Peripheral blood biomarkers of cell-specific autoimmunity. Studies in children at increased risk for type 1 diabetes.

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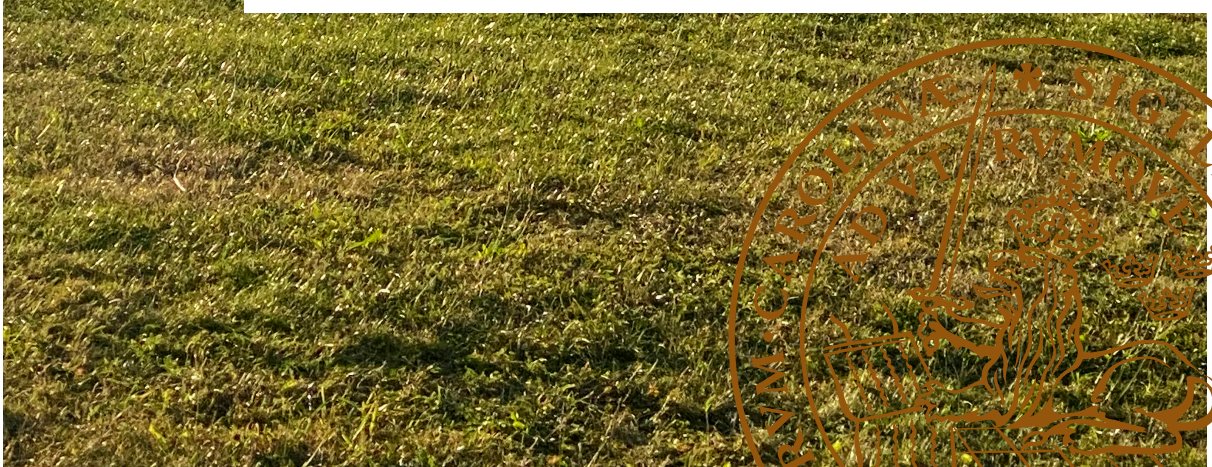

Peripheral blood biomarkers of cell-specific autoimmunity

Studies in children at increased risk for type 1 diabetes

Falastin Salami

LUND
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DOCTORAL DISSERTATION

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Abstract

While the incidence of children suffering from autoimmune type 1 diabetes (T1D) is increasing in Sweden and worldwide, the underlying etiology and cellular mechanisms behind this remain unknown. The predisposition of the high-risk HLA DR-DQ genotype and as yet unknown environmental triggers lead to autoimmunity and the onset of T1D, which is preceded by islet beta-cell autoantibodies acting as markers for ongoing autoimmunity. This study aims to identify peripheral blood biomarkers to predict and explain cellular autoimmune processes leading to beta-cell loss before and after seroconversion. We also investigate whether immune tolerance treatment with GAD-alum affects T-cells in nondiabetic children at increased genetic risk of T1D prospectively followed in longitudinal studies.

Children participating in the Swedish TEDDY cohort with or without islet beta-cell autoantibodies were studied. Complete blood count in these children was analyzed and related to autoantibody status, gender, HLA genotype, and glucose metabolism measures. HbA1c, a predictive biomarker for a subsequent autoantibody or T1D, was analyzed in the TEDDY cohort from Finland, Germany, Sweden, and the US. HbA1c trajectories were also studied in the progression from developing a single autoantibody to diagnosing T1D Children aged 4–17.99 years at enrollment participating in the DiAPREV-IT2 clinical trial were studied and different T-cells were immunophenotyped to investigate the immune tolerance treatment with GAD-alum.

A reduction in neutrophil counts primarily in boys and children with the HLA-DR3-DQ2/DR4-DQ8 genotype, and a reduction of red blood cell counts, hemoglobin, and hematocrit primarily in girls and in children with HLA-DR3-DQ2/DR4-DQ8 were inversely associated with autoimmunity and the number of beta-cell autoantibodies. A reduction in red blood cell indices (MCH and MCV) was associated with increased HbA1c, by increasing number of beta-cell autoantibodies. Reduction in red blood cell count, hemoglobin, and hematocrit levels were associated with increased fasting blood glucose. Increased red blood cell counts and hemoglobin, hematocrit, and MCH were associated with increased fasting insulin. Increased HbA1c was associated with an increased risk of T1D regardless of the number and type of autoantibodies. The development of IA-2A as a second or fourth autoantibody was associated with decreased HbA1c levels. The HbA1c trajectories presented a more rapid increase of HbA1c as the number of autoantibodies increased from one to three. GAD-alum-treated children had lower T-helper cell (CD3+ CD4+ T-cells) and cytotoxic T-cell (CD3+ CD8+ T-cells) levels 18–24 months after two immunizations with GAD-alum.

Reductions in neutrophil levels, red blood cells, and red blood cell parameters and increased levels of HbA1c are all associated with multiple autoantibodies, reflecting a prominent islet autoimmune burden. The reduction in different complete blood counts with increasing numbers of beta-cell autoantibodies may suggest an unknown effect of impaired beta-cell function on haematopoiesis. Predicted trajectories of HbA1c could be used to further develop a model to predict the time to T1D diagnosis in children with multiple autoantibodies. The decrease in HbA1c associated with the appearance of IA-2A may be a consequence of aggressive autoimmune destruction of beta-cells leading to insulin leakage into the bloodstream. These results should prove helpful for understanding the pathogenesis of T1D and better predicting the onset of T1D in seroconverted children. Immunization with GAD-alum has a long-term effect on T-cells 18–24 months after treatment.

Key words: Type 1 diabetes, biomarkers, children, CBC, autoimmunity, neutrophils, red blood cells, hemoglobin, HbA1c T-cells, prospective follow-up, GAD-alum,

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To my family

رَّبِّ زِدْنِي عِلْمًا

"My Lord, increase me in knowledge."

(Holy Quran. Surat Taha, verse 114)

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II. **Salami F**, N Tamura R, Elding Larsson H, Lernmark Å, Törn C; TEDDY Study Group. Complete blood counts with red blood cell determinants associate with reduced beta-cell function in seroconverted Swedish TEDDY children. Endocrinol Diabetes Metabolism. 2021 May 3;4(3):e00251. © 2021 Salami et al. Endocrinology, Diabetes & Metabolism, published by John Wiley & Sons Ltd.

III. **Salami F**, Tamura R, You L, Lernmark Å, Larsson HE, Lundgren M, Krischer J, Ziegler AG, Toppari J, Veijola R, Rewers M, Haller MJ, Hagopian W, Akolkar B, Törn C; TEDDY Study Group. HbA1c as a time predictive biomarker for an additional islet autoantibody and type 1 diabetes in seroconverted TEDDY children. *Submitted Manuscript*

IV. **Salami F**, Spiliopoulos L, Maziarz M, Lundgren M, Brundin C, Bennet R, Hillman M, Törn C, Elding Larsson H. Long-Term GAD-alum Treatment Effect on Different T-Cell Subpopulations in Healthy Children Positive for Multiple Beta Cell Autoantibodies. Journal of Immunology Research. 2022 May 25;2022:3532685. © 2022 Falastin Salami et al.

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I. Martinez MM, Spiliopoulos L, Salami F, Agardh D, Toppari J, Lernmark Å, Kero J, Veijola R, Tossavainen P, Palmu S, Lundgren M, Borg H, Katsarou A, Larsson HE, Knip M, Maziarz M, Törn C; and the TEDDY-Family (TEFA) Study Group. Heterogeneity of beta-cell function in subjects with multiple islet autoantibodies in the TEDDY family prevention study - TEFA. Clin Diabetes Endocrinol. 2022 Jan 5;7(1):23.

II. Lind A, Salami F, Landtblom AM, Palm L, Lernmark Å, Adolfsson J, Elding Larsson H. Immunocyte single cell analysis of vaccine-induced narcolepsy. Eur J Immunol. 2021 Jan;51(1):247-249.

III. Martinez MM, Salami F, Larsson HE, Toppari J, Lernmark Å, Kero J, Veijola R, Koskenniemi JJ, Tossavainen P, Lundgren M, Borg H, Katsarou A, Maziarz M, Törn C; TEDDY Family (TEFA) Study Group. Beta cell function in participants with single or multiple islet autoantibodies at baseline in the TEDDY Family Prevention Study: TEFA. Endocrinol Diabetes Metab. 2020 Nov 5;4(2):e00198.

IV. Salami F, Abels M, Hyöty H, Vaziri-Sani F, Aronsson C, Vehik K, Delli A, Hagopian W, Rewers M, Ziegler A, Simell O, Akolkar B, Krischer J, She J, Lernmark A; the TEDDY study group. DETECTION OF LACTOBACILLI IN MONTHLY MAIL-IN STOOL SAMPLES FROM 3-18 MONTHS OLD INFANTS AT GENETIC RISK FOR TYPE 1 DIABETES. Int J Probiotics Prebiotics. 2012 Aug;7(3-4):135-144.

Abstract

Objective

While the incidence of children suffering from autoimmune type 1 diabetes (T1D) is increasing in Sweden and worldwide, the underlying etiology and cellular mechanisms behind this remain unknown. The predisposition of the high-risk HLA DR-DQ genotype and as yet unknown environmental triggers lead to autoimmunity and the onset of T1D, which is preceded by islet beta-cell autoantibodies acting as markers for ongoing autoimmunity. This study aims to identify peripheral blood biomarkers to predict and explain cellular autoimmune processes leading to beta-cell loss before and after seroconversion. We also investigate whether immune tolerance treatment with GAD-alum affects T-cells in nondiabetic children at increased genetic risk of T1D prospectively followed in longitudinal studies.

Methods

Children participating in the Swedish TEDDY cohort with or without islet beta-cell autoantibodies were studied. Complete blood count in these children was analyzed and related to autoantibody status, gender, HLA genotype, and glucose metabolism measures. HbA1c, a predictive biomarker for a subsequent autoantibody or T1D, was analyzed in the TEDDY cohort from Finland, Germany, Sweden, and the US. HbA1c trajectories were also studied in the progression from developing a single autoantibody to diagnosing T1D. Children aged 4–17.99 years at enrollment participating in the DiAPREV-IT2 clinical trial were studied and different T-cells were immunophenotyped to investigate the immune tolerance treatment with GAD-alum.

Results

A reduction in neutrophil counts primarily in boys and children with the HLA-DR3-DQ2/DR4-DQ8 genotype, and a reduction of red blood cell counts, hemoglobin, and hematocrit primarily in girls and in children with HLA-DR3-DQ2/DR4-DQ8 were inversely associated with autoimmunity and the number of beta-cell autoantibodies. A reduction in red blood cell indices (MCH and MCV) was associated with increased HbA1c, by increased number of beta-cell autoantibodies. Reduction in red blood cell count, hemoglobin, and hematocrit levels were associated with increased fasting blood glucose. Increased red blood cell counts and hemoglobin, hematocrit, and MCH were associated with increased fasting insulin. Increased HbA1c was associated with an increased risk of T1D regardless of the number and type of autoantibodies. The development of IA-2A as a second or fourth autoantibody was associated with decreased HbA1c levels. The HbA1c trajectories

presented a more rapid increase of HbA1c as the number of autoantibodies increased from one to three. GAD-alum-treated children had lower T-helper cell (CD3+ CD4+ T-cells) and cytotoxic T-cell (CD3+ CD8+ T-cells) levels 18–24 months after two immunizations with GAD-alum.

Conclusion

Reductions in neutrophil levels, red blood cells, and red blood cell parameters and increased levels of HbA1c are all associated with multiple autoantibodies, reflecting a prominent islet autoimmune burden. The reduction in different complete blood counts with increasing numbers of beta-cell autoantibodies may suggest an unknown effect of impaired beta-cell function on haematopoiesis. Predicted trajectories of HbA1c could be used to further develop a model to predict the time to T1D diagnosis in children with multiple autoantibodies. The decrease in HbA1c associated with the appearance of IA-2A may be a consequence of aggressive autoimmune destruction of beta-cells leading to insulin leakage into the bloodstream. These results should prove helpful for understanding the pathogenesis of T1D and better predicting the onset of T1D in seroconverted children. Immunization with GAD-alum has a long-term effect on T-cells 18–24 months after treatment.

