7)	0.016	0.607
7. KCNJ11 rs5215					
CC	C	35	0.4 (0.0, 0.6)		
CT		86	0.4 (0.2, 0.9)		
TT		75	0.5 (0.3, 0.7)	-0.033	0.235
8. KCNQ1 rs2237892					
CC	C	173	0.4 (0.2, 0.7)		
CT		25	0.5 (0.4, 0.9)		
TT		1	0.0 (0.0, 0.0)	-0.029	0.612
9. MTNR1B rs1387153					
TT	T	17	0.4 (0.1, 0.7)		
CT		82	0.4 (0.0, 0.7)		
CC		96	0.5 (0.3, 0.8)	-0.006	0.841
10. PPARG rs1801282					
CC	C	152	0.4 (0.2, 0.7)		
CG		42	0.5 (0.2, 0.9)		
GG		3	0.4 (0.3, -)	-0.032	0.451
11.SLC30A8 rs13266634					
CC	C	116	0.4 (0.2, 0.8)		
CT		92	0.4 (0.0, 0.6)		
TT		13	0.3 (0.0, 0.7)	0.053	0.054
12. TCF7L2 rs4506565					
TT	T	19	0.5 (0.3, 1.0)		
AT		79	0.4 (0.0, 0.6)		
AA		101	0.4 (0.2, 0.8)	0.023	0.447
13. THADA rs11899863					
CC	C	164	0.4 (0.2, 0.7)		
CT		35	0.5 (0.2, 0.9)	-0.005	0.929

14. WFS1 rs1801214					
TT	T	77	0.4 (0.2, 0.9)		
CT		95	0.4 (0.2, 0.7)		
CC		28	0.4 (0.3, 0.8)	0.026	0.377
15. GCKR rs780094					
GG	G	62	0.4 (0.0, 0.8)		
AG		97	0.4 (0.2, 0.7)		
AA		38	0.5 (0.4, 0.8)	-0.030	0.292

Differences in fasting C-peptide between genotypes were calculated with the linear regression model and adjusting for age, gender and T1D duration. P <0.003 indicates significance (Bonferroni correction). N is the number of participants.

Table 19. Analysis of the effect of T1D associated SNPs in fasting C-peptide (ng/ml) at screening and month 18.

Polymorphism	Risk allele	N	Fasting C-peptide (ng/ml) Median (IQR)		p value Adjusted for age, gender, diabetes duration and intervention group	
			Screening	*Month 18*	*p value* (screening)	*p value* (month 18)
1. INS						
AA	A	63	0.9 (0.3, 1.4)	0.5 (0.3, 1.0)		
AT		27	0.8 (0.6, 1.3)	0.5 (0.6, 0.7)		
TT		3	0.8 (0.4, -)	0.5 (0.1, -)	0.254	0.539
2. PTPN22 rs2476601						
AA	A	8	0.95 (0.70, 1.28)	0.5 (0.1, 0.7)		
AG		31	0.70 (0.44, 1.10)	0.4 (0.3, 0.6)		
GG		56	0.90 (0.44, 1.47)	0.6 (0.3, 1.3)	0.355	0.046
3. IL2 rs4505848						
GG	G	14	0.7 (0.3, 1.4)	0.5 (0.2, 1.2)		
AG		31	1.0 (0.6, 1.6)	0.6 (0.3, 1.3)		
AA		42	0.8 (0.4, 1.3)	0.5 (0.3, 0.9)	0.995	0.790
4. SH2B3 rs3184504						
TT	T	28	0.7 (0.3, 1.3)	0.5 (0.2, 1.0)		
TC		42	0.9 (0.6, 1.5)	0.4 (0.2, 0.8)		
CC		25	1.1 (0.3, 1.5)	0.5 (0.3, 1.3)	0.574	0.410
5. ERBB3 rs2292239						
AA	A	13	1.1 (0.4, 1.5)	0.6 (0.4, 1.0)		
AC		49	0.90 (0.35, 1.4)	0.5 (0.2, 0.9)		
CC		33	0.9 (0.6, 1.4)	0.5 (0.3, 1.0)	0.857	0.796
6. COBL rs4948088						
CC	C	88	0.9 (0.5, 1.4)	0.5 (0.3, 0.9)		
CG		7	0.6 (0.5, 1.4)	0.8 (0.4, 1.0)	0.488	0.382
7. PTPN2 rs1893217						
CC	C	1	0.8 (0.6, 1.3)	0.5 (0.3, 0.9)		
CT		23	0.9 (0.44, 1.4)	0.5 (0.3, 0.9)		
TT		71	0.8 (0.6, 1.4)	0.5 (0.3, 1.2)	0.860	0.756
8. CTLA4 rs3087243						
AA	A	28	0.8 (0.4, 1.5)	0.4 (0.2, 0.6)		
AG		28	1.0 (0.7, 1.6)	0.7 (0.4, 1.0)		
GG		39	0.7 (0.4, 1.2)	0.5 (0.2, 0.9)	0.984	0.389
9. IL18RAP rs917997						
AA	A	2	0.3 (0.3, -)	0.30		
AG		30	0.9 (0.6, 1.4)	0.5 (0.2, 1.0)		
GG		63	0.9 (0.4, 1.5)	0.5 (0.3, 1.0)	0.738	0.565
10. IL10 rs3024505						
CC	C	65	0.9 (0.5, 1.4)	0.5 (0.2, 1.0)		
CT		29	0.8 (0.3, 1.4)	0.5 (0.3, 0.9)	0.564	0.717
TT		1	0.9	-		
11. IFIH1 rs1990760						
TT	T	38	0.9 (0.6, 1.5)	0.6 (0.3, 1.3)		
TC		46	0.9 (0.4, 1.4)	0.5 (0.2, 0.8)		
CC		11	0.9 (0.4, 1.1)	0.5 (0.3, 0.6)	0.061	0.086
12. CD25 rs11594656						
TT	T	47	0.9 (0.4, 1.3)	0.5 (0.3, 0.7)		
TA		36	1.0 (0.5, 1.5)	0.7 (0.4, 1.4)		
AA		6	0.7 (0.5, 0.8)	0.2 (0.1, 0.5)	0.552	0.227
13. KIAA0350 rs12708716						
AA	A	40	0.8(0.4, 1.3)	0.5 (0.3, 0.9)		
AG		39	1.0 (0.6, 1.6)	0.6 (0.3, 1.1)		
GG		16	0.7 (0.3, 1.2)	0.5 (0.3, 1.0)	0.816	0.800

14. IL7R rs6897932						
CC	C	57	0.8 (0.4, 1.3)	0.5 (0.3, 0.9)		
CT		31	0.9 (0.6, 1.5)	0.5 (0.2, 1.0)		
TT		6	1.3 (0.5, 2.7)	0.6 (0.2, 2.6)	0.596	0.791

Differences in fasting C-peptide between genotypes were calculated with the linear regression model and adjusting for age, gender and T1D duration. P <0.004 indicates significance (Bonferroni correction). N is the number of participants.

Table 20. Analysis of the effect of T2D associated SNPs in fasting C-peptide (ng/ml) at screening and month 18.

Polymorphism	Risk allele	N	Fasting C-peptide (ng/ml) Median (IQR)		p value Adjusted for age, gender, diabetes duration and intervention group	
			Screening	*Month 18*	*p value* (screening)	*p value* (month 18)
1. CDKAL1 rs10946398						
CC	C	4	1.1 (0.8, 1.8)	1.2 (0.7, 2.0)		
CA		39	0.8 (0.4, 1.4)	0.6 (0.3, 1.1)		
AA		35	0.9 (0.4, 1.3)	0.5 (0.2, 0.8)	0.894	0.031
2. CDKN2A rs2383208						
AA	A	48	0.9 (0.4, 1.4)	0.5 (0.2, 1.0)		
AG		25	1.0 (0.5, 1.4)	0.5 (0.3, 1.2)		
GG		5	0.7 (0.5, 0.9)	0.6 (0.4, 0.9)	0.784	0.348
3. FTO rs8050136						
AA	A	13	1.3 (0.5, 1.6)	0.9 (0.4, 2.1)		
CA		41	0.8 (0.4, 1.3)	0.5 (0.2, 0.6)		
CC		38	0.9 (0.5, 1.3)	0.5 (0.3, 1.0)	0.276	0.225
4. HHEX/IDE rs10882102						
GG	G	32	0.9 (0.4, 1.5)	0.5 (0.3, 1.0)		
CG		34	0.9 (0.5, 1.3)	0.5 (0.2, 0.8)		
CC		12	0.8 (0.3, 1.4)	0.7 (0.2, 1.3)	0.488	0.745
5. KCNQ1 rs2237892						
CC	C	69	0.8 (0.4, 1.4)	0.5 (0.2, 1.0)		
CT		9	0.9 (0.7, 1.4)	0.5 (0.3, 1.0)	0.826	0.833
TT		0				
6. HMGA2 rs1531343						
CC	C	1	1.6	0.7		
CG		2	0.7 (0.2, -)	0.2 (0.1, -)		
GG		75	0.8 (0.4, 1.3)	0.5 (0.3, 1.0)	0.270	0.546
7. IGF2BP2 rs4402960						
TT	T	4	0.8 (0.3, 1.1)	0.3 (0.1, 0.5)		
TG		38	0.9 (0.4, 1.5)	0.5 (0.2, 1.1)		
GG		36	0.7 (0.4, 1.3)	0.6 (0.3, 1.0)	0.664	0.146
8. KCNJ11 rs5215						
CC	C	13	0.9 (0.5, 1.6)	0.8 (0.3, 1.0)		
CT		32	0.8 (0.5, 1.1)	0.4 (0.1, 0.8)		
TT		33	1.0 (0.3, 1.4)	0.5 (0.3, 1.4)	0.494	0.520
9. MTNR1B rs1387153						
TT	T	8	0.3 (0.2, 0.6)	0.3 (0.1, 0.5)		
CT		36	0.9 (0.5, 1.4)	0.5 (0.3, 1.1)		
CC		34	1.1 (0.5, 1.6)	0.5 (0.3, 1.0)	0.022	0.457
10. PPARG rs1801282						
CC	C	56	0.9 (0.4, 1.3)	0.5 (0.3, 1.0)		
CG		21	0.9 (0.5, 1.6)	0.6 (0.24, 0.9)		
GG		1	0.4	0.1	0.894	0.863
11. SLC30A8 rs13266634						
CC	C	7	1.4 (0.2, 3.2)	0.5 (0.1, 0.8)		
CT		10	0.9 (0.6, 1.3)	0.5 (0.3, 0.7)	0.503	0.567
TT		0	-	-		
12. TCF7L2 rs4506565						
TT	T	7	0.7 (0.4, 1.0)	0.5 (0.3, 0.6)		
AT		30	0.9 (0.5, 1.4)	0.5 (0.3, 0.9)		
AA		41	0.9 (0.4, 1.4)	0.5 (0.2, 1.1)	0.477	0.657
13. THADA rs11899863						
CC	C	69	0.9 (0.5, 1.4)	0.5 (0.3, 1.0)		
CT		9	0.4 (0.3, 1.4)	0.3 (0.1, 0.8)	0.433	0.306

14. WFS1 rs1801214						
TT	T	30	0.8 (0.4, 1.1)	0.5 (0.3, 0.9)		
CT		33	0.9 (0.4, 1.4)	0.4 (0.2, 1.0)		
CC		15	1.0 (0.5, 1.6)	0.7 (0.4, 1.4)	0.563	0.634
15. GCKR rs780094						
GG	G	26	0.7 (0.4, 1.2)	0.6 (0.3, 1.0)		
AG		38	1.0 (0.5, 1.4)	0.5 (0.2, 1.0)		
AA		14	0.8 (0.7, 1.1)	0.5 (0.3, 0.9)	0.879	0.661

Differences in fasting C-peptide between genotypes were calculated with the linear regression model and adjusting for age, gender and T1D duration. P <0.003 indicates significance (Bonferroni correction). N is the number of participants.

FIGURES

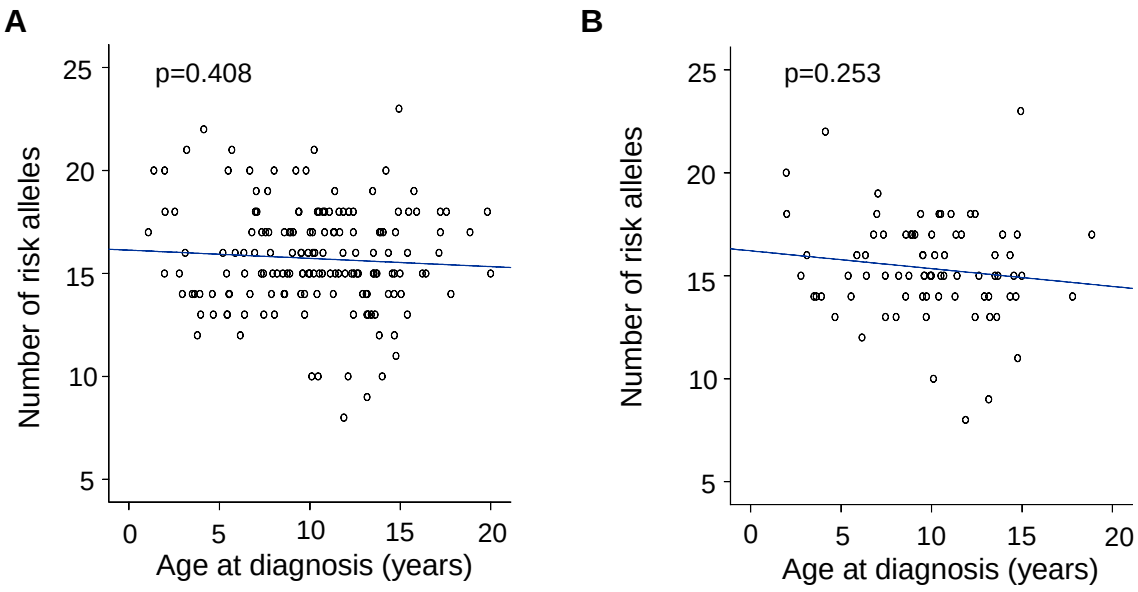

Figure 7. Correlation between the number of risk alleles and age at diagnosis in the whole cohort (Figure A, n=165 children) and in children carrying the HLA risk genotypes (Figure B, n=78 children). The score includes all 14 examined loci *CD25, IL7R, COBL, CTLA4, ERBB3, IFIH1, IL2, IL10, IL18RAP, KIAA0350, PTPN2, PTPN22, PTPN22, SH2B3* and *INS*.

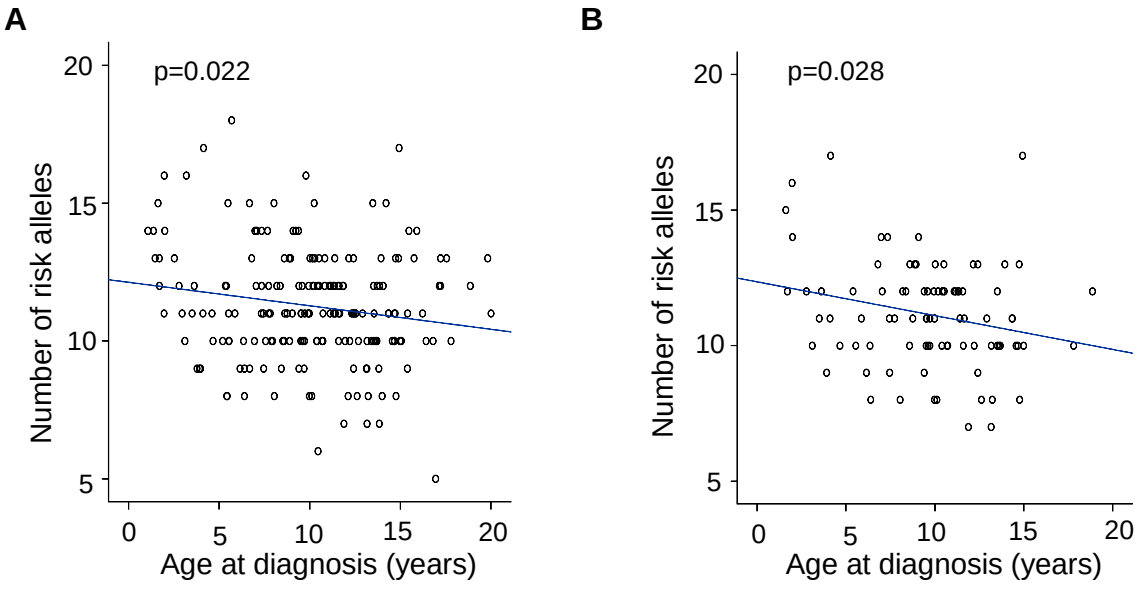

Figure 8. Correlation between the number of risk alleles and age at diagnosis in the whole cohort (Figure A, n=168 children) and in children carrying the HLA risk genotypes (Figure B, n=88 children). The score includes the loci *SH2B3, PTPN2, COBL, IL18RAP, CD25, IL2, IL7R, CTLA4, KIAA0350* and *IFIH1*.