Abbreviations

ADAP	Assays and multiplex agglutination-PCR
APC	Antigen presenting cell
CAR	Coxsackie-adenovirus receptor
CBC	Complete blood count
DDL	Diabetes Diagnostic Laboratory
DiAPREV-IT2	Diabetes Prevention Immune Tolerance 2
DiPiS	Diabetes Prediction in Skåne
DIPP	Diabetes Prediction and Prevention
DKA	Diabetic ketoacidosis
DPTRS	Diabetes Prevention Trial–Type 1 Risk Score
FDR	First degree relative
FPIR	The first phase insulin response
FSC	Forward scatter
GADA	Glutamic acid decarboxylase autoantibody
GRS	Genetic risk scores
GWAS	Genome-wide linkage analysis studies
HbA1c	Glycated hemoglobin A1c
HLA	Human leukocyte antigen
IAA	Insulin autoantibody
IA-2A	Insulinoma antigen-2 autoantibody
IASP	Islet Autoantibody Standardization Program
IGT	Impaired glucose tolerance
IL2RA	Interleukin 2 receptor alpha
IVGTT	Intravenous insulin tolerance test
LADA	Latent autoimmune diabetes in the adult

LD	Linkage disequilibrium
LIPS	Luciferase immune precipitations system
LYP	Lymphoid-specific phosphatase
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
MHC	Major histocompatibility complex
MODY	Maturity onset diabetes in the young
NGS	Next generation sequencing
OGTT	Oral glucose tolerance test
PMT	Photomultiplier tubes
PTP	Protein tyrosine phosphatase
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RDW	Red cell distribution width
RBA	Radiobinding assay
SSC	Side scatter
TEDDY	The Environmental Determinants of Diabetes in the Young
T1D	Type 1 diabetes
VNTR	Variable number of tandem repeats
ZnT8A	Zink transporter 8 autoantibody

Introduction

Type 1 diabetes

Type 1 diabetes (T1D) is a chronic autoimmune disease, usually affecting children or young adults but may also appear at any age. The condition occurs when the pancreatic islet beta-cells are destroyed by autoreactive immune cells leading to insulin deficiency and dysglycaemia, thus requiring lifelong insulin administration. Frederick Banting discovered insulin in 1921, which transformed the prognosis of T1D from a fatal condition to a survivable condition lasting for a lifetime.

Children with T1D and their parents have a challenging everyday life with an enormous burden to keep the child's blood glucose within safe levels by monitoring blood glucose, administrating proper amounts of insulin through injections or insulin pumps, closely watching their food intake, and in many cases, also dealing with anxiety and illness beliefs. The blood glucose levels should be kept within normal ranges to avoid hyperglycemia and hypoglycemia that may cause life-threatening acute or long-term diabetic complications. Despite the insulin treatment for glucose level management, lifestyle management with a healthy diet and exercise is important for children with T1D to maintain health and prevent long-term complications. In over 100 years since the discovery of insulin, neither a cure nor a full understanding of the etiology of T1D been achieved. However, T1D diabetes research has so far improved the health and life quality of persons with T1D, and researchers pursue their work worldwide to predict, prevent, improve treatment, and strive to cure T1D.

Incidence

The incidence of T1D has been increasing globally with an average annual increase of 3–4% over the past three decades, especially among children, with approximately 90,000 diagnosed yearly (Fig.1) (1-3). The worldwide incidence of T1D is 15 per 100,000 people (4). The incidence among children varies geographically, with the highest incidence observed in industrialized countries in the northern hemisphere, specifically the Nordic countries and other countries in Europe, with Finland ranked in first place (52/100,000) (5) and Sweden in second (49/100,000) (6). Countries in the southern hemisphere have low T1D incidences; the lowest rates are in China and Venezuela (0.1/100,000) (7). The geographic variation of the incidence follows populations' ethnic and racial distribution worldwide. Caucasoid populations have the highest incidences as compared with other major ethnic groups. This is reflected in nations with different ethnicities, such as the USA, where the incidence ranges between 7/100,000 (Native Americans) to 27/100,000 (Non-Hispanic Whites) (8). However, the incidence of T1D can vary substantially between neighbouring areas in North America and Europe within populations with similar descent and genetic backgrounds, representing an epidemiological enigma. One example is the two-third higher T1D incidence in Finland than in neighbouring Estonia, which could be explained by unknown environmental factors (9). Through improved worldwide T1D surveillance and documentation programs, new high incidence countries like Kuwait (41/100,000)(10) and Saudi Arabia (33.5/100,000)(11) have been identified, as well as the rapid increase of incidence among children and adolescents in low incidence countries, for example, China and Egypt (12, 13).

The disease may appear at any age, but the incidence increases by age, with two peaks occurring around 5–7 years and around 10–14 years in proximity to puberty (14-16). The incidence is higher among boys than girls in high -incidence countries, while the opposite is observed in low-incidence countries (17). The incidence also varies with season and birth month, as several studies in high incidence countries have shown a higher incidence of T1D among children during autumn and winter and in children born in the spring (18-20).

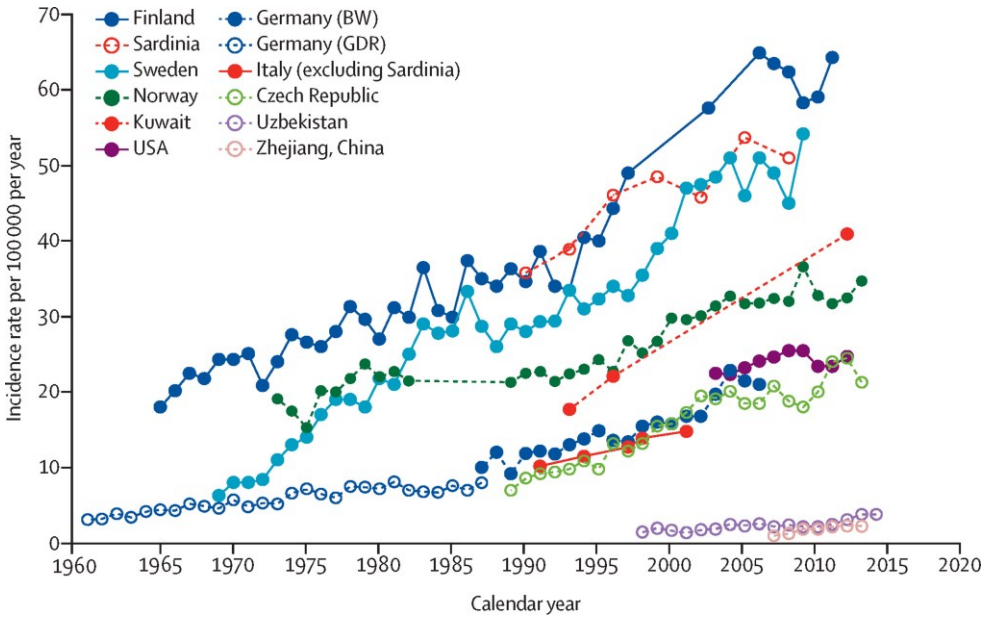

Figure 1. Global time trend of T1D incidence. Adapted and reproduced with permission from Norris et al. (2).

Clinical presentation

Diabetes is as a chronic metabolic disease recognized by hyperglycemia and characterized by the loss of beta cell function leading to insulin deficiency (type 1 diabetes) or insulin resistance (type 2 diabetes). The major types of diabetes are type 1, type 2, and gestational diabetes, and the other rarer types include different types of monogenic diabetes [such as neonatal diabetes and Maturity onset diabetes in the young (MODY), diseases of the exocrine pancreas (for instance cystic fibrosis), and drug- or chemical-induced diabetes]. T1D accounts for 5–10% of diabetes cases and is defined by the appearance of autoantibodies against pancreatic islet beta-cell autoantigens before diagnosis and loss of beta-cell function (21). However, a minority of T1D patients, often of Asian or African descent, fall in the category of idiopathic diabetes with no genetic HLA association, lacking autoantibodies and suffering from episodic ketoacidosis requiring insulin treatment for survival (22). Latent autoimmune diabetes in the adult (LADA) is another type of autoimmune diabetes defined by the presence of glutamic acid decarboxylase antibodies (GADA) distinguished from T1D by more preserved beta-cell residues, much slower disease progression, and no insulin treatment demand in the months from clinical onset (23, 24).

The lack of endogenous insulin secretion due to the loss of beta-cell mass leads to hyperglycemia and the manifestation of clinical T1D. Pathophysiological

disturbances, signs and symptoms resulting from beta-cell dysfunction, insulinopenia, and metabolic derangement include diabetic ketoacidosis (DKA), lack of energy, frequent thirst and urination, constant hunger, bed-wetting, fatigue, nausea, weight loss, and blurred vision. The most severe and life-threatening presentation is DKA, more common in children < 5 years old with increasing prevalence by decreasing age, occurring in more than one-third of diagnosed children worldwide (25-27). Children living in developing countries and families with lower socioeconomic status and lower education have a higher risk for DKA (28, 29). However, early T1D diagnosis reduces DKA risk and preserves long-term beta-cell function (30, 31).

The management of glucose levels to normal levels is challenging with exogenous insulin administered peripherally, since patients lack the endogenous insulin action from beta-cells giving feedback or suppression of insulin release when levels of glucose decrease (1, 32). Despite the constant development of better fast and long-term insulin analogues enabling near-physiological insulin delivery, technical devices for glucose monitoring, insulin pumps, and improving glycemic control, T1D patients still suffer from long-term complications due to hyperglycemia and hypoglycemia remains a major burden (33, 34). Severe secondary long-term diabetic complications affecting T1D patients include cardiovascular diseases, peripheral artery diseases, kidney diseases, neuropathy, and vascular retinopathy. Maintaining the blood sugar significantly reduces the risk of these complications; however, the risk increases with younger age, specifically for cardiovascular diseases (35). The major cause of premature morbidity and mortality in T1D patients is cardiovascular diseases (36, 37).

Etiology of T1D

Genetic factors

Genetic and environmental factors contribute to the still elusive etiology of T1D. The HLA locus residing on the short arm of chromosome 6 accounts for nearly 40-50% of T1D inheritance (38, 39) and is thus believed to constitute the major susceptibility genes. T1D is a polygenetic disease with a complex genetic background mapped by extensive international collaborative studies using candidate gene association studies and genome-wide linkage analysis studies (GWASs) (40, 41). In addition to HLA risk genes around 60 non-HLA risk genes associated with T1D have been identified (41, 42). The risk of T1D in the general population is 0.4%, while the risk in first-degree relatives (FDRs) is higher. Monozygotic twins have a 30–70% genetic risk for T1D or islet autoimmunity if one develops the disease, whereas dizygotic twins or siblings have only 6–10 % genetic risk (43-45).

The risk in children with a father with T1D is comparable with the sibling risk, and the risk for children with a mother with T1D is marginally less (46-48). Hence, this strong genetic predisposition of T1D is not sufficient to induce autoimmunity; an environmental trigger is certainly required.

HLA

HLA is the human term for the major histocompatibility complex (MHC) encoding antigen-presenting glycoprotein receptors that bind and present antigens to T-cell receptors initiating the adaptive immune response and taking part in the peripheral and central immune tolerance. The HLA locus has a dense clustering of genes. There are three subclasses of HLA, subclass I, II, and III. The different HLA classes are associated with different phases in the pathogenesis of T1D, primarily the development of islet autoantibodies or the progression to T1D diagnosis after the appearance of autoantibodies (49). HLA genes are the most polymorphic genes in the whole human genome and also exhibit strong linkage disequilibrium (LD), considered when the alleles at different genes are non-randomly associated in a given population (50).

HLA class II

HLA class II comprises the HLA-DR,-DQ, and -DP genes encoding the MHC class II heterodimer of an α and a β polypeptide chain expressed on cell membranes of antigen-presenting cells (APC)(B-cells, macrophages, and dendritic cells). Each of the α and β polypeptide chains has four domains, the peptide binding domains, which are highly polymorphic, the immunoglobulin-like domain, the transmembrane region, and a cytoplasmic tail. Peptides presented by MCH class II are recognized by CD4+ T-helper cells (Fig. 2). HLA-DR and -DQ are the most important risk loci for T1D. Allelic polymorphism in these loci affects the binding grooves. Thus, distinct selections of epitopes are presented to CD4+ T-helper cells that induce the immune reaction against microorganisms and allergens (49). The alleles DRA1/DRA2 or DQA1/DQA2 encode the α-chain while DRB1/DRB2 or DQB1/DQB2 alleles encode the β chain of the heterodimer. The HLA-DR and DQ DQ exhibit strong LD; thus, detecting one allele allows for estimating the other allele on the same haplotype.