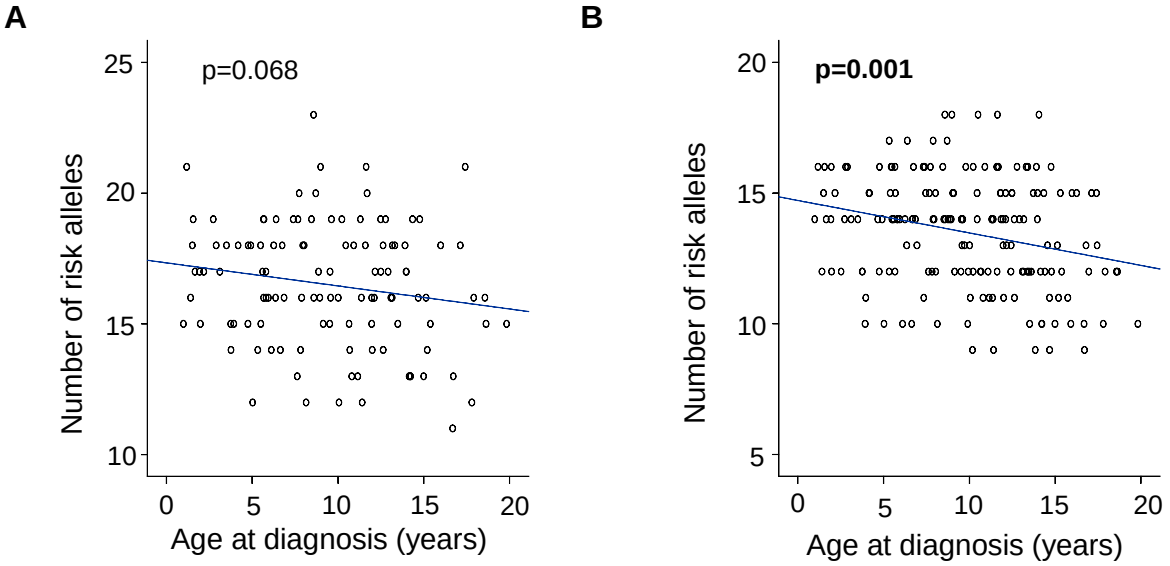

Figure 9. Correlation between the number of T2D risk alleles and age at diagnosis. (A) Risk score includes all examined T2D associated loci (*CDKAL1, CDKN2A, FTO, GCKR, HHEX/IDE, HMGA2, IGF2BP2, KCNJ11, KCNQ1, MTNR1B, PPARG, SLC30A8, TCF7L2, THADA* and *WFS1).* (B) Risk score includes *HHEX/IDE, GCKR, FTO, CDKN2A, CDKAL1, KCNQ1, MTNR1B, PPARG, TCF7L2, THADA* and *WFS1.*

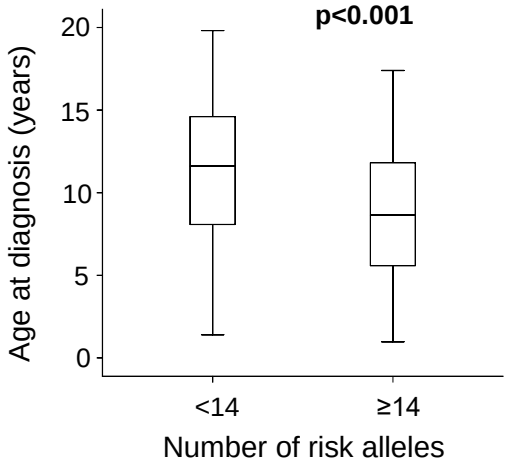

Figure 10. Median age at diagnosis in two different risk allele strata: <14 (n=75 children) and ≥14 risk alleles (n=89 children). The T2D risk allele score includes *HHEX/IDE, GCKR, FTO, CDKN2A, CDKAL1, KCNQ1, MTNR1B, PPARG, TCF7L2, THADA AND WFS1.*

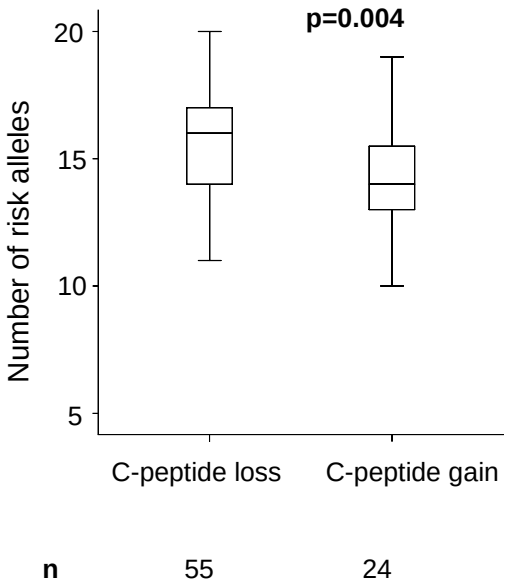

Figure 11. Plot of the median number of risk alleles for the two patient groups. Risk score includes all 14 examined T1D associated loci. P adjusted for age, gender, diabetes duration, screening values and intervention group.

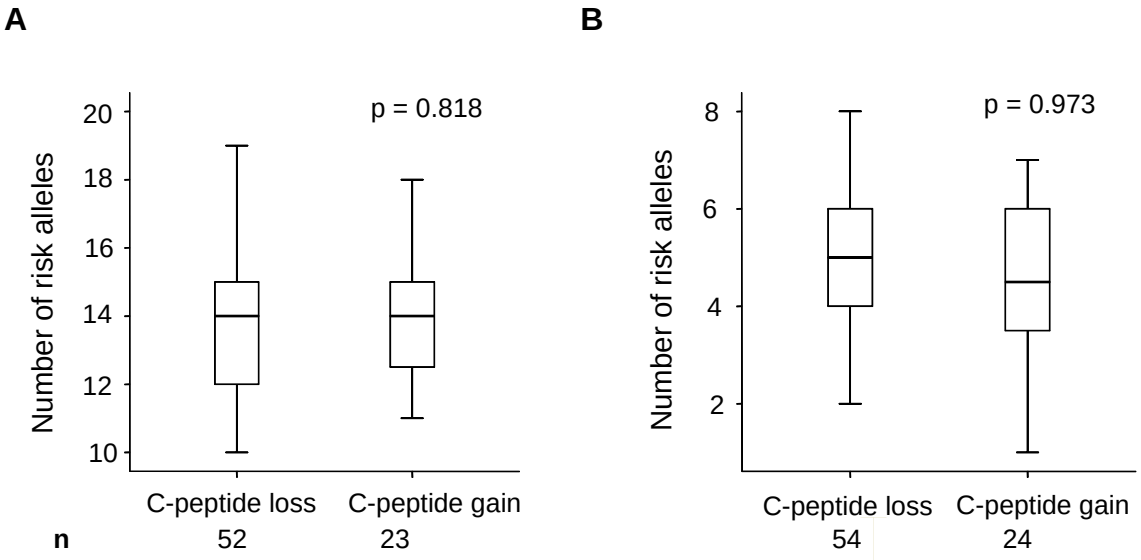

Figure 12. Plot of the median number of T2D associated risk alleles for the two patient groups. (A) The risk score includes all examined T2D associated loci (B) The risk score includes all T2D loci, known to be associated with impaired β cell function and reduced insulin secretion *(CDKAL1, CDKN2A, IGF2BP2, KCNJ11, MTNR1B, SLC30A8* and *TCF7L2)*. P adjusted for age, gender, diabetes duration, screening values and intervention group.

4.2 Infusion of autologous umbilical cord blood in children with new onset T1D

4.2.1 Characteristics of study population

As of February 2013, 17 newly diagnosed children were enrolled in the trial. Of these, 7 underwent an autologous cord blood infusion and 10 were followed as controls (Table 21). One participant withdrew consent voluntarily 6 months post-infusion. The participants of the control group were significantly older compared to children who received an infusion (median age 3.02 vs. 6.60 years, respectively, p=0.005). Both groups were similar in terms of diabetes duration (p=0.813) and baseline values of metabolic and immune parameters (Tables 22, 23, 24).

Table 21. Patient demographics and cord blood characteristics.

	Cases	**Controls**
N	7	10
Age (years)	3.02 (1.82-5.38)	6.60 (3.59-10.85)
Sex		
Male	5	4
Female	2	6
Time since diagnosis (days)	101 (73-348)	139 (63-410)
Cord blood characteristics:		
TNC/kg (cells x 10^7)	3.89 (0.73-5.24)	-
Volume (ml)	56.3 (33.0-70.9)	-
Viability TNC (%)	80.3 (73.0-87.5)	-
$CD34^+$ cells (cells x 10^6)	1.27 (0.43-2.27)	-
Amount DMSO (g)	3.4 (2.0-4.2)	-
Hb (mg/dl)	124.0 (89.0-142.0)	-

Data are shown as median (range) and n is the number of participants. TNC: total nucleated cells, DMSO: dimethyl sulfoxide, Hb: hemoglobin.

Median infused total nucleated cell count was 3.89 x 10^7 / kg body weight and median viability of the total nucleated cells (TNC) was 80.30%. The median volume of the final sample was 56.6 ml and contained a median total number of CD34+ cells

of 1.27 x 10^6 (Table 21). All aliquots had negative gram stains and none grew pathogenic organisms when cultured for virus, bacteria or fungus.

Table 22. Metabolic outcome in cases and controls during the first year of follow-up.

Metabolic outcome and time point	Cases	Controls	*P* value (unadjusted)	*P* value (adjusted)
AUC C-peptide (ng/ml x 120 min)	111.3 (0.0-146.3)	162.1 (0.0-224.3)	0.193	0.376
Baseline	101.0 (0.0-144.6)	-		
Month 3	60.6 (0.0-112.1)	104.6 (0.0-183.9)	0.193	0.915
Month 6	25.4 (0.0-83.3)	-		
Month 9	23.9 (0.0-107.6)	59.3 (0.0-222.3)	0.435	0.244
Month 12				
Peak C-peptide (ng/ml)				
Baseline	1.15 (0.00-1.48)	1.51 (0.0-2.33)	0.270	0.415
Month 3	0.92 (0.00-1.78)	-		
Month 6	0.67 (0.00-1.20)	1.02 (0.00-2.14)	0.315	0.995
Month 9	0.24 (0.00-1.00)	-		
Month 12	0.25 (0.00-1.06)	0.55 (0.27-2.76)	0.524	0.228
HbA1c (%)				
Baseline	7.1 (5.8-8.1)	6.9 (5.3-7.6)	0.740	0.771
Month 3	7.4 (6.2-7.9)	-		
Month 6	7.3 (6.5 -7.7)	7.3 (5.4-8.0)	0.962	0.429
Month 9	7.1 (6.6-7.3)	-		
Month 12	7.2 (6.7-7.9)	7.0 (5.8-9.1)	0.428	0.525
Daily insulin dose (IU/day/kg)				
Baseline	0.50 (0.17-1.46)	0.41 (0.23-0.95)	0.669	0.611
Month 3	0.54 (0.31-0.98)	-		
Month 6	0.55 (0.21-1.01)	0.69 (0.14-0.95)	0.270	0.403
Month 9	0.57 (0.32-1.33)	-		
Month 12	0.68 (0.34-1.45)	0.76 (0.27-1.00)	0.713	0.820

Sample size: n ≤ 7 for cases and n ≤ 10 for controls. Data are shown as median (range). Unadjusted comparisons were performed by Mann Whitney U test and adjustment for age, gender, disease duration and baseline levels was done by linear regression. AUC: area under the curve.

4.2.2 Safety assessment

No infusion-related serious adverse events were observed during or after the procedure, so that all participants could be dismissed after a monitoring period of 5 to 6 hours. None of the 7 children developed fever, hypo- or hypertension, nausea or vomiting or clinical relevant changes in serum creatinine, in liver parameters or

complete blood counts. No clinically significant changes in laboratory parameters were observed during the post-infusion period.

4.2.4 Metabolic outcome

Median AUC C-peptide decreased gradually in both groups (Figure 14A), in infused children by 78.53% and in controls by 63.42% 1 year after enrollment. There were no significant differences between the two groups at the 6-month (p=0.193 and adjusted p=0.915) and 12-month visits (p=0.435 and adjusted p=0.809, Table 22). Similarly, peak C-peptide declined in both groups over the 12-month follow-up period (Figure 14B). The changes in median peak C-peptide from baseline to months 6 and 12 were comparable in both groups (Table 22). Two children, one from each group, had undetectable levels of C-peptide (both fasting and stimulated) already at baseline.

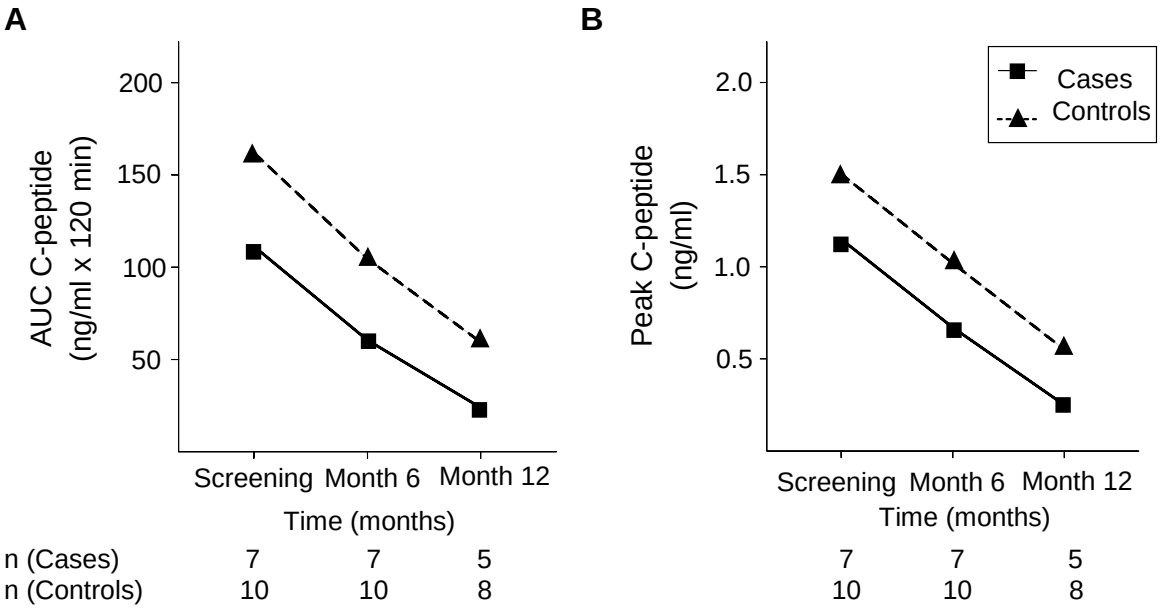

Figure 13. Median AUC C-peptide (A) and median peak C-peptide (B) from baseline to month 6 and 12 in cases and controls.

Median daily insulin requirements remained stable during the first 6 months post-infusion in the case groups but increased 12 moths post-infusion by 0.18 units (Figure 15A). Controls had increased insulin requirements in all follow-up visits in comparison to baseline, but their 6- and 12-month levels were not significantly higher in contrast to cases (6-month visit: p=0.270 and adjusted p=0.403 / 12-month visit: p=0.713 and adjusted p=0.672, Table 22). HbA1c peaked in both groups at month 6 and declined at month 12 (Figure 15B), but median 6- and 12-month HbA1c levels were comparable in both groups (6-month visit: p=0.962 and adjusted p=0.429 / 12-month visit: p=0.428 and adjusted p=0.908, Table 22). Sensitivity analyses with complete cases and controls yielded similar results (data not shown).

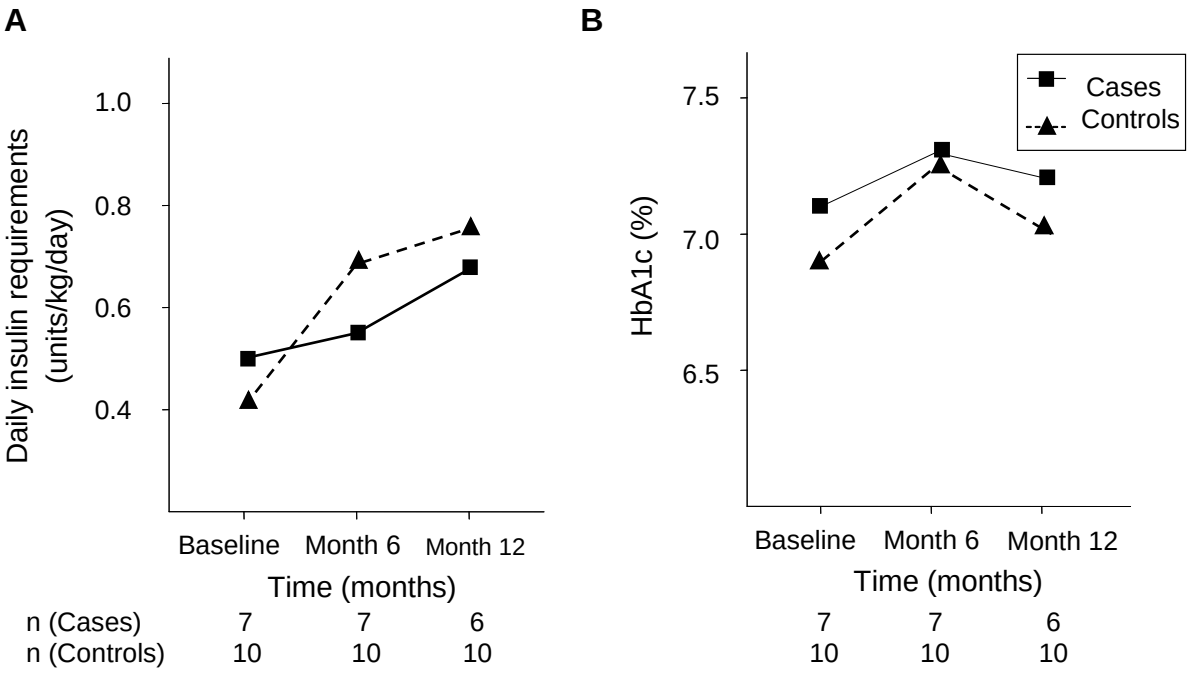

Figure 14. Median daily insulin requirements/kg (A) and median HbA1c (B) from baseline to month 6 and 12 in cases and controls.

4.2.3 Immune outcome

There were no significant differences between cases and controls in the titers of type 1 diabetes associated autoantibodies during follow-up (Table 23).

Table 23. Immune outcome in cases and controls during the first year of follow-up.