The haplotypes conferring the highest risk are DRB1*03:01-DQA1*05:01-DQB1*02:01 (DR3-DQ2), and DRB1*04:01/02/04/05/08-DQA1*03:01-DQB1*03:02/04 (DR4-DQ8) and the heterozygosity of these haplotypes (DR3-DQ2/DR4-DQ8) represents the highest risk (OR = 16.59; 95% CI, 13.7–20.1) (51). The odds ratio varies between studies. This interaction effect is explained by the ability to form DQ heterodimers encoded in *trans* and is a leading hypothesis for why the T1D risk conferred by the DR3/DR4 genotype is greater than the risk for the two haplotypes (52). The four-digit number in the gene term, e.g, DRB1*03:01, represents the unique HLA protein sequence. Nearly 90% of all T1D pediatric

patients have one or both risk haplotypes but only 10 % of children with one of these haplotypes will develop T1D (41, 53). In Sweden, around 50 % of the population carries one of the risk haplotypes (DR3-DQ2 or DR4-DQ8), and only 3% carry both (54). HLA DR/DQ genotypes, in addition to the risk, could also confer a protective and neutral effect. The DQB1*06:02 allele has the most protective effect (OR 0.03) among other protective genes (55). T1D risk HLA class II genotypes are also shared with other autoimmune diseases, including autoimmune thyroid disease (Hashimoto's and Grave's diseases) (DR3), celiac disease (DR3-DQ2), Addison's disease (DR3-DQ2, DR4-DQ8 (DRB1*0404)), and other rare autoimmune diseases (56).

The genetics of T1D have mainly been studied and evaluated in Caucasians because of the high prevalence of T1D in this ethnic group (57). T1D-associated HLA class II haplotypes and genotype frequencies vary among ethnic groups and their susceptible or protective effects on risk (58, 59). For example, the African-specific DR3 haplotype DRB1*03:02-DQA1*04:01-DQB1*04:02 has shown to be protective contrary to the known high risk conferring DR3 haplotype DRB1*03:01-DQA1*05:01-DQB1*02:01 most often observed in other ethnic groups (60). Therefore, it is important to consider the T1D ethnic and racial genetic heterogeneity in creating valid genetic risk predictive models. However, the increased incidence of T1D among children worldwide with a heightened proportion of lower-risk genotypes may be explained by environmental factors' impact on the disease's development (58, 61).

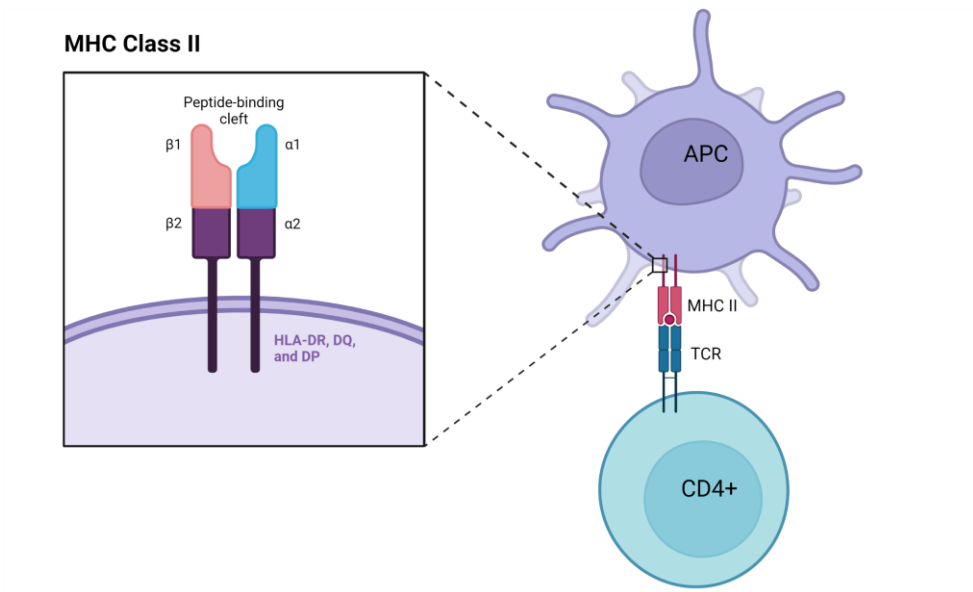

Figure 2. MHC class II antigen presentation on the antigen-presenting cell (APC) and schematic illustration of MHC class II. Created with BioRender.com.

HLA class I

HLA class I encode MHC molecules expressed on all nucleated cells comprising the α polypeptide chain that forms a heterodimer consisting of a heavy chain of adjunct with β-2 microglobulin protein encoded by another gene on chromosome 15 (49). The MHC class I receptor presents epitopes recognized by cytotoxic CD8+ T-cells that mediate the destruction of virus-infected cells and are the major infiltrating cells during insulitis associated with T1D (62). HLA class I genotypes associated with acceleration of the progression to T1D diagnosis are HLA-A*24,HLA* B*18, and B*39, while HLA-B*57:01 confers a protective effect. The strongest association with T1D risk is conferred by HLA*B* 39:06 (OR=10.31). Linkage disequilibrium (LD) association studies between HLA class I genes and DR-DQ locus have shown that B*18 effects are associated with the DR3-DQ2 haplotype and that A*24 and B*39 effects are associated with the DRB1*08-DQB1*04 haplotype (63-67).

Non-HLA risk T1D genes

Non-HLA risk genes confer subtle risks of T1D compared to HLA genotypes and are mostly implicated in immune cell function (42, 68). The exact function of many of these non-HLA candidate genes in the pathogenesis of T1D is still not clearly understood and remains to be characterized. Further understanding of these heterogeneous genetic risk variants and HLA risk and their role in the pathogenesis of T1D allows for better disease prediction and more specific disease intervention approaches. The strongest non-HLA risk factors are INS, PTPN22, IL2RA,and CTLA-4 briefly summarized below.

INS

The non-HLA gene conferring the highest T1D risk of 10 % is the INS (insulin) gene located on chromosome 11p15 encoding pre-proinsulin (69). The increased risk is attributed to polymorphism in INS SNPs residing outside the coding sequence, indicating that modulation of the INS transcription may cause diabetes susceptibility (70). Polymorphism in the short class I, a variable number of tandem repeats (VNTR) alleles (in the IDDM locus) in the insulin promoter, contributes to T1D risk (71). These susceptible VNTRs affect insulin gene expression and are associated with poor expression of insulin in the thymus leading to an impaired selection of the autoreactive insulin-specific T-cells in the thymus escaping from thymic destruction and thus decreasing central tolerance (52, 72).

PTPN22

The protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (PTPN22) gene on chromosome 1p13 is the second susceptible non-HLA gene after the INS gene (73). PTPN22 encodes lymphoid-specific phosphatase (LYP) that suppresses T-cell activation, T-cell receptor activation, and promotes autoreactive T-cell escape from

the thymus (74, 75). Moreover, the PTPN22 allele C1858T with a single amino acid substitution R620W has been associated with several autoimmune diseases including T1D (76). Individuals with this variant are protected from pulmonary tuberculosis and cancer suggesting a role in promoting effector cells and responses at the cost of immune regulation (77-79). However, the function of PTPN22 effects remains unclear.

IL2RA

Multiple SNPs that are independently associated with the risk of T1D have been found in the IL2RA (interleukin 2 receptor alpha) gene encoding IL-2 receptor subunit α (CD25) (80). The IL-2 receptor activates intracellular signaling upon interaction with mainly IL-2 in T-regs and impacts the suppressive function of the cell. Reduced T-reg potency will inhibit the control of T-effector cells promoting the activation of these cells (81). DNA methylation has been evident in several IL2RA SNPs and methylation at CpGs within the promoter of the IL2RA gene is higher in T1D patients than in controls, indicating that epigenetic changes may contribute to the IL2RA risk alleles (82).

CTLA-4

The CTLA-4 gene resides on chromosome 2q33.2 and encodes cytotoxic T-lymphocyte associated protein 4, a transmembrane co-receptor expressed on T-cells, an important negative regulator of adaptive immunity (83). Several autoimmune diseases including T1D are associated with variations of the CTLA-4 gene region (84). However, the mechanisms behind these risk SNPs and how they contribute to autoimmunity remain elusive. CTLA-4 co-receptors promote T-reg function and inhibit T-cell effector activation needed to maintain tolerance and prevent autoimmunity, while the risk variants result in reduced expression of CTLA-4 in T-regs leading to reduced T-reg suppressive potency and a decreased control of the T-effector cells (85). Studying autoreactive T-cells with risk CTLA-4 alleles has revealed downregulation of CTLA-4 expression on these cells (86).

Genetic prediction

The rapid evolution of DNA sequencing by the next generation sequencing (NGS) approach allows researchers to obtain high-resolution typing data at an allelic level. This has improved the characterization of HLA diversity and polymorphism in populations and the genetic risk assessment and prediction. In addition to HLA, genetic risk scores (GRSs) have evolved in the recent years to include non-HLA risk genes and SNPs, improving the genetic prediction of T1D and discriminating diabetes type in patients (87, 88). One of these GRSs, termed T1D GRS2 was developed incorporating 67 SNPs, HLA-DR-DQ interactions, and non-HLA loci and was highly discriminative for T1D (area under the ROC curve, AUROC, 0.92) and early onset of T1D (AUROC 0.96) resulting in an improved T1D GRS. Such

GRSs are used in newborn screening for the involvement in T1D prediction surveillance programs (89) or primary prevention trials (90) as well as in the clinic for classification of the adult incident diabetes type (91).

Environmental factors

Epidemiological and clinical studies have presented strong evidence for the involvement of environmental factors in the etiology of T1D. The strongest evidence from epidemiological studies is the increased incidence of T1D in children in genetically stable populations (92), in countries with low-risk HLA genotypes (59, 93), in people migrating from low-incidence to high-incidence countries, and especially in young children or in children born in the new country (94, 95), and in people from countries with rapid economic development (96).

The Environmental Determinants of Diabetes in the Young (TEDDY) study is the greatest multinational prospective study in modern times investigating environmental factors associated with islet autoimmunity and T1D onset in children at genetic risk from birth until 15 years of age (97). As the incidence of T1D is increasing and proven to be influenced by environmental factors and a plethora of identified genetic factors, prospective studies such as TEDDY are important for the education of the etiology, prediction, and prevention of the disease, and further for the development of necessary translational medical research aiming to prevent, delay, or cure T1D (98). TEDDY has thus provided the largest evaluation of candidate environmental determinants, including viral and bacterial infections, microbiome, nutrients (eg., vitamin D, breastfeeding, cow milk and gluten), and probiotics associated with both islet autoimmunity and T1D (97, 99-101). The results of all these evaluations suggest the existence of many pathways leading to the autoimmune destruction of beta-cells. Environmental risk determinants with the most supportive evidence to date in children are the low diversity microbiome and enterovirus infections (102-104). These major environmental determinants and other less risk-associated factors are presented in Figure 3 (105).

Viral infection

Various viruses (e.g., rotavirus, adenovirus, enterovirus, and norovirus) associated with gut infections have been linked to T1D, and the most frequently associated are the enteroviruses specifically the coxsackievirus B serotype (CVB) (102, 106-108). While the incidence of T1D is seasonal among children in Europe, with increasing cases during fall and winter, viral infections are also more common in the colder seasons and are associated with insulin resistance and increased risk for T1D (109, 110). Previous studies have demonstrated an association between enterovirus infections and autoimmunity or T1D in pregnancy or newborn, in prediabetic subjects, and following enteroviral epidemics (111-114). These studies indicate that enterovirus infections possibly initiate and speed up all three stages of T1D

pathophysiology. The TEDDY study has, in recent years, shown that chronic shedding in stool predicted islet autoimmunity, primarily insulin autoantibodies (102, 103). Given that enteroviral infections are common in children emphasizes the enterovirus risk first in combination with genetic susceptibility and the immune-inflammatory response against the enterovirus. Spreading and replication of the enterovirus in the body occur via the upper respiratory and gastrointestinal tract. The presence of enterovirus in pancreatic islets has been demonstrated in newly diagnosed T1D patients (115). A postulated mechanism of enterovirus starts with viral beta-cell infection by binding to the Coxsackie-adenovirus receptor (CAR) that precedes perturbed viral clearance and stimulation of chemokine responses from beta-cells that in turn triggers islet autoimmunity through molecular mimicry, inflammation, and T-cell suppression (106, 116). A Coxsackie B virus vaccine, developed in Finland to mitigate the increased incidence of T1D among children, is currently being examined in a clinical trial (PROVENT trial: NCT04690426) (117). The rotavirus vaccine, included in vaccination programs in Europe and the US (118) to prevent infant rotavirus infections, was recently tested to see if it also reduced autoimmunity in children. However, several cohort studies in Europe and the US did not show any preventive effect of the rotavirus vaccine on T1D (119-121).