Immune outcome and time point	Cases	Controls	*P* value (unadjusted)	*P* value (adjusted)
WBC (cells x10^6)				
Baseline	5.3 (3.3 -10.0)	5.6 (4.1-7.7)	0.887	0.350
Month 3	5.0 (4.3-7.4)	-		
Month 6	5.2 (4.3-6.2)	5.5 (3.7-8.3)	0.837	0.607
Month 9	5.7 (3.8-7.0)	-		
Month 12	4.5 (3.6-6.1)	5.9 (3.7-9.2)	0.328	0.045
IA-2A (units)				
Baseline	104.2 (0.0-281.9)	105.5 (0.0-333.4)	0.601	0.392
Month 3	92.6 (0.4-403.2)	-		
Month 6	87.1 (0.0-280.0)	128.9 (0.0-404.7)	0.669	0.327
Month 9	94.35 (0.0-335.6)	-		
Month 12	41.1 (0.0-330.9)	54.0 (0.0-529.5)	0.699	0.366
GADA (units)				
Baseline	25.2 (3.1-297.1)	19.2 (0.1-738.8)	0.962	0.770
Month 3	49.5 (0.0-425.2)	-		
Month 6	8.7 (0.1-349.4)	14.5 (0.1-348.8)	0.887	0.883
Month 9	51.1 (0.0-490.4)	-		
Month 12	14.8 (0.1-276.1)	47.1 (0.1-729.8)	0.898	0.437
IAA (units)				
Baseline	262.5 (121.0-958.0)	219.0 (0.42-750.0)	0.864	0.936
Month 3	301.7 (193.0-484.4)	-		
Month 6	340.3 (165.5-1160.9)	191.3 (58.30-2046.9)	0.364	0.900
Month 9	331.5 (147.6-548.3)	-		
Month 12	92.3 (62.3-415.6)	251.0 (7.30-2419.2)	0.364	0.308
ZnT8Arg (units)				
Baseline	53.2 (0.1-1594.5)	134.5 (0.1-1763.0)	0.887	0.760
Month 3	62.2 (0.1-958.8)	-		
Month 6	18.4 (0.1-1095.4)	77.7 (0.1-2130.3)	0.962	0.157
Month 9	28.31 (0.1-1353.8)	-		
Month 12	27.4 (0.1-431.2)	167.7 (0.1-5554.0)	0.518	0.289
ZnT8Tryp (units)				
Baseline	148.4 (0.1-2241.6)	100.8 (0.1-1658.0)	0.740	0.314
Month 3	59.4 (0.1-585.3)	-		
Month 6	38.1 (0.1-1285.4)	90.5 (0.1-2097.4)	0.740	0.206
Month 9	56.6 (0.1-1004.5)	-		
Month 12	92.4 (10.3-829.5)	115.7 (0.1-11663.8)	0.797	0.172

Sample size: n ≤ 7 for cases and n ≤ 10 for controls. Data are shown as median (range). Unadjusted comparisons were performed by Mann Whitney U test and adjustment for age, gender, disease diabetes duration and baseline levels was done by linear regression. WBC: white blood cells, GADA: glutamic acid decarboxylase autoantibodies, IAA: insulin autoantibodies, IA-2A: islet antigen-2 autoantibodies, Treg: regulatory T cell, ZnT8RA: zinc transporter 8 R325 autoantibodies, ZnT8WA: zinc transporter 8 W325 autoantibodies

Peripheral blood Tregs declined until month 12 in both groups (Table 24). Memory Tregs remained at stable levels in cases and decreased in the control group during the first year of follow-up; however, median levels of 6- and 12-month peripheral blood and memory Tregs did not differ significantly between cases and controls (Table 24). Recently activated T cells and CD4+ to CD8+ T cell ratio remained at similar levels at all study visits in both groups (Table 24). Sensitivity analyses with complete cases and controls yielded similar results (data not shown).

Table 24. T cell repertoire in cases and controls during 1st year of follow-up.

Immune outcome and time point	Cases	Controls	*P* value (unadjusted)	*P* value (adjusted)
FOXP3+ Treg (%)				
Baseline	6.53 (4.2-9.10)	6.05 (1.29-9.00)	0.905	0.365
Month 3	6.14	-		
Month 6	4.44 (1.46-7.11)	5.31 (3.46-7.88)	0.622	0.448
Month 9	4.81 (2.37-8.95)	-		
Month 12	4.30 (2.53-6.83)	3.78 (2.41-7.30)	0.833	0.520
Memory Treg (%)				
Baseline	1.66 (0.60-2.42)	1.53 (0.65-2.60)	0.905	0.586
Month 3	1.97	-		
Month 6	1.62 (0.64-4.22)	1.48 (0.77-3.70)	0.950	0.531
Month 9	1.41 (1.03-1.94)	-		
Month 12	1.63 (0.60-1.97)	1.15 (0.85-3.11)	0.943	0.982
Recently activated T cells (%)				
Baseline	0.21 (0.20-0.26)	0.16 (0.15-0.65)	0.167	0.398
Month 3	0.32	-		
Month 6	0.50 (0.11-1.04)	0.27 (0.07-0.66)	0.145	0.219
Month 9	0.20 (0.17-0.69)	-		
Month 12	0.17 (0.06-0.53)	0.17 (0.09-0.70)	0.699	0.615
CD4+ to CD8+ T cell ratio				
Baseline	2.22 (0.42-3.35)	1.97 (1.22-5.83)	0.918	0.860
Month 3	2.28 (1.36-3.08)	-		
Month 6	1.85 (1.27-4.37)	1.97 (1.31-2.97)	0.536	0.686
Month 9	1.95 (1.63-4.30)	-		
Month 12	1.82 (1.47-3.14)	1.65 (1.45-3.31)	0.529	0.370

Sample size: n ≤ 7 for cases and n ≤ 10 for controls. Data are shown as median (range). Unadjusted comparisons were performed by Mann Whitney U test and adjustment for age, gender, type 1 diabetes duration and baseline levels was done by linear regression. WBC: white blood cells. FOXP3+ Treg, memory Treg and recently activated T cells are expressed in % of CD4+ T cells.

4.2.5 Cell dose and metabolic outcome

Absolute changes for peak and AUC C-peptide from baseline to month 6 were calculated for all infused children and then correlated with the number of total nucleated cells/ kg body weight and the number of infused CD34+ cells. There was a tendency that those infused children who received more nucleated cells/kg showed a better course regarding AUC C-peptide preservation in the first 6 months post-infusion (Pearson's r=0.751, p=0.052, Figure 16A). The absolute change of AUC C-peptide correlated significantly with the number of infused CD34+ cells (Pearson's r=0.931, p=0.002, Figure 16B). 6-month change in peak C-peptide correlated significantly with the number of CD34+ cells (Pearson's r= 0.802, p=0.030, n=7) but not with the number of infused nucleated cells/kg (Pearson's r=0.599, p=0.155, n=7).

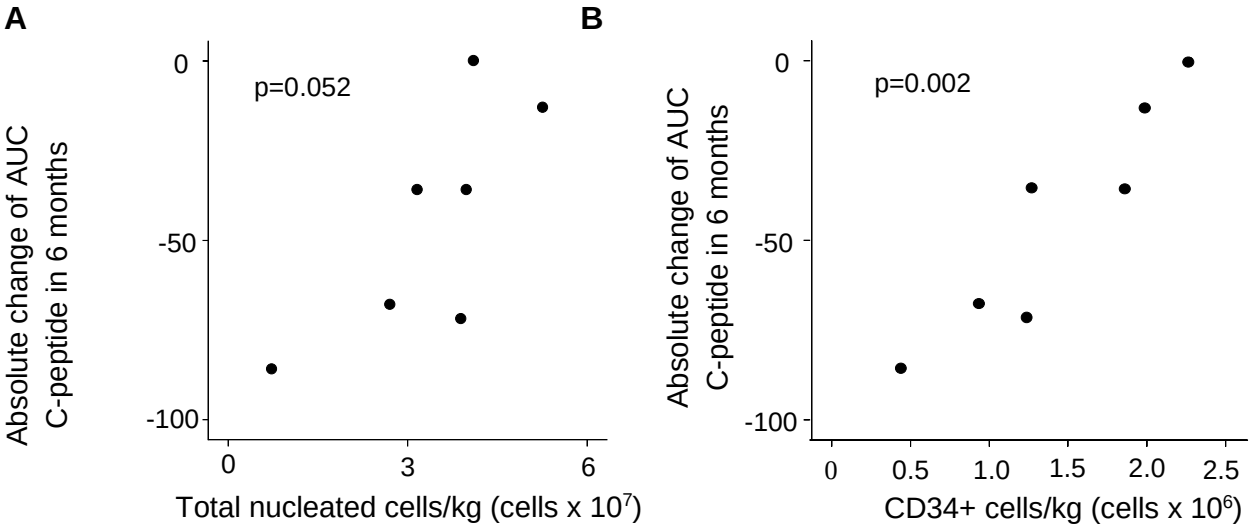

Figure 15. Correlation between the absolute change of peak C-peptide, the number of infused nucleated cells/kg (A, Pearson's r=0.751, p=0.052) and the number of infused CD34+ cells (B, Pearson's r=0.931, p=0.002).

Median change in daily insulin use did not correlate with the number of nucleated cells (Figure 17A, Pearson‘s r=-0.026, p=0.956, n=7) or the number of CD34+ cells significantly (Figure 17B, Pearson‘s r=-0.008, p=0.986, n=7). No significant correlation was observed between the median 6-month change in HbA1c and the number of infused nucleated cells/kg (Figure 18A, Pearson‘s r=-0.052, p=0.912, n=7) or CD34+ cells (Figure 18B: Pearson‘s r=-0.176, p=0.706, n=7).

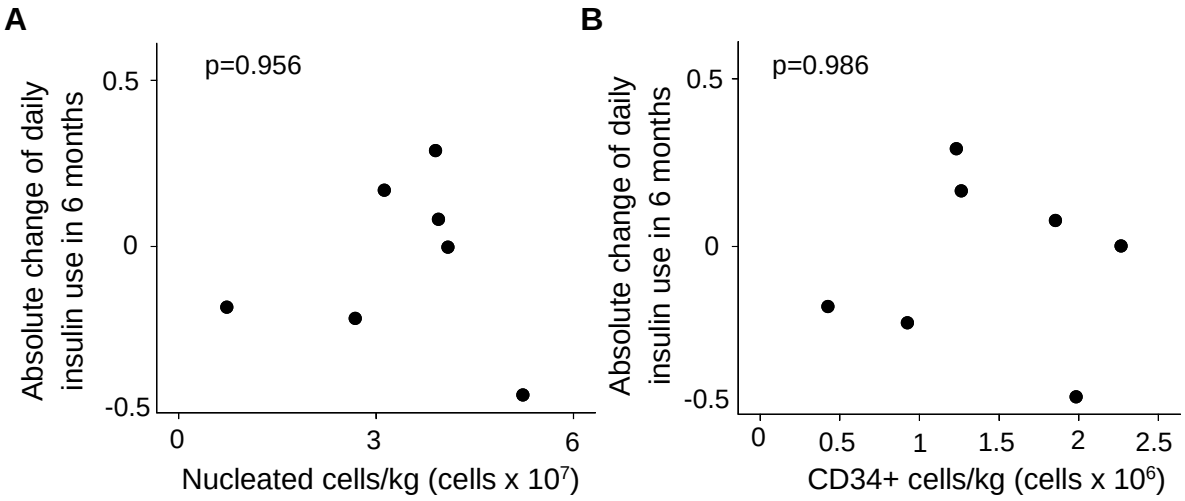

Figure 16. Correlations between the absolute change of daily insulin use (units/kg/day), the total number of infused nucleated cells (A) and the number of infused CD34+ cells (B).

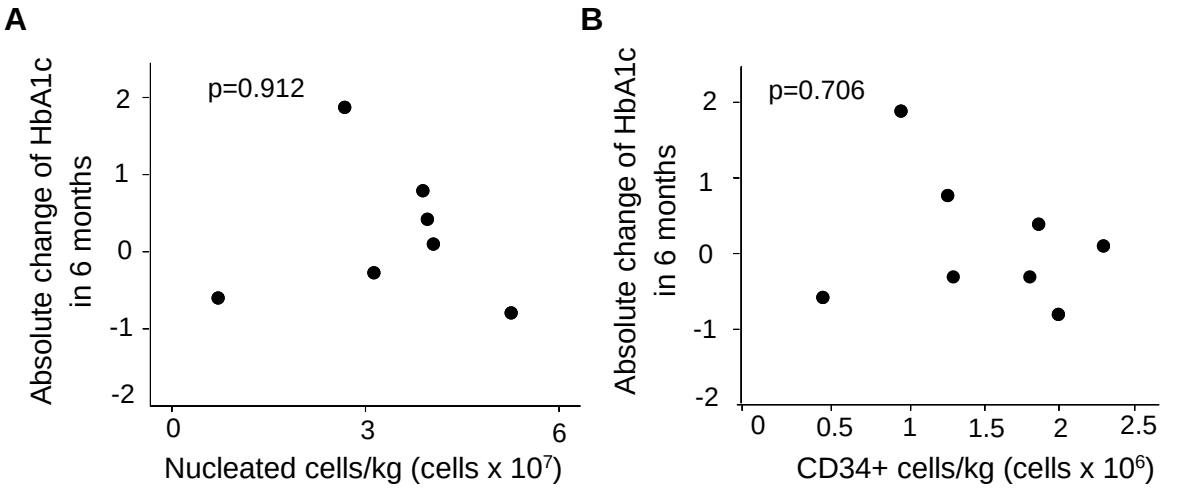

Figure 17. Correlations between the absolute change of HbA1c (%), the total number of infused nucleated cells (A) and the number of infused CD34+ cells (B).

5. DISCUSSION

5.1 Genetic determinants of T1D diagnosis age and β cell function

a. Genetic associations with age at diagnosis

Early age at diagnosis is an unfavorable prognostic factor for T1D as it has been associated with worse metabolic control and early appearance of end organ complications (Karvonen et al 2000, Svensson et al 2006). Studies indicate that late-onset patients have better preserved β cell function than early-onset patients as well as a longer symptomatic period before diagnosis (Palmer 2009).

While it is established that HLA susceptibility genes DR3/DR4-DQ302 are associated with early age of T1D manifestation (Caillat-Zucman et al 1992), little is known about the contribution of non-MHC susceptibility genes on the age when T1D develops. The aim of this thesis was to determine 14 non HLA susceptibility loci and in addition 15 T2D loci to test whether they contribute to the age of T1D onset and whether a score can be developed by combining all these genes for predicting age of onset.

In the present analysis, the association between HLA high risk genotypes and early disease onset was confirmed in the DiMelli cohort. These genotypes were significantly associated with age at diagnosis ≤15 years. Regarding T1D associated SNPs, their effect on age at diagnosis was not significant in the DiMelli cohort. Previous studies have investigated the association between T1D associated genes and age at onset in larger cohorts (Table 25). *PTPN22* risk allele carriers have been

associated with a younger diagnosis age in several reports (Nielsen et al 2007, Kordonouri et al 2010), while in others no significant association was found (Smyth et al 2004, Kahles et al 2005). Polymorphisms in chromosome region 12q13 (e.g. *rs773107, rs2292239, rs10876864*) have been associated with younger age at diagnosis in a group of T1D Spanish patients aged 0-65 years (Hakonarson et al 2008, Espino-Paisan et al 2011). Other associations reported in literature include also a polymorphism in the *PTPN2* gene (*rs2542151*) (Espino-Paisan et al 2011) as well as in the *IL2RA* gene (*rs11594656*); however the latter analysis was performed only in a late-onset patient group, aged 15-40 years (Klinker et al 2010).

Table 25. Associations with age at onset in previous studies.

Gene	Sample size cases/controls	Age at onset (years)	Number of analyzed genes	Journal (Reference)
1. PTPN22	253 / 354	0.6-16.6	1	Int J Immunogenet (Nielsen et al 2007)
2. *PTPN22*	243 / 209	0.1-17.8	1	Hum. Immunol (Kordonouri et al 2010)
3. *PTPN22*	220 / 239	1-44	1	Eur. J. Endocrinol. (Kahles et al 2005)
4. *PTPN22*	1573 / 1718 and 1388 T1D families	<16	1	Diabetes (Smyth et al 2004)
5. *ERBB3*	444 / 861	0-65	3	Diabetologia (Espino-Paisan et al 2011)
6. *PTPN2*	439 / 861	0-65	1	Immunogenetics (Espino-Paisan et al 2011)
7. *INS*, *PTPN22*, *IFIH1* and *IL2RA*	591 / 1538	15-40	5	Immunogenetics (Klinker et al 2010)

While in the present analysis the effect of each polymorphism on age at diagnosis was rather inconsiderable, a high load of risk alleles was found to be associated with young age at diagnosis significantly. The summation of risk alleles in the form of a risk score is a powerful approach in order to investigate associations

between genetic factors and clinical parameters, because a genetic risk score can integrate synergistic effects of individual markers (Linder et al 2012). In particular, in the present analysis it was shown that a risk score calculated from 10 significant T1D-related SNPs (*SH2B3, PTPN2, COBL, IL18RAP, CD25, IL2, IL7R, CTLA4, KIAA0350* and *IFIH1)* is associated with early disease onset significantly. This result suggests that a high genetic load leads to an accelerated manifestation, probably because of a faster development of autoimmunity or progression from autoimmunity (Howson et al 2011). A second possible explanation would be that as the immune system matures and ages, the effect of specific genes in it becomes weaker.

Despite the differences in basic pathogenetic mechanism, both T1D and T2D are the result of metabolic consequences of inadequate β cell function and insulin secretion (American Diabetes Association 2012). An impact of genes affecting β cell function on the risk to develop T1D has been suggested (Wilkin 2001) but has not yet been examined systematically. A further aim of the thesis was to investigate whether genetic susceptibility for impaired β cell function and insulin secretion deriving from known T2D related SNPs, leads to early manifestation of T1D.