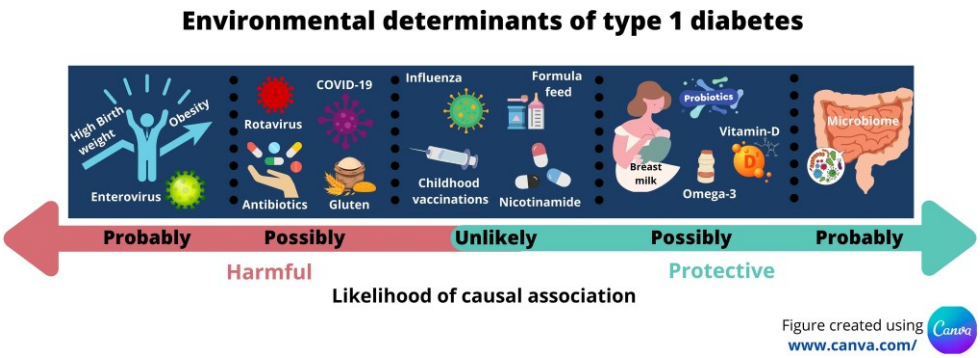

Figure 3. Key environmental determinants of T1D and their likelihood of contributing to causality. Adapted and reproduced with permission from Lauren M. Quinn *et al.* (105). Copyright © 2021 Quinn, Wong, and Narendran.

Gut Microbiome

The human microbiota is settled by 3 years old and until then will fluctuate by changes affected by early life factors, such as birth method, breastfeeding, exposure to gluten, cow milk and solid food, and antibiotics. These factors are shaping the microbiome and have also been suggested as environmental factors associated with

T1D and thus been evaluated in several studies demonstrating contradictory findings (Figure 5.)(101, 105, 122). People with T1D or at risk have an altered microbiota with increased frequency of Bacteroides, decreased bacterial diversity, reduced microbiota stability, and decreased Lactobacillus, Prevotella, and Bifidobacteria (123). This promotes beta-cell autoimmunity due to increased intestinal inflammation, loss of barrier function, increased permeability, and excessed exposure to dietary antigens (124). The huge microbiome study of TEDDY children prospectively followed from 3 to 46 months old demonstrated an increased expression of Bifidobacterium genes in healthy children compared to children with diabetes or at genetic risk (101). These genes stimulate fermentation and short-chain fatty acids that stimulate the production and activation of regulatory T-cells preventing autoimmunity (124, 125). Fecal transplantation and the use of probiotics to maintain the microbiota have been proposed as preventive treatments for halting or reducing T1D progression with controversial results (126, 127).

Childhood obesity and infant growth

Obesity, increased birth weight, weight increase after seroconversion, and increased childhood BMI are all associated with increased risk of T1D (128-131). Moreover, an obesogenic environment with low physical activity and an unhealthy diet leads to an increased demand for insulin, dysbiosis, inflammation, and insulin resistance, leading to increased beta-cell burden and beta-cell stress, and progression to T1D onset in children at genetic risk for T1D (132-134). Obesity and overweight have previously been associated with type 2 diabetes, but in recent years, evidence is growing to include an even risk for T1D progression. Different physical activity programs have shown a decrease in insulin resistance and the preservation of beta-cell function in T1D patients (135).

Vitamin D

Vitamin D deficiency has been linked to T1D not least through the increased incidence of T1D in the colder northern countries and during fall and winter with less exposure to sunlight. While results from many studies have been contradictory, results from TEDDY and a recent dose-response meta-analysis of several studies confirmed that higher plasma vitamin D correlated with lower risk for islet autoimmunity (136, 137). Vitamin D is known to protect against autoimmunity through its anti-inflammatory effect and immunomodulatory effects on stimulating of regulatory T-cells (138).

Taking all this information together, none of the environmental triggers evaluated to date explain the causality of islet autoimmunity leading to T1D. They reflect a combination of pathways involved in the pathogenesis of T1D.

Pathogenesis

The autoimmune nature of T1D was described with a landmark model by George Eisenbarth in 1986 presenting an autoimmune response that caused a linear decrease of beta-cell mass until the manifestation of T1D, when 80–90% of the beta-cells mass is lost (139). In the first state, genetic predisposition, together with still unknown environmental factors, promote the triggering of autoimmunity, and in the next state, the progressive autoimmune destruction of beta-cells by autoreactive T-cells leads to the progressive loss of beta-cell function and insulin deficiency. This is hallmarked by insulitis, an inflammation process when immune cells infiltrate the pancreatic islets (140). Most infiltrating cells are cytotoxic CD8+ cells but also CD4+ T-cells, B-cells together with macrophages are found in children with T1D, and the genetic predisposition of common Human leukocyte antigen class II (HLA II) genes representing the T-cell mediated autoimmunity of T1D (141, 142). The profiles of infiltrating immune cells in the insulitis lesions are heterogeneous and may underlie disease severity, progression, and age at clinical onset (143). One example is the high prevalence of B cells in the insulitis lesion associated with early age at diagnosis while children older than 13 years had a low B-cell profile insulitis (143, 144). A higher prevalence of B-cells in insulitis may therefore mark earlier autoimmunity or a more rapid loss of beta-cell function. Several studies have shown that insulitis does not appear in all islets simultaneously, suggesting insulitis is an evolving process (145). Later research with improved technologies and better availability of pancreatic autopsies and biopsies have resulted in better education and estimation of insulitis, which has challenged the Eisenbarth model. Insulitis commonly occurs in children with recent T1D (<1 year). However, insulitis is present in those islets positive for insulin while insulin-negative islets lack insulitis (146, 147). The statement in the Eisenbarth model about a 90% loss of beta-cell mass at diagnosis may apply only to younger children, as recent evidence reported on teenagers having at least 40–60% of their islets positive for insulin (143, 148). In addition, several studies have shown that individuals with T1D may preserve the ability to produce endogenous insulin and that the vast majority of T1D patients have detectable endogenous C-peptide years after diagnosis, indicating some preservation of beta-cell function after diagnosis (149, 150).

The immunopathogenesis of T1D is illustrated in Figure 4. It starts with the antigen-presenting cells migrating to the lymph nodes and presenting beta-cell antigens to autoreactive CD4+ T-cells that stimulate the activation of autoreactive CD8+ cytotoxic T-cells, migrate to the islet and lyse beta-cells by binding to the MHC class I receptor on beta-cells presenting beta-cell antigens. The inflammation is worsened by releasing cytokines and reactive oxygen species from lymphocytes and innate immune cells e.g., macrophages and neutrophils. Defects in T-regs prevent its potent suppression of autoimmunity. B-cells in pancreatic lymph nodes are also stimulated to produce autoantibodies against beta-cell antigens (151, 152).

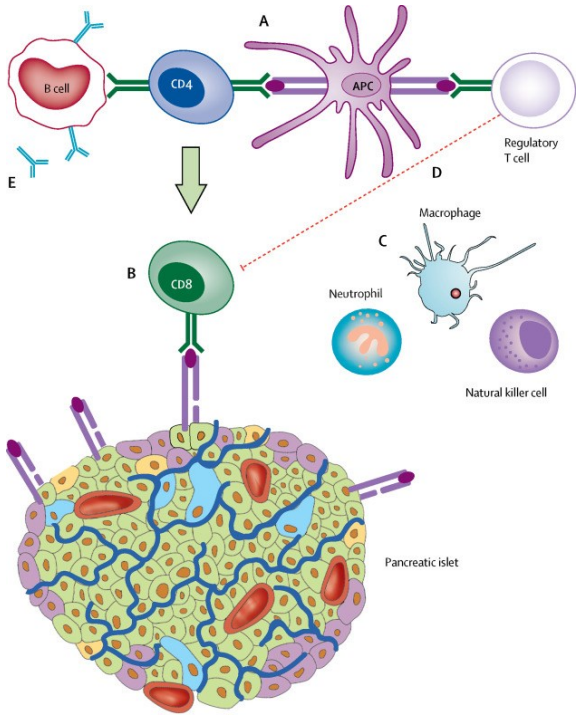

Figure 4. Hypothesized immunopathogenesis of T1D. A) The process starts in the pancreatic lymph node when APCs present beta-cell antigens to autoreactive CD4+ T-cells that activate autoreactive CD8+ cytotoxic T-cells that migrate to the islet and lyse beta-cells by binding to the MHC class I receptor on beta-cells, presenting beta-cell antigens. B) The inflammation is worsened by releasing cytokines and reactive oxygen species from lymphocytes and innate immune cells. C) T-regs are defected and have lost their ability to suppress autoimmunity. D) B-cells are also stimulated to produce autoantibodies against beta-cell antigens. E) Islet autoantibodies are produced. Adapted and reproduced with permission from DiMeglio et al. (152).

Staging of T1D

To dissect the progressive chronic autoimmunity and identify individuals for secondary prevention therapies to intervene, delay, or even prevent the onset of symptomatic T1D, a staging system for asymptomatic T1D has been suggested based principally on the previous Eisenbarth T1D pathogenesis model. During the autoimmune process, autoreactive B-cells are exposed to beta-cell antigens leading to the production of islet beta-cell autoantibodies which are used as biomarkers predicting T1D (described later in the etiology section). The detection of more than one of these autoantibodies confers a high risk for the development of clinical T1D (153). The disease progression continuum preceding symptomatic T1D is divided into three stages, stage 1 is presymptomatic with more than one autoantibody and normoglycemia, while dysglycaemia is evident at stage 2, and stage 3 is symptomatic indicating the onset of T1D (Figure 5) (154). The dysglycemia at stage

2 is defined as impaired glucose tolerance (IGT) with 2-h plasma glucose values ≥7.8 mmol/L or high glucose levels at intermediate time points of oral glucose tolerance test (OGTT) plasma glucose values ≥11.1 mmol/L and/or glycated hemoglobin A1c (HbA1c) ≥39 mmol/mol.

The risk of developing symptomatic diabetes in children with genetic HLA risk at stage 1 is nearly 44% in 5 years, 70% in 10 years, and 100% in a lifetime, and at stage 2 is 60% in two years and 75% in 5 years (153, 155, 156). The staging system is, however, incomplete; the time between the stages varies from days to years, the progression from one of the stages to the other is highly heterogeneous and the time frame for T1D diagnosis development could be up to 20 years (157). Individuals are identified in the three stages through their participation in longitudinal prospective natural history followed by regular monitoring for dysglycemia and detection of autoantibodies. Advantages of the staging have been earlier diagnosis, earlier treatment with insulin, and significant reduction of DKA, improving glucose/insulin management and delaying diabetic long-term complications (30, 158).

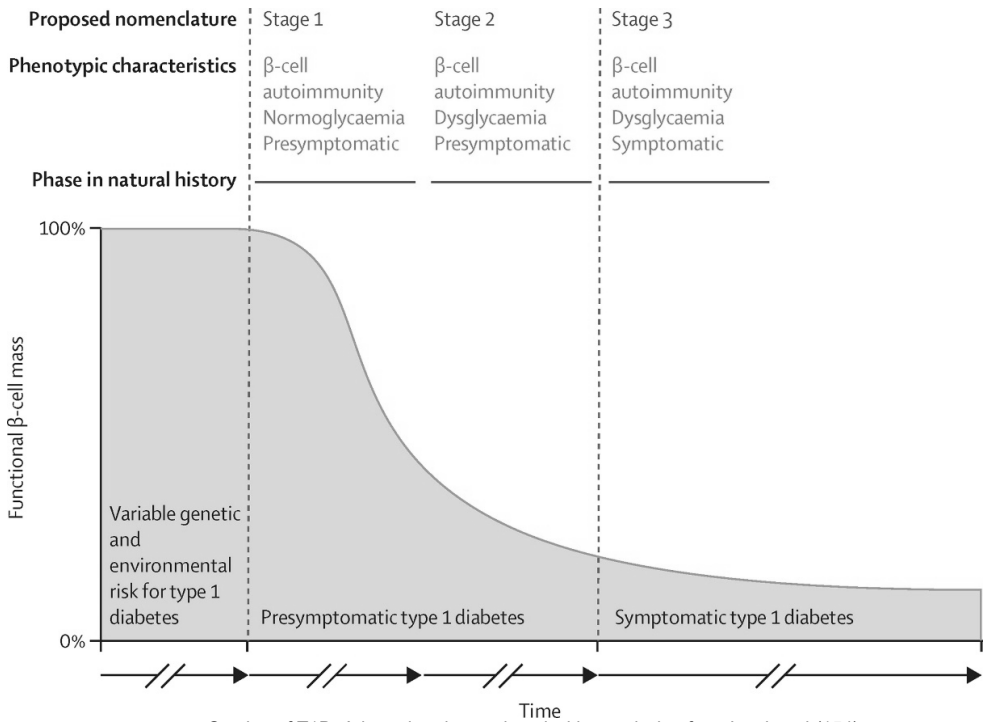

Figure 5. Staging of T1D. Adapted and reproduced with permission from Insel et al (154)

Prediction of T1D

Islet autoantibodies

T1D is predicted by seroconversion of islet beta-cell autoantibodies to four beta-cell autoantigens; insulin (IAA), glutamic acid decarboxylase (GADA), insulinoma antigen-2 (IA-2A), and zinc transporter 8 (ZnT8A). These are biomarkers for beta-cell autoimmunity, and the best predictors of T1D detected in serum. The role of these four autoantibodies in beta-cell destruction is still unclear. One or several of these markers are detected in most newly diagnosed T1D patients and only 2–4% of T1D patients, are seronegative (159). Thanks to these autoantibodies, both islet autoimmunity and T1D could be predicted in follow-up studies with healthy FDR or healthy children screened for HLA risk and followed from birth. These studies have been important for understanding the natural history of T1D, autoantibodies, and their risks. The appearance of autoantibodies is associated with an increased risk of T1D that, in turn, differs by number, type, and combination of these autoantibodies (153).