In the present analysis, there was a significant association between age at diagnosis and *rs10882102 (HHEX/IDE)*; risk allele carriers (G) had a significantly younger age at diagnosis in comparison to the rest. *HHEX* (hematopoietically expressed hemeobox) / *IDE* (insulin degrading enzyme) genes are strong candidate susceptibility genes for T2D (Pascoe et al 2007). This finding suggests that younger disease onset probably results from accelerated progression of β cell destruction. In previous reports the same polymorphism was associated with low birth weight (Winkler et al 2009) as well as reduced BMI and weight at age 8 years in offspring

with a family history of T1D (Winkler et al 2010). Interestingly, lower birth weight has been reported to be associated with earlier T1D onset (Khan et al 1994, Dabelea et al 2006). Consequently, HHEX/IDE could be regarded as one of the genetic factors, responsible for early disease onset.

While each of the examined T2D associated loci individually did not seem to have a significant effect on age at diagnosis in the studied cohort, a combination of SNPs was suggested to increase the power of results. A risk score consisting of 11 important T2D loci (*HHEX/IDE, GCKR, FTO, CDKN2A, CDKAL1, KCNQ1, MTNR1B, PPARG, TCF7L2, THADA* and *WFS1)* was introduced for the first time, in which a high load of risk alleles was found to be associated with younger age at diagnosis significantly, probably due to impairment of β cell function or insulin secretion. Contrary to the results from previous reports (Qu et al 2008, Raj et al 2009), it is suggested in the present trial that T2D loci may have an effect on childhood-onset T1D.

b. Genetic associations with fasting C-peptide levels

The ability to continue to secrete endogenous insulin is a second critical factor in T1D progression. High risk HLA genotypes (DRB1*03-DQB1*0201/DRB1*04-DQB1*0302) associate not only with younger age of disease onset but also with reduced β cell function (Petrone et al 2005, Spoletini et al 2007), but little is known whether loci outside the HLA region can affect the secretion of endogenous insulin in individuals with newly diagnosed T1D. Therefore, the present work focused on the

association between T1D and T2D related genetic variants and C-peptide levels at disease onset.

In the studied childhood-onset population, age and gender associated with fasting C-peptide levels significantly, results which were consistent with previous reports (Ludvigsson et al 1976, Kibirige et al 2003, Howson et al 2012, Samuelsson et al 2013). Young patients presented lower C-peptide levels and female participants had higher C-peptide levels in contrast to males. Differences in immune function between boys and girls (Cohen-Solal et al 2008, Okuyama et al 2011) as well as the fact that girls enter puberty earlier than boys (Wheeler 1991), and therefore increased insulin resistance could result in better C-peptide at diagnosis, can explain the effect of gender on C-peptide levels.

There was no significant effect on fasting C-peptide levels coming either from T1D or T2D associated SNPs in the present analysis. In literature only a few associations have been reported including *SLC30A8*, *PTPN22* and *INS*. *Rs13266634 (SLC30A8)* risk allele is reported to be associated with preserved β cell function (stimulated C-peptide) in a population of 257 children, aged <16 years, with new onset T1D (Nielsen et al 2011). *Rs13266634* risk allele (C) has also been associated with reduced insulin requirements in 177 T1D patients, aged <18 years, in a study performed by Moosavi et al. (Moosavi et al 2012). *PTPN22* 1858T gene variant has been associated in a group of 120 new onset patients, aged 5 to 36 years, with reduced residual β cell function as well as with poor metabolic control 12 months after disease manifestation (Petrone et al 2008), however this result could not be confirmed in the present study, not only in the young but also in the older population. In addition, *INS VNTR* class III alleles have been associated with

preservation of β cell function in a group of 257 children, with diagnosis age 9.1±3.7 (mean±SD) (Nielsen et al 2006), but in the present study cohort no association between *INS* and fasting C-peptide was observed (0.890, adjusted for age, gender and time to diagnosis).

c. Rate of β cell function loss and genetic risk scores

A further aim of this thesis was to investigate whether preservation of β cell function after disease onset has a genetic background. Analyzing the genetic determinants of β cell function in a late-onset population (Altered Peptide Ligand NBI-6024, Calcitriol trials and ChAglyCD3 Study), interesting associations were found in the present work. It is worthwhile to note that that a high load of T1D related risk alleles (*CD25, IL7R, COBL, CTLA4, ERBB3, IFIH1, IL2, IL10, IL18RAP, KIAA0350, PTPN2, PTPN22, PTPN22, SH2B3, INS),* was found to be associated with reduced β cell function 18 months after disease manifestation significantly. This finding suggests for the first time that a high load of genes, which are responsible for the autoimmune destruction of β cells, leads to a more massive impairment of β cell secretory ability. It seems, also, that these loci are not only involved in the autoimmune process that lead to the development of T1D (prediabetic phase), but continue to affect the course of the disease months after clinical manifestation. In the present analysis, although T1D susceptibility loci were found to have an important impact on β cell function, the effect of T2D associated SNPs was not considerable. Probably, once the autoimmune destruction has started, the activity of T2D related loci on the β cell is probably modified or restricted.

The present analysis has several strengths and limitations. For the first time, different gene polymorphisms are combined in order to form scores, able to predict the age of disease manifestation and β cell function status. The present findings provide insight into genetic determinants of T1D progression and show how the combination of specific loci, that have actually a very small effect on disease development individually, can be used successfully for risk stratification. Identification of genetically susceptible individuals, who will have a faster disease progression either by developing T1D very early in life or by losing their ability to secrete insulin within a few months after diagnosis, would be a rational factor in designing new individualized intervention trials. As a further strength, the DiMelli cohort consists of all registered newly diagnosed cases within Bavaria, so the subjects are not selected according to criteria, such as defined metabolic status or high genetic risk. Therefore, the described associations are not limited to specific patient groups and may be representative of childhood T1D.

A limitation of the trial involves the limited number of participants and as a result the main findings should be confirmed and validated by larger independent trials. A second limitation is that not all of the known T1D or T2D associated susceptibility were included in the analysis. Third, C-peptide was measured in different laboratories regarding the examined intervention trials (Altered Peptide Ligand NBI-6024, Calcitriol and CD3). Thus, a bias toward more sensitive and/or less specific measurement of C-peptide could be present due to assay differences.

In conclusion, these data contribute to a better understanding of the genetic component in T1D and suggest that various T1D and T2D susceptibility genes cannot only accelerate the age at diagnosis but continue to play a role on T1D

progression after disease manifestation. This knowledge will allow us to design intervention strategies which will try to decelerate or even halt the progress of the disease very early in the pathogenesis of T1D.

5.2 The effect a single autologous cord blood infusion on disease progression

The second part of this this thesis investigated the potential effect of a single autologous cord blood infusion on β cell function and glucose control in a group of children with newly diagnosed T1D. Although there were no significant safety issues or adverse events related to the infusion, a single infusion of autologous cord blood did not prevent from the natural loss of residual β cell function, did not cause significant changes in daily insulin requirements and glucose control and was not found to change the T cell repertoire significantly.

The administration of cord blood in newly diagnosed children with T1D has already been found to be feasible and safe in previous reports from Haller et al (Haller et al 2011), nevertheless it was unknown if infused children presented different courses in contrast to controls. In this trial, infused children had a similar decline rate of C-peptide over 1 year of follow-up as controls. However, it should be noted that the participants of the control group were significantly older contrary to the children who underwent an infusion, therefore the initial measurements of β cell residual function (assessed by C-peptide) were considerably different at time of inclusion. When comparing residual β cell function among new onset young patients, there are a few points that need to be discussed. Loss of endogenous insulin

secretion is the main effect of T1D, with various natural history studies showing a decline before and after clinical manifestation until a complete absence of insulin production occurs (Greenbaum et al 2009). Unfortunately a considerable amount of β cell function is lost between surveillance diagnosis of the diseases and symptomatic diagnosis. According to previous reports, within the first year of diagnosis more than 80% of the patients have detectable levels of C-peptide whereas 5 years after manifestation only 10-20% of subjects retain significant levels of β cell function (Greenbaum et al 2009). Residual β cell function in patients suffering from T1D has an extremely different course among individuals and is shown to be associated with factors, such as age at diagnosis, number of positive antibodies present at diagnosis, degree of metabolic control as well as genetic (high HLA risk, PTPN C1858T variant etc) and immunological parameters (Palmer 2009).

Regarding age, older patients do not only appear to have higher levels of C-peptide at diagnosis but also present a slower, progressive loss of β cell function after the clinical presentation of the disease in contrast to younger patients. This inverse correlation of age with greater loss of residual pancreas function suggests that T1D follows a more aggressive course in younger children (Karjalainen et al 1989, Palmer 2009, Greenbaum et al 2012). Younger children seem to have lower C-peptide levels at diagnosis as well as a shorter remission phase compared to children older than 10 years, as reported by Dost et al (Dost et al 2007). Children younger than 5 years have a higher rate of diabetic ketoacidosis at diagnosis when compared to older ones, a fact that indicates the rapid and extensive β cell damage taking place before diagnosis (Bonfanti et al 1998, Bowden et al 2008).

Regarding changes in metabolic control (insulin use and HbA1c), there were no significant differences between cases and controls. Usually children with new onset T1D require an initial total insulin dose of circa 0.5 to 1 units/kg. Younger and prepubertal children require lower dose, while hormonal influences of increased growth hormone and sex hormone secretion during puberty dictate higher doses up to 1.5 units/kg per day (Silverstein et al 2005). In the present trial, this fact should be taken into consideration as 4 of the participants of the control group entered puberty during the trial. Regarding guidelines for T1D in childhood (Silverstein et al 2005), HbA1c target values are slightly higher for younger children because of the instable glucose levels. Indeed in the present trial, children of the intervention group had slightly higher levels; however, they presented a more stable course over time.

In other diseases, the total number of infused nucleated cells and CD34+ cells is found to correlate with the clinical outcome (Perez-Simon et al 1999, Bittencourt et al 2002, Rocha et al 2002), however with regard to T1D such findings have not been documented so far. In the present trial the median number of infused nucleated cells/kg was higher in comparison to Haller's study (3.89×10^7 vs. 1.8×10^7 cells, respectively) (Haller et al 2011), which can be explained by the fact that participants of the present trial were of younger age (median age 3.02 vs. 5.10 years). Children who received a richer in stem cells cord blood sample were found to have a better preservation of C-peptide 6 (assessed by AUC C-peptide) months post-infusion. This finding could be useful for future follow-up trial in which it may be necessary to initially define the suitable final product that affects clinical outcome. Storing as well as thawing procedures should be improved in the future in order to prevent great loss of cells or vital ingredients in the final product. Regarding adult patients, where

the number of cells infused per kilo is even smaller, cell expansion techniques should also be considered. In addition, noninvasive measures for stem cell tracking via magnetic resonance imaging, bioluminescence, or fluorescence resonance energy transfer will provide a quantitative evaluation on the survival and fate of the transplanted cells (Driscoll et al 2011).

The mechanisms of action, the optimal therapeutic window and the best route of cord blood infusion for T1D remain unfortunately unknown. Proposed mechanisms include the: a. migration of cord blood stem cells to the pancreas and differentiation into β cells b. proliferation of new islets from remaining viable tissue or c. suppression of effector T cells by regulatory T cells. The last mechanism comes in agreement with the results from the cord blood trial held by Haller et al., where the total number of CD4+CD25+FOXP3+ regulatory T cells was found to be significantly increased 6 months after cord blood infusion in subjects with T1D (Haller et al 2011). However, it could not be clearly established if there was a direct correlation between number of infused nucleated cells and C-peptide change (Haller et al 2011). In the present trial, there was no significant increase in Tregs after infusion as reported by Haller et al, while memory Tregs remained rather stable.

A main strength of this analysis is the recruitment of a control group, which for the first time allows comparing the disease course after infusion with a “natural” course. The use of the same storing and thawing procedures for all infused cord blood samples improves comparability of examined parameters between individuals. Limitations include the small number of participants as well as the significant age difference between cases and controls. The inclusion criteria of this trial and the experimental nature of the trial in combination with the fact that many interested

families possessed only one autologous cord blood unit, narrowed the potential target group significantly. The main reason for all eligible families that declined participation was the use of the cord blood in a future, promising treatment that would definitely result in a better glycemic control or maybe bring a complete cure of the disease. Nevertheless, the matter of exhausting the whole cord blood unit in a research project could have implications for the design of future studies (Driscoll et al 2011). Once cell expansion techniques as well as cell differentiation methods are developed and perfected, storage of multiple aliquots would enable more parents to participate in research trials. Parents of younger children, who did not possess an autologous cord blood sample, decided not to participate as controls, mainly because of their wish to take part in intervention trials. As a further limitation, some T cell measurements were taken from stored samples and could therefore not be used for statistical analysis.

The collection as well as private banking of umbilical cord blood displays a number of ethical and regulatory challenges that remain to be addressed in the future. These challenges include issues related to consent, ownership, commercialization, the need for fair and equitable harvesting of and access to placental blood and embryo cloning for the purpose of producing an identical tissue donor (Petrini 2010). Obviously, these issues need to be considered by medical ethicists in order to assist clinicians when informing future parents. Healthcare providers should inform the interested birthing parents about the advantages and possible disadvantages of cord blood banking and related future cord blood usage.

In conclusion, these findings demonstrate that an autologous cord blood infusion in young children with newly diagnosed T1D is safe, but does not seem to

protect from the natural loss of β cell function during the first year post-infusion. A single infusion of minimally manipulated cord blood is probably not able to diminish the vast autoimmune destruction present in T1D; further mechanistic analysis of the changes in T cell repertoire will provide insight into possible immunosuppressive mechanisms and the content of CD34+ cells in the blood sample might offer potential for improvement of future cell therapies. Although progress is encouraging, multiple therapeutic avenues as well as combinational therapies will need to be explored in order to achieve the goal of safely diverting, or even preventing T1D.

6. SUMMARY

The rising incidence of type 1 diabetes (T1D) has led to a continuously growing interest in investigating the specific causes, genetic or environmental, that are responsible for T1D manifestation and disease progression. Little is known whether non HLA genes affect the age of disease manifestation and whether they continue to play an essential role in disease progression after diagnosis, by influencing the rate of loss of β cell function.

The present thesis investigated whether T1D and type 2 diabetes (T2D) associated single nucleotide polymorphisms (SNPs) influenced T1D progression, either by accelerating the age at diagnosis or by affecting the rate of β cell function loss after disease manifestation. A further objective was to examine for the first time whether risk allele combinations from different SNPs, that had only a small effect on disease development by its own, could be used in order to identify individuals at risk for developing the disease early in life or individuals with predisposition of losing their endogenous insulin secretory ability within months after diagnosis.

Analysing the effect of each T1D and T2D SNP on age at diagnosis separately in a cohort of 357 children with T1D, only T2D associated *rs10882102* (*HHEX/IDE*) risk allele was found to associate with early disease onset significantly (p=0.001). Interestingly, a high load of risk alleles, coming from the combination of 10 T1D susceptibility genes (*SH2B3, PTPN2, COBL, IL18RAP, CD25, IL2, IL7R, CTLA4, KIAA0350* and *IFIH1)*, associated with early disease onset significantly (p=0.022 in the whole cohort, p=0.028 within patients carrying the HLA high risk genotypes). Similarly, a higher number of risk alleles in a genetic score containing 7

T2D associated SNPs (*THADA*, *TCF7L2, MTNR1B, HHEX/IDE, GCKR, CDKN2A* and *CDKAL1)* correlated with younger age at diagnosis significantly (p=0.002).

A second genetic analysis was performed in a group of 86 adolescent and adult individuals with new onset T1D. Patients who presented a C-peptide decline over the first 18 months after disease onset were found to carry more risk alleles in a risk score, consisting of all 14 examined T1D susceptible loci (*ERBB3, PTPN22, KIAA0350, IL10, SH2B3, PTPN2, COBL, IL18RAP, CD25, IL2, IFIH1, IL7R* and *CTLA4)*, compared with patients with C-peptide preservation (p=0.004, adjusted for age, gender, diabetes duration, screening C-peptide and intervention group). T2D SNPs alone or in form of a risk score did not associate with loss of β cell function in T1D patients significantly.

In conclusion, these findings suggest that a high genetic load leads to an accelerated disease progression, probably because due to a faster development of autoimmunity or progression from autoimmunity. Various T1D and T2D susceptibility genes seem not only to accelerate age at diagnosis but are found to continue to play a role in T1D progression after disease manifestation. Furthermore, these findings show that the combination of specific loci, that have actually a very small effect on disease development individually, can be added in form of a risk score and used successfully for risk stratification. This knowledge will allow further intervention strategies to be contemplated, which will try to decelerate or even halt the progress of the disease very early in the pathogenesis of T1D.

In the second part of the thesis a non-randomized, open-label, controlled intervention trial was performed, with the starting hypothesis that a single autologous umbilical cord blood infusion administered to young children with newly diagnosed T1D could

preserve residual β cell function and improve blood glucose control. The application of autologous cord blood in children with type 1 diabetes has been recently found to be safe, but not to preserve β cell function in a previous study, which, however, had not included a control group. The objective was to compare the changes of metabolic (area under the curve [AUC] C-peptide, insulin use and HbA1c) and immune function (islet autoantibody titer and T-cell repertoire) over one year of follow-up between 7 cord blood infused children and 10 natural controls.

Cases and controls were compared regarding metabolic and immune outcome, adjusted for age, gender, diabetes duration and baseline levels. There were no significant adverse events related to the infusion. Metabolic parameters were not significantly different at 12 months follow-up between the two groups (adjusted p=0.244 for AUC C-peptide, adjusted p=0.228 for peak C-peptide, adjusted p=0.820 for insulin use, adjusted p=0.525 for HbA1c). Islet autoantibody titers and T cell repertoire did not differ significantly between cases and controls during the first year of follow-up. 6-month change of AUC C-peptide correlated significantly with the number of infused CD34+ cells (r=0.931, p=0.002).

These findings indicate that an autologous cord blood infusion does not seem to affect the natural course of metabolic and immune parameters after disease onset. However, the content of CD34+ cells in the blood sample might offer potential for improvement of future cell therapies.

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