T1D is a heterogeneous disease with many different approaches reflected in the appearance of autoantibodies that initiates autoimmunity and peak at different ages associated with different genetic risk factors. The common detected first autoantibodies are IAA or GADA. IAA as the first autoantibody is common in children with HLA-DR4-DQ8 haplotype and the first years of life and peaks at the age of 1–2 years and declines rapidly over the next 5 years. In contrast, the appearance of GADA as the first autoantibody is common in children with HLA-DR3-DQ2 haplotype and begins to rise after the second year of life to retain a relatively constant incidence. IAA and GADA first appear simultaneously before 3 years old and are not as common as the appearance of a single autoantibody of each first (Figure 6) (97). IAA first is rare in older children and adults while GADA first is the most common at all ages. The risk for T1D increases among children by increasing numbers of autoantibodies (160). Children with two or more autoantibodies have a 70% risk of progressing to T1D diagnosis within 10 years compared with 15% of children having only one autoantibody (153). However, autoantibodies might not be persistent upon appearance as they can also disappear in reverters. The condition of islet autoimmunity could be described in three types, mild in reverted children, less aggressive in children with a single persistent autoantibody, and more aggressive in children with multiple autoantibodies (161). The staging of T1D starts with the appearance of two or more autoantibodies and the rate of progression to the clinical onset is in addition to autoantibody number and combination associated with the titer, autoantibody affinity, and age at seroconversion, and importantly, the younger, the higher rate of progression (154, 162, 163).

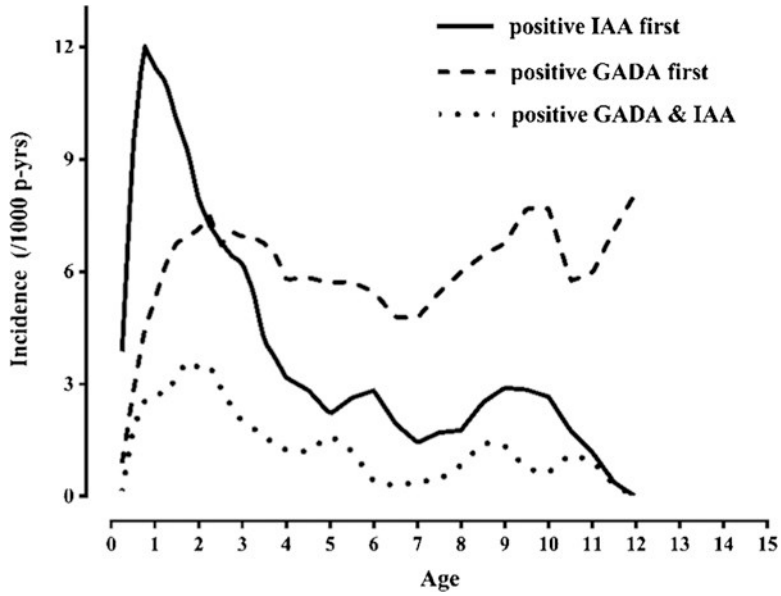

Figure 6. The incidence of IAA and GADA islet beta cell autoantibodies. Reprinted with permission from Rewers and colleagues (97).

The appearance of IA-2A or ZnT8A is associated with the faster progression of the disease and could therefore be used to identify individuals at higher risk for disease progression (164). Even though the majority with a single autoantibody do not develop T1D, seroconversion to IA-2A as the first autoantibody is associated with a 5-year progression rate to T1D, representing a more aggressive autoimmune destruction of the islet beta-cells in young children (160). All four autoantibodies are detected in serum by radiobinding assay (RBA) with high sensitivity and specificity (165-168). However, other simpler, faster, low-volume, non-radioactive, methods are warranted in the area to replace RIA. Two non-radioactive alternative methods have been developed in recent years, luciferase immune precipitations system (LIPS) assays and multiplex agglutination-PCR (ADAP), allowing for the analysis of the four antibodies in a single test (169, 170).

IAA

Insulin is expressed both in pancreatic beta-cells and thymic medullary epithelial cells. The proven insulin mRNA and protein levels are low but relevant for the negative selection of autoreactive T-cells (171). IAA binds conformational epitopes of insulin where a receptor could bind but not in any denatured or reduced form. High-affinity IAA binds residues 8 to 13 of the insulin A chain and low-affinity IAA

binds residues 28–30 of the B chain. The high-affinity IAA can also bind pro-insulin (172).

GADA

GAD has two isoforms, the GAD65 and GAD67, and is a major enzyme in synthesizing of gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter of the central nervous system (173). The GAD65 isoform is expressed in the central and peripheral nervous system and beta-cells found within the synaptic-like micro-vesicles. The function of GADA recognizes the middle and COOH-catalytic domains while binding poorly to the NH2-terminal (174, 175). GABA is a glutamate product, and its signals increase insulin secretion and stimulate beta-cell proliferation. GABA also has an immunomodulatory effect on T-cells by decreasing the production of inflammatory cytokines in T-cells and thus inhibiting T-cell proliferation (176, 177).

IA-2A

IA-2 belongs to the receptor-type protein tyrosine phosphatase (PTP) family, found within the insulin secretory granules in the beta cell. It consists of an extracellular domain, a transmembrane region, and an intracytoplasmic domain, the latter recognized by IA-2A is expressed in the neuroendocrine cell, including pancreatic beta-, alpha-, and gamma-cells, but their clear function remains elusive in beta-cells signifying involvement in insulin regulation pathways in two ways, first by promoting insulin secretory granule mobilization to the plasma membrane for further release of insulin and secondly by translocation to the nucleus to regulate genes participating in insulin secretion (178, 179). Expression of IA-2 has also been demonstrated in splenocytes in the thymus suggesting implications in the negative selection of self-reactive immune cells (180).

ZnT8A

ZnT8 is a transmembrane protein highly expressed in beta-cells localized on the insulin secretory granules. It has an important role in supplying Zn+ ions from the β-cell cytoplasm into insulin secretory granules, which is crucial for the biosynthesis, storage, and secretion of insulin. ZnT8 is also moderately expressed in alpha-cells and kidney cells (181). There are three ZnT8A variants directed specifically against three epitopes at amino acid position 325; arginine (R), glutamate (Q), and tryptophan (W) (182, 183). A ZnT8A positive subject could have one or several of these variants.

Prediction by glucose metabolism

In addition to the autoantibodies, loss of glucose tolerance following a glucose challenge of an OGTT or an intravenous glucose tolerance test (IVGTT), loss of

first phase insulin response (FPIR) to IVGTT, loss of C-peptide and elevated HbA1c precede T1D diagnosis by months to years (184). In prospective follow-up studies, these factors are measured regularly after seroconversion.

FPIR measures beta-cell function calculated by the sum of insulin concentration at 1 and 3 min in the IVGTT. A decline of FPIR is a sign of impaired beta-cell function preceding T1D several years before onset with a rapid decline detected in the last 1.5 years (185). Another metabolic predictor reflecting the beta-cell function is the C-peptide, a byproduct of proinsulin. The difference in 0 to 30 min C-peptide level from OGTT correlates with FPIR and starts to decline more than 2 years before T1D diagnosis mirroring the decline of FPIR (154, 186). A third metabolic predictor is HbA1c, which indicates the mean blood glucose level over the previous 8–12 weeks. We have shown in TEDDY children an increase (within normal limits) of HbA1c in progressors years before onset (187). The Diabetes Prediction and Prevention (DIPP) study has reported that a 10 % increase of HbA1c in two consecutive tests increased the risk 6-fold and predicted the clinical diagnosis (188). The threshold value for HbA1c for diabetes is ≥6.5% (48 mmol/mol), as determined by the World Health Organization and American Diabetes Association, which has been adapted from Type 2 diabetes. Thus, an HbA1c threshold specific for T1D has not been set and might therefore be set on a lower threshold due to the HbA1c increase years before onset still within normal limits. These markers predict the disease best in combination. The TEDDY study and the Diabetes Prevention Trial–Type 1 Risk Score (DPTRS), among others have each developed a combined risk score improving the prediction of the disease 2 years ahead (89, 189).

The search for biomarkers

As discussed earlier, the islet beta-cell autoantibodies are the best biomarkers of the autoimmune progression predicting T1D. However, the etiology is still unknown and the pathogenic processes leading to beta-cell loss and T1D are very heterogenous, implicating the need for more biomarkers especially because the time between the different stages of T1D varies from months to years. Biomarkers are needed for a better understanding of the etiology and the pathogenesis, improved prediction for better prevention studies (both primary and secondary), and better monitoring of therapeutic interventions.

Aims

The overall aim of this thesis was to investigate biomarkers in the periphery that may predict seroconversion or different stages in the pathogenic process leading to T1D and to investigate the longitudinal impact of the antigen-specific immunotherapy with GAD-alum on T-cell profiles in the periphery through longitudinal follow-ups of children at increased genetic risk for T1D.

The specific aims are as follows:

I. To investigate whether complete blood count (CBC) is associated with seroconversion or the number of islet beta-cell autoantibodies and whether the association differed by gender and HLA DR-DQ genotype in 4–12-year-old children from the Swedish TEDDY study cohort.

II. To investigate whether CBC in seroconverted Swedish TEDDY children is associated with glucose metabolism measures (OGTT) and hemoglobin A1c (HbA1c) and whether trajectories of these parameters differ between positivity for a single autoantibody or multiple autoantibodies.

III. To investigate HbA1c as a predictive marker for the progression to a subsequent autoantibody (second, third, and fourth) or T1D in seroconverted TEDDY children during follow-up and if so to further investigate whether there is a difference between the two endotypes of IAA or GADA as the first appearing autoantibody.

IV. To investigate whether immunization with recombinant GAD65 conjugated hydroxide (GAD-alum) treatment affected peripheral T-cell subpopulations in children positive for multiple beta-cell autoantibodies participating in the Diabetes Prevention–Immune Tolerance 2 (DiAPREV-IT 2) clinical trial during 2 years of follow-up post-treatment.

Methods

Study population

The Environmental Determinants of Diabetes in the Young (TEDDY) study

The TEDDY study is a multicenter prospective observational cohort study aiming to identify environmental and genetic factors that trigger or protect against autoimmunity as the first outcome or T1D as the second. The study is conducted in six centers, three in Europe (Finland, Sweden, and Germany) and three in the US (Colorado, Georgia/Florida, and Washington). More than 400,000 newborns with an FDR or from the general population in these six centers were screened for T1D HLA class II high-risk genes (190), and eligible children were then asked to participate in the intensive prospective follow-up of which 8676 were enrolled.

The HLA analysis was performed at the different centers, and eligibility was confirmed after 9 months at the Central Reference Laboratory in Oakland. Most TEDDY children (89%) represent the general population without any FDR with T1D. Enrolled children are prospectively followed from birth until 15 years of age or T1D diagnosis with clinical visits at TEDDY clinics in the different centers. The visits start at 3 months and continue every third month until 4 years of age and biannually until 15 years of age or each third-month post seroconversion. Much data are collected during the visits, including blood samples (such as plasma, serum, mononuclear cells, mRNA, DNA, erythrocytes, and buffy coats), toenail clippings, drinking water, nasal swabs, salivary cortisol, stool samples, urine, and complete blood count (CBC) sample (only Sweden), dietary records, diaries with important life events, growth measurements (weight, length, and puberty signs), physical activity, and psychosocial questionnaires. Analysis intended for the processed blood includes autoantibody analysis, genotyping and epigenetic analysis, viruses, vitamin D, dietary antioxidants, red blood cell membrane fatty acid and cellular phenotyping and cellular analysis. In addition, clinical measurements of glucose metabolism and beta-cell function by OGTT and HbA1c tests are performed after seroconversion. The islet beta-cell autoantibodies (IAA, GADA, and IA-2A) are assayed and confirmed persistent if concordant at two laboratories, one at Bristol University and the other at Barbara Davis Center for Childhood Diabetes at the University of

Colorado at two consecutive visits (97, 191). The enrollment in TEDDY started in 2004 and ended in 2010 and the study follow-up will continue until 2025. Paper I and II included participants from the Swedish TEDDY cohort only (Figure 7). Paper III included all seroconverted children from all sites to IAA first, GADA first, both IAA and GADA, or GADA, IAA, and ZnT8A (Figure 8).

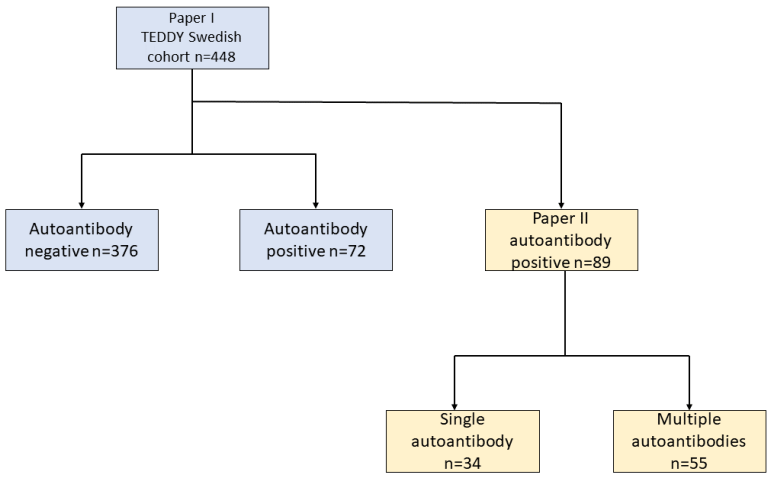

Figure 7. Flowchart for papers I and II with children from the Swedish TEDDY cohort.

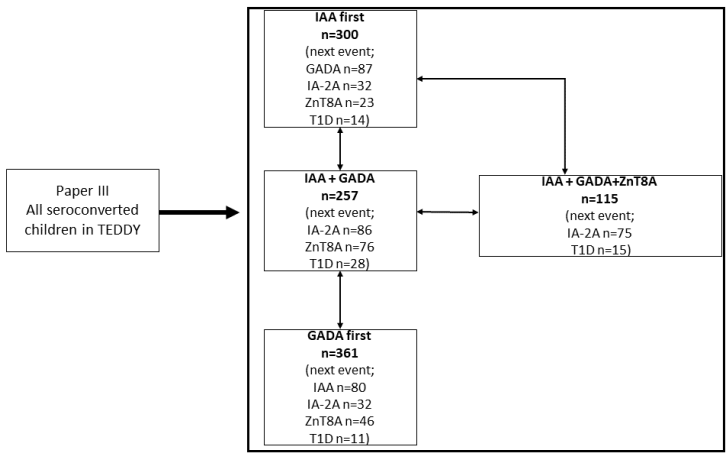

Figure 8. Flowchart for paper III with all seroconverted children to the combination of autoantibodies presented in the boxes. Doubled arrows indicate not mutually exclusive groups.

Diabetes Prevention Immune Tolerance 2 (DiAPREV-IT 2) study

The DiAPREV-IT 2 study is a randomized, double-blind investigator-initiated placebo-controlled clinical trial (NCT02387164) aiming to prevent or delay the autoimmune process in T1D. Antigen-Specific immunotherapy was used with the GAD autoantigen conjugated to alum (GAD-alum) produced as Diamyd® drug in combination with vitamin D supplement to boost the immune system. Diamyd® is well tolerated and safe (192). In the current study, two 20 µg doses of GAD-alum (or Alhydrogel for placebo treatment) were subcutaneously injected as prime and booster with one month apart, combined with a daily administered vitamin D (2000 IU) during the 24 months of study follow-up. The DiAPREV-IT 2 study was preceded by the DiAPREV-IT study that failed to prove the efficacy of GAD-alum (192) which led to premature termination of the participant enrollment when only 26 out of the 80 intended participants were enrolled (Figure 9). These 26 participants were recruited from three ongoing studies, TEDDY, Diabetes Prediction in Skåne (DiPiS), and TrialNET, and randomized for GAD-alum treatment (13 participants) or placebo (13 participants). The randomization was stratified by normal or impaired glucose metabolism at baseline evaluated with IVGTT or OGTT. The participants were asymptomatic for T1D, positive for GADA and at least one more autoantibody, and 4–17.99 years old followed in the study for 24 months with 3 months between each visit following the second treatment visit. The study follow-up included glucose tolerance tests (IVGTT, OGTT), HbA1c tests, complete blood count (CBC) tests, phenotyping of T-cells, autoantibody analysis, and physical examination.

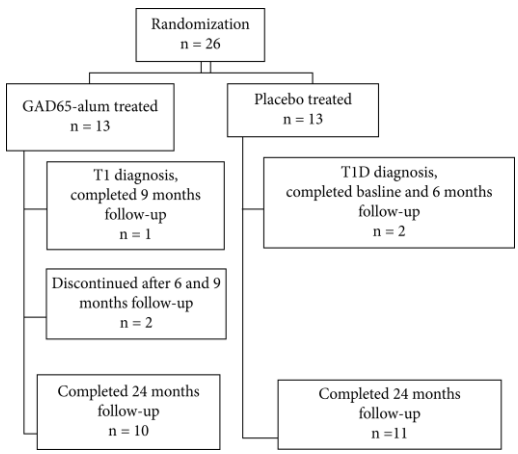

Figure 9. Flow-chart for paper IV.

Ethical permits

Ethical approvals for **papers I, II, and III** were obtained from the following ethical institution review boards; the Swedish Regional Ethical Review Board in Lund (DNR 2004/217, 2017/667) and the Swedish Ethical Review Authority (2019/04405) in addition an annual review by the Lund University Committee for Continuing Ethical Review, the Hospital District of Southwest Finland Committee, the Ethik-Kommission der Bayerischen Landesärztekammer (Germany), the Colorado Multiple Institutional Review Board, the University of Florida Health Center Institutional Review Board, the Augusta University Institutional Review Board (Georgia), the University of South Florida Institutional Review Board, and the Western Institutional Review Board (Washington). The TEDDY study was also evaluated and observed by an external advisory board on behalf of the National Institute of Health in the US. The study in **paper IV** was approved by the Regional Ethical Review Board in Lund and the Swedish Medical Product Agency and conducted in accordance with the Declaration of Helsinki (EUCTR 2014-003755-64). Written informed consents were obtained from all participants enrolled in each of the four papers.

Laboratory Methods

HLA class II analysis (Paper I, II, III)

The TEDDY high-risk HLA-DR-DQ genotype screening was performed on cord blood samples or, in some cases (University of Washington, Seattle, WA site), on capillary blood from heel stick until the age of four months to increase the number of FDR participants. As previously published, a dried blood spot or a small volume of whole blood lysate sample was used for genotyping at each TEDDY center (190, 191). The HLA-DR-DQ genotyping of the eligible children was confirmed at 9 months old by the central HLA Reference Laboratory at Roche Molecular Systems, Oakland, CA, using high-resolution genotyping of HLA-DRB1, DQA1, and DQB1 (163, 190).

Islet autoantibody analysis

In TEDDY (Paper I, II, and III)

Islet autoantibodies (IAA, GADA and IA-2A) were analyzed every third month between 3 and 48 months of age and continue biannually for autoantibody negative children and every third month for seroconverted children until the age of 15 years or diagnosis of T1D. The autoantibody analysis was performed in either the Barbara Davis Center for Childhood Diabetes, University of Colorado, in the US or the University of Bristol in the UK, and all positive autoantibodies and 5% of the negative samples were confirmed after that in the other reference laboratory. These laboratories have high specificity, sensitivity, and concordance (166). A confirmed autoantibody was considered persistent if detected in two consecutive confirmed samples. These premises define persistent islet autoimmunity in TEDDY from the date of the draw when the first positive sample was taken (191). The ZnT8A was analyzed only in one of the two reference laboratories once seroconverted to one or several of GADA, IAA, or IA-2A (193).

The islet autoantibodies were analyzed by RBA as described elsewhere (167, 194, 195). In brief, the autoantigens were produced by in vitro transcription and translation and radiolabeled to be incubated with the blood serum from a participant. The radiolabeled antigens bind autoantibodies in the serum and form complexes that will precipitate to be collected and analyzed in a β-counter.

DiAPREV-IT 2 (Paper IV)

In the DiAPREV-IT 2 study, each islet autoantibodies IAA, GADA, IA-2A, and the three amino acid variants of ZnT8A (R/W/Q) were analyzed at baseline and every third month during the 24-month follow-up at the laboratory at CRC, Lund

University. The CRC laboratory participates in the Islet Autoantibody Standardization Program (IASP) workshop.

GADA and IA-2A

GAD65 and IA-2 antigens were labeled with [35S]methionine (Perkin Elmer). Serum samples were analyzed in duplicates in 96-well plates and incubated overnight at 4°C. The autoantibody-antigen complex was precipitated with 50% protein A-Sepharose in a filter plate, washed in a microplate washer (BioTek 405 LS, Bio Tek Instruments, Winooski, USA), dried, and resuspended in supermix scintillation cocktail (Perkin Elmer, Boston, MA, USA). GADA and IA-2A levels were expressed in U/mL derived WHO standard 97/550 (196) measured in a Wallac Microbeta Trilux (Perkin Elmer) β counter (197). The cut-off value for GADA is 34 U/mL and 5 U/mL for IA-2A positive samples.

IAA

IAA was analyzed with two methods, a non-competitive RBA for screening the positive samples and a competitive RBA to estimate the IAA levels more accurately in positive samples as described previously (198). In the non-competitive assay, sera from participants were incubated for 48 h at 4°C with radiolabeled ^{125}I-labeled human recombinant insulin (NEX420050UC, Perkin Elmer). Unbound radiolabeled insulin was separated from the autoantibody bound insulin by Protein A-Sepharose, washed, dried, and suspended in a Supermix Scintillation Cocktail (Perkin Elmer). The radioactivity was analyzed in the β-counter. The cut-off for an IAA positive sample is 3.6 U/mL. The positive samples were further analyzed in the competitive assay by incubating serum from the positive participants with radiolabeled insulin added to four wells and unlabeled insulin (Actrapid; Novo Nordisk) added to two of these wells. The samples were further processed as described above in the non-competitive assay. The final IAA titer levels were calculated by subtracting the radioactivity measured in the wells with radiolabeled and not labeled insulin and the wells with only radiolabeled insulin.

ZnT8-R/W/Q autoantibody

The three variants of ZnT8A (R/W/Q) were analyzed in a ZnT8-TripleA RBA with only 5 µl serum and a 60 µl mixture with all three radiolabeled ZnT8-R/W/Q as previously described (183). The cut-off value for positive ZnT8-RA, ZnT8-QA, and ZnT8-WA levels were 63, 99, and 74 Um/L, respectively.

Complete blood count (CBC) (Papers I, II, III, and IV)

CBC is a hematological analysis method and one of the most commonly used blood tests in the clinical laboratory presenting the counts (cells × 109/L) of each of the different white blood cell types (total leukocytes, neutrophils, lymphocytes, monocytes, basophils, eosinophils, and platelets) in the blood together with red

blood cell counts (cells × 1012/L), hemoglobin (HGB) (g/L), and different red blood cell indices [hematocrit (HCT) (L/L), mean corpuscular hemoglobin (MCH) (pg), mean corpuscular hemoglobin concentration (MCHC) (g/L), mean corpuscular volume (MCV) (fL), and red cell distribution width (RDW) (% coefficient of variation)]. CBC was analyzed in a multi-parameter automated hematology analyzer (CELL-Dyn Ruby; Abbott Laboratories, Diagnostic Division). The CELL-Dyn Ruby uses Multi-Angle Polarized Scatter Separation (MAPSS™) technology that uses four light scatter signals analyzed by detectors at four different angles for granularity (90° depolarized), nuclear lobularity (90°), intracellular complexity (10°) and size (0°) to separate the different blood cell types. The hemoglobin concentration was determined in the CELL-Dyn Ruby by monitoring the light absorbance in hemoglobin which is proportional to the hemoglobin concentration (199). The blood samples analyzed in the four papers were measured for CBC within 8 h from blood draw with only 100 µl of whole blood per CBC test.

HbA1c (Paper II and III)

The HbA1c testing in TEDDY starts when a child develops her or his first autoantibody. If the child reverts and loses the autoantibody to a negative autoantibody status, HbA1c testing will be halted. The HbA1c is collected at each clinical site in TEDDY and sent to the Diabetes Diagnostic Laboratory (DDL) at the University of Missouri. HbA1c is analyzed by Tosoh G8 HPLC using the National Glycohemoglobin Standardization Program (NGSP) certified method (200).

OGTT (Paper II)

Autoantibody-positive children in TEDDY at ≥ 3 years of age are asked to complete a 2 h OGTT every 6 months. The child has to be fasting (except for water) for up to 8 h before OGTT. The child is given an oral glucose solution with 1.75 g glucose/kg body weight to be consumed within 5 min. A two-point OGTT was performed in TEDDY by collecting blood samples (using venipuncture) for glucose, insulin, and C-peptide analysis at 0 and 120 min. The two-point OGTT is the most commonly used in TEDDY, where more than 90% of all done OGTTs are with two points. A few children completed a six-point OGTT. The test is used to investigate the beta-cell function and diagnose T1D; therefore, glucose monitoring is done at each TEDDY center using different methods. T1D was determined according to the American Diabetes Association criteria for diagnosis (201). Insulin C-peptide and Insulin from OGTT are analyzed at the core laboratory at the Northwest Lipid Metabolism and Diabetes Research Laboratories, University of Washington, Seattle, Washington. The glucose level in mg/dL was determined by an enzymatic "in vitro" test using Roche hexokinase reagent on a Roche Double Modular P Analytics automated analyzer. Levels of C-peptide were measured by Tosoh

reagents on a TOSOH 2000 autoanalyzer (Tosoh Bioscience Inc., San Francisco, California). Insulin levels were determined by an immuno-enzymometric assay on the TOSOH 2000 autoanalyzer.

Flow cytometric phenotyping of T-cells (Paper IV)

Flow cytometry was utilized to assess different T-cell phenotypes in paper IV. The different T-cells were phenotyped based on their cellular expressed protein markers stained by monoclonal antibodies conjugated to fluorophores that are analyzed by the laser-based technology of a flow cytometer. The technology consists of three components, a fluidic system, an optic system, and an electronic system, all allowing the fast stream of cells over the laser beams to measure the optical traits of the cells. The fluidic system transports the stained cells from a suspension by hydrodynamic focusing with sheath fluid that focuses the cells through a tiny stream of fluid to be hit by the laser beam in the optic system one by one. Different detectors then measure the scattered light, forward scatter (FSC), and side scatter (SSC) from the cells providing information about cell size and granularity, and fluorescence detectors measure the fluorescence emitted by conjugated fluorophores simultaneously on the stained cells. Different lasers emit light at different wavelengths and different interrogation points. Emitted fluorescence from each fluorophore is isolated by their respective optical emission filters, which block certain wavelengths while transmitting others. Then, dichroic filters with mirrors direct the emitted fluorescence to the appropriate detector. The photomultiplier tubes (PMT) in flow cytometers are detectors that convert light into voltage pulses or electronic signals by the electronic system. These signals are later processed and analyzed in the flow cytometer software. Upon excitation, each fluorochrome releases energy or emits photons of light with higher wavelengths and its own emission spectrum, which may overlap in some extension with another fluorochrome spectrum, causing “spill over” in different channels. This could be a problem when staining with multicolor fluorescent dyes. A compensation process is performed to overcome this spillover by eliminating spectral overlap between the channels for a specific fluorophore by mathematic deconvolution programmed in the flow cytometer (202, 203). VersaComp antibody capture compensation beads were used for compensation in this study according to instructions by the manufacturer (Beckman Coulter, Inc, Brea, CA, USA). For more accurate gating, fluorescent minus one (FMO) controls were used when the multicolor panel of this study was set. In addition to the unstained cell negative gate, FMO could help to distinguish the real negative from the positive population by staining all the fluorophores minus the one in question. FMO controls are useful to put the correct gate when the expression level of a specific marker is low and to exclude background signals from spectral overlap (204). Flow cytometric analysis of T-cells was done every 6 months.

Statistical methods

Linear mixed-effects models

Linear mixed-effects models were used in papers I, II, and III to model the repeated measurements over time while accounting for correlation between the measurements collected on the same individual. The heterogeneity in a population, both within and between individuals, is considered in the model using both fixed and random effects (thereby mixed effects model) for the longitudinal response. The mean responses for the population parameters are modeled as fixed effects, and subject-specific effects are modeled as random effects. The model allows for analyzing within- and between-subject correlation, as well as the mean response in the population over time, and to predict individual response trajectories. In paper I, a mixed-effects model was used to assess the association between the number of autoantibodies (0, 1, 2, and 3) and each CBC parameter, adjusting for age. Age and autoantibody number or status were treated as fixed effects in the model with a random intercept and slope. A similar model was used in paper II to assess the association between different CBC parameters and HbA1c, adjusting for the number of autoantibodies, age, gender, and HLA genotype. In the models for each of the CBC parameters, CBC was set as the dependent variable (outcome), and the independent covariates were age at baseline, gender, HLA category and the time-dependent covariates were the number of positive islet autoantibodies and HbA1c. The association between HbA1c and autoantibody number was also studied using a mixed effects model with HbA1c as the outcome with autoantibody status as the time-dependent main predictor. Linear unbiased predictions were obtained from these mixed effects models for HbA1c and neutrophils. The association between different CBC parameters and glucose metabolism measures from OGTT was also assessed using mixed effects models with CBC parameters as the outcome, fixed effects for age, gender, and HLA category with a random intercept and slope. Two-sided p-values of less than 0.05 were considered statistically significant for papers I and II. In paper III, mixed-effects models were used in the trajectory analysis of HbA1c and BMI-z scores. A $p<0.01$ was considered statistically significant.

Wilcoxson's rank sum test

Wilcoxson's rank sum test is a non-parametric statistical test for two independent groups of samples comparing the medians in these paired independent observations. The test is used when normality in the outcome variable cannot be assumed. This is often the case in small data sets. This test was used in paper I to compare the age of the subjects at the time of the initial CBC measurement between those positive and negative for islet autoantibodies.

Fisher's exact test

Fisher's exact test is a non-parametric test used to analyze contingency tables of the number of samples or subjects categorized using two categorical covariates. The number of girls vs. boys with and without the HLA-DR3-DQ2/DR4-DQ8 genotype was compared using this test in paper I.

Dynamic prediction model

A dynamic prediction model was used in paper III to predict the time of occurrence of the next event, where the event was either the occurrence of a subsequent autoantibody or the onset of T1D using the HbA1c level as the main predictor. The model was constructed using joint models which model the hazard function using a competing risk model and the longitudinal data using mixed effects models. The competing risk model is a model used in survival analysis to study the association between the covariates of interest and the risk of transition from a non-event state to one of two or more events. In our study, we were interested in transitioning from having one autoantibody state to having two or more autoantibodies or being diagnosed with T1D. A proportional hazard was assumed for competing events. Covariates in the study included HLA (DR3/DR4 yes or no), gender, country, BMI-z score, and HbA1c.

t-test

A t-test is used to test whether there is a statistically significant difference between the means of sets of data from two groups. The test assumes that the data in each group is normally distributed. The t-test was used in paper IV to compare the different measures (CBC, levels of different T-cell subsets and autoantibody titers) between the GAD-alum treated and placebo-treated group at each visit during the 24 months of follow-up in the DiAPREV-IT 2 study. A p-value <0.05 was considered marginally significant, and a p-value <0.01 was considered significant participants in the DiAPREV-IT 2 study.

Results

Paper I

The association of CBC and the number of autoantibodies

The characteristics and islet autoantibody status at the first CBC measurement of the 448 TEDDY children from Sweden who participated in this study are presented in Table 1. Effects by age on CBC were determined using the mixed-effects model on the 376 autoantibody negative children. The mixed-effects models investigating the differences in CBC between autoantibody positive and negative children were adjusted for age.

Table 1. Characteristics of TEDDY children investigated for CBC.

Islet autoantibody	Negative *n*=376	Positive *n*=72
Children, *n* (%)	376 (84)	72 (16)
Sex, n Girls	182	30
Boys	194	42
Age at first CBC (months), median(min-max)	91 (52-145)	101.5 (59-139) n.s
Girls	91 (53-144)	103 (59-139)
Boys	94 (52-145)	99 (59-137)
CBC measures per child min-max	1-6	1-9
Months of follow up min-max	1-30	1-30
Islet autoantibody, n 1	0	25
2	0	16
3	0	31
Change in IA status	none	none
HLA DR-DQ, n (%) DR3/4 -DQ2/8	151 (40.2)	39 (54.1)
DR4/4-DQ8/8	87 (23.1)	12 (16.7)
DR4/8-DQ8/4	40 (10.6)	12 (16.7)
DR3/3-DQ2/2	91 (24.2)	9 (12.5)
DR4/1-DQ8/5	4 (1.1)	0
DR4/13-DQ8/6	2 (0.5)	0
HLA ineligible	1 (0.3)	0

The main findings of this study were the reduction in white blood cells (p= 0.007) due to the reduction in neutrophils primarily found in boys (p=0.004) and children with HLA-DR3- DQ2/DR4-DQ8 (p=0.01) associated with positivity for three autoantibodies compared to the autoantibody negative children (Table 2). A marginal reduction of lymphocytes was also found in all children with three autoantibodies (p=0.038). Reductions in red blood cell counts, hemoglobin, and hematocrit were found in children with multiple autoantibodies, primarily in girls (p=0.002, p=0.0007, p=0.0007) and children with HLA-DR3-DQ2/DR4-DQ8 (p=0.006, p=0.031, p=0.004).

Table 2. Summary of the main results. The association between CBC and the number of autoantibodies.

CBC	n IA	Estimate	Standard error	p value
White blood cells (10E9 cells/L)				
All	3	-0.613	0.225	0.007
Boys	3	-0.789	0.332	0.019
HLA-DR3-DQ2/DR4-DQ8	3	-0.729	0.360	0.045
Neutrophils (10E9 cells/L)				
All	3	-0.427	0.141	0.003
Boys	3	-0.634	0.216	0.004
HLA-DR3-DQ2/DR4-DQ8	3	-0.572	0.216	0.010
Lymphocytes (10E9 cells/L)				
All	3	-0.175	0.0840	0.038
Girls	2	0.368	0.175	0.038
Red Blood cells (10E12 cells/L)				
All	2	-0.200	0.089	0.026
Girls	2	-0.471	0.142	0.002
HLA-DR3-DQ2/DR4-DQ8	3	-0.320	0.113	0.006
Hemoglobin (g/L)				
All	2	-0.635	0.235	0.008
Girls	2	-1.248	0.335	0.0007
HLA DR3-DQ2/DR4-DQ8	3	-0.710	0.326	0.031
Hematocrit (L/L)				
All	2	-1.801	0.640	0.006
All	3	-1.034	0.525	0.050
Girls	2	-3.635	0.990	0.0007
HLA-DR3-DQ2/DR4-DQ8	3	-2.514	0.853	0.004

Paper II

The 89 autoantibody positive Swedish TEDDY children included in this study are characterized in Table 3 and were followed for CBC measurements from June 2014 until April 2019. The major findings in this study were the significant association between the levels of red blood cell counts, parameters, and indices with glucose

metabolism measures, such as fasting glucose, fasting insulin and HbA1c, reflecting the islet beta-cell function.

Table 3. Characteristics of the 89 single and multiple autoantibody positive children included in paper II.

Characteristics	Single Autoantibody n=34	Multiple Autoantibodies n=55
Gender, n (%)		
Girls	15 (44)	22 (40)
Boys	19 (56)	33 (60)
HLA-DR-DQ		
DR3-DQ2/DR4-DQ8	17 (50)	30 (55)
DR4-DQ8/DR4-DQ8	3 (8)	14 (25)
DR4-DQ8/DR8-DQ4	7 (21)	7 (13)
DR3-DQ2/DR3-DQ2	7 (21)	4 (7)
IA at first CBC, n (%)		
0	4 (12)	2 (4)
1	30 (88)	5 (9)
2	0	21 (38)
3	0	27 (49)
Age at First CBC Obs (yrs)		
Median (SD)	9.3 (1.5)	8.1 (1.8)
Min-Max	5.2-12.0	5.0-11.4
CBC follow-up, years		
Median (SD)	2.5 (1.8)	2.0 (1.6)
Min-Max	0.0 – 4.7	0.0 - 4.9

CBC association with HbA1c, fasting glucose, and fasting insulin

The mixed repeated measure model with CBC as the dependent variable presented an inverse association between HbA1c and both MCH (Estimate (SE) = −1.51 (0.3)) ($p < 0.001$) and MCV (Estimate (SE) = −3.67 (0.57)) ($p < 0.001$). These associations were also affected by high-risk HLA-DR-DQ genotypes ($p=0.019$, $p < 0.001$) and the first appearing autoantibody of GADA or IAA ($p < 0.001$, $p =0.004$). The association between different CBC parameters and glucose metabolism measures obtained from OGTT in children with multiple autoantibodies (GADA, IAA, IA-2A, ZnT8A) are presented in Table 4. Inverse significant associations between the CBC measurements of red blood cell counts, hemoglobin, hematocrit, or MCH levels and fasting glucose levels were found, as well as the association between increased levels of these CBC parameters and increased fasting levels insulin. A decrease in RDW was associated with an increase in fasting insulin. Increased levels of white blood cells, lymphocytes, neutrophils, and basophils were associated with an increased level of 120 min glucose measure.

Table 4. Associations between red blood cell counts, parameter levels and indices with OGTT measures in children with multiple autoantibodies.

CBC	n	Glucose (0) estimate (SE)	Glucose (120 min) estimate (SE)	Insulin (0) estimate (SE)
Red blood cells	49	- 0.023 (0.008) p=0.003	-	0.13 (0.03) p<0.001
Hematocrit	49	− 0.002 (0.0006) p=0.002	-	0.011 (0.002) p<0.001
Hemoglobin	49	−0.58 (0.20) p=0.006	-	3.9 (0.8) p<0.001
MCV	50	-	-	-
MCH	54	-	-	0.13 (0.05) p=0.021
RDW	54	-	-	−0.55 (0.024) p=0.021
White blood cells	50	-	0.007 (0.002) p=0.002	-
Neutrophils	50	-	0.004 (0.002) p=0.016	-
Lymphocytes	50	-	0.003 (0.001) p=0.008	-
Basophils	50	-	0.00008 (0.00004) p=0.021	-

Predicted trajectories

The mixed-effects model with HbA1c as the dependent variable revealed an association between increased levels of HBA1c and the number of autoantibodies ($p < 0.001$). Predicted trajectories over the 2–4.9 years of follow-up for HbA1c and CBC were estimated from the respective mixed-effects models. Predicted trajectories were stratified by the number of islet autoantibodies (single or multiple) to further investigate any difference between children with single or multiple islet autoantibodies at the initial observation. Differences were found in predicted trajectories of HbA1c and neutrophil counts ($p < 0.001$). In agreement with our earlier findings in paper I, predicted neutrophil counts were lower in children with multiple autoantibodies ($p < 0.001$) and decreased with the number of autoantibodies compared to children with a single autoantibody, and age did not affect the predicted neutrophil counts. While both age and number of autoantibodies had an impact on the predicted HBA1c levels with higher HBA1c in children with multiple autoantibodies ($p < 0.001$), increasing by age ($p < 0.001$) (Figure 10).

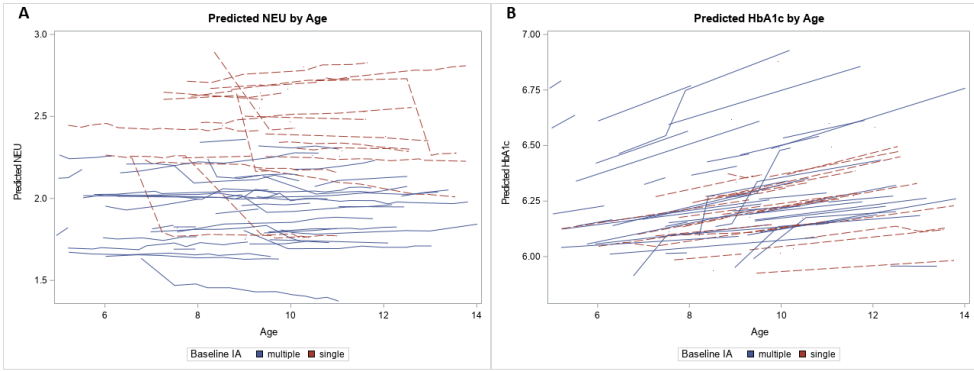

Figure 10. Predicted trajectories for neutrophil counts (A) and HbA1c (B) stratified by the number of islet beta-cell autoantibodies. Each trajectory represents one child.

Paper III

Autoantibody-positive children in the TEDDY cohort were investigated in four subcohorts, starting with the following combination: 1) IAA as the first autoantibody, 2) GADA as the first autoantibody, 3) GADA and IAA positivity, and 4) IAA, GADA, and ZnT8A positivity. These are followed with HbA1c measures until a subsequent state of autoantibody development or T1D diagnosis. The demographic data and number of HbA1c for these four subcohorts is summarized in Table 5.

HbA1c association with autoantibodies and T1D

The main results of this study obtained by the dynamic prediction model were the association between increased HbA1c and the clinical onset of T1D ($p<0.001$) with an augmented rate of increase of HbA1c in progressors in proximity to the clinical onset of T1D (quadratic estimate 0.47, SE (0.092), $p <0.001$), visualized in landmark plots going back 5 years before the clinical onset of T1D (Figure 11). The landmark plots present the predicted mean trajectories of HbA1c. This linear increase of HbA1c in progressors was not affected by any beta-cell autoantibody or combination of autoantibodies. No association was found between increasing HbA1c and any additional subsequent autoantibody, such as a second, third, or fourth. Another important finding of this study is the association between decreased HbA1c and the development of IA-2A either as a second autoantibody following GADA as the first (HR 0.85, 95% CI [0.75,0.97], $p=0.017$) or the fourth autoantibody following GADA, IAA, and ZnT8A (HR 0.90, 95% CI [0.82,0.99],

p=0.036). Moreover, HLA DR3/DR4 heterozygosity was associated with GADA as the second autoantibody following IAA (HR 1.89, 95% CI [1.27,2.800], p=0.002) and IAA as the second following GADA (HR 2.16, 95% CI [1.43,3.26], p=0.001). Females had a higher risk of developing IA-2A as the third autoantibody following GADA and IAA (HR 1.81, 95% CI [1.17,2.79], p=0.007). First-degree relatives with IAA had a higher risk of developing two or more autoantibodies (HR 3.707, 95% CI [1.754,7.834], p<0.001).

Table 5. Demographic data, number of HbA1c measurements, and number of T1D diagnoses as the next event in the four subcohorts.

Subcohorts	**IAA first n=30**	**GADA first n=361**	**AA+GADA n=257**	**IAA+GADA+ZnT8A n=115**
Gender, n (%)				
Female	139 (46)	168 (47)	116 (45)	54 (47)
Male	161 (54)	193 (53)	141 (55)	61 (53)
HLA genotype, n (%)				
DR4/DR3	144 (48)	177 (49)	161 (63)	66 (57)
DR4/DR4	54 (18)	56 (16)	39 (15)	20 (17)
DR4/DR8	62 (21)	43 (12)	28 (11)	14 (12)
DR3/DR3	26 (9)	79 (22)	15 (6)	8 (7)
Other	14 (5)	6 (2)	14 (5)	7 (6)
Baseline Age, median (min-max)	2.0 (0.3–13.7)	5.0 (0.3–14.0)	2.8 (0.5–14.6)	5.2 (1.2–13.8)
Number HbA1c* , median (min-max)	9 (1-43)	8 (1-34)	3 (1–40)	5 (1-43)
Number of T1D**	14	11	28	15

*number of HbA1c measures until the next state

**number of T1D diagnoses for next state

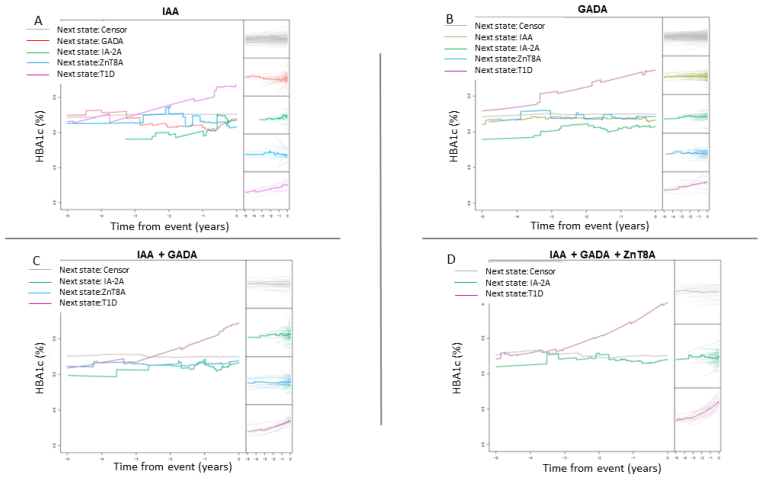

Figure 11. Landmark plots of predicted mean HbA1c going back 5 years from each event (a subsequent antibody state or T1D). Right-hand panels in the diagrams present trajectories for each subject. The four plots represent each subcohort: A) IAA single autoantibody positives, B) GADA single autoantibody positives, C) IAA and GADA positives, and D) IAA, GADA, and ZnT8A positives. Censored grey lines present subjects that were lost to follow-up before the transition into the next event of autoantibody development or T1D.

Paper IV

The baseline characteristics of the 26 children treated with GAD-alum (13 subjects) or placebo (13 subjects) are presented in Table 6.

Long term effect of GAD-alum on T-cells

The main finding of this study was the lower levels of both naïve and effector memory T-cells of both CD3+/CD4+ T-helper cells and CD3+/CD8+ cytotoxic T-cells detected 18 to 24 months post-treatment with GAD-alum compared to the placebo treatment. The different T-cell subpopulations with significantly lower levels associated with GAD-alum treatment 18 to 24 months after treatment are presented in Table 7. No correlation was found between the GADA titers and the treated group's lower T-cell levels. A significant increase of GADA titers at 6 and 12 months following the two doses of GAD-alum were found in the GAD-alum treated group compared to the placebo group (Figure 12).

Table 6. Baseline characteristics of the participants.

	GAD-alum n=13	Placebo n=13
Gender, n (%)		
Female	7 (53.8)	4 (30.8)
Male	6 (46.2)	9 (69.2)
First degree relatives, n (%)		
Yes	4 (30.8)	2 (15.4)
No	9 (69.2)	11 (84.6)
High risk HLA haplotypes, n (%)		
DR3-DQ2	5 (38.4)	7 (53.8)
DR4-DQ8	13 (100)	12 (92.3)
Positive beta-cell autoantibodies, n (%)		
2	1 (7.7)	2 (15.4)
3	3 (23.1)	5 (38.5)
4	4 (30.8)	3 (23.1)
5	4 (30.8)	1 (7.7)
6	1 (7.7)	2 (15.4)
[2]GADA titers, mean (SD), (min-max) (U/mL)	916 (916), (58-2645)	1198 (1626), (72-6006)
Age, mean (SD), (min-max)	9.0 (2.9), (4.6-13.8)	9.4 (2.7), (4.6-13.0)

Table 7. GAD-alum treatment associations with lower levels of different T-cell subpopulations, 18–24 months (visit 8–10) post-treatment.

Phenotyped T-cell populations	[1]Visit	[2]Estimate	95% CI	p-value
T-cells CD3+	8 10	-0.41 -0.36	-0.7, -0.12 -0.66, -0.06	0.008 0.022
CD3+CD4+	8 10	-0.24 -0.20	-0.43, -0.06 -0.39, 0	0.014 0.048
CD4+ CD45RA+ CD45RO-	8	-0.18	-0.33, -0.03	0.019
CD4+ CD45RA+ CD45RO+	8	-0.03	-0.06, 0	0.038
CD4+CD45RA+CD62L+	8	-0.19	-0.34, -0.05	0.013
CD4+CD45RA+CD62L-	8	-0.01	-0.02, 0	0.039
CD4+CD62L+	8	-0.25	-0.46, -0.05	0.017
CD4+CD62L-	8	-0.03	-0.05, -0.01	0.008
CD3+CD8+	8 10	-0.15 -0.12	-0.28, -0.02 -0.22, -0.02	0.023 0.018
CD8+ CD45RA+ CD45RO-	8 10	-0.11 -0.11	-0.22, -0.01 -0.20, -0.01	0.035 0.028
CD8+CD45RA+CD62L+	8 10	-0.11 -0.11	-0.2, -0.02 -0.18, -0.03	0.020 0.011
CD8+CD62L+	10	-0.09	-0.16, -0.01	0.023
CD8+CD62L-	8	-0.06	-0.12, -0.01	0.032

[1]Visits 8 and 10 equal 18, respectively, 24 months of follow-up after first treatment dose. [2]Estimated difference between GAD-alum-treated children and placebo-treated children. 95% CI: 95% confidence interval.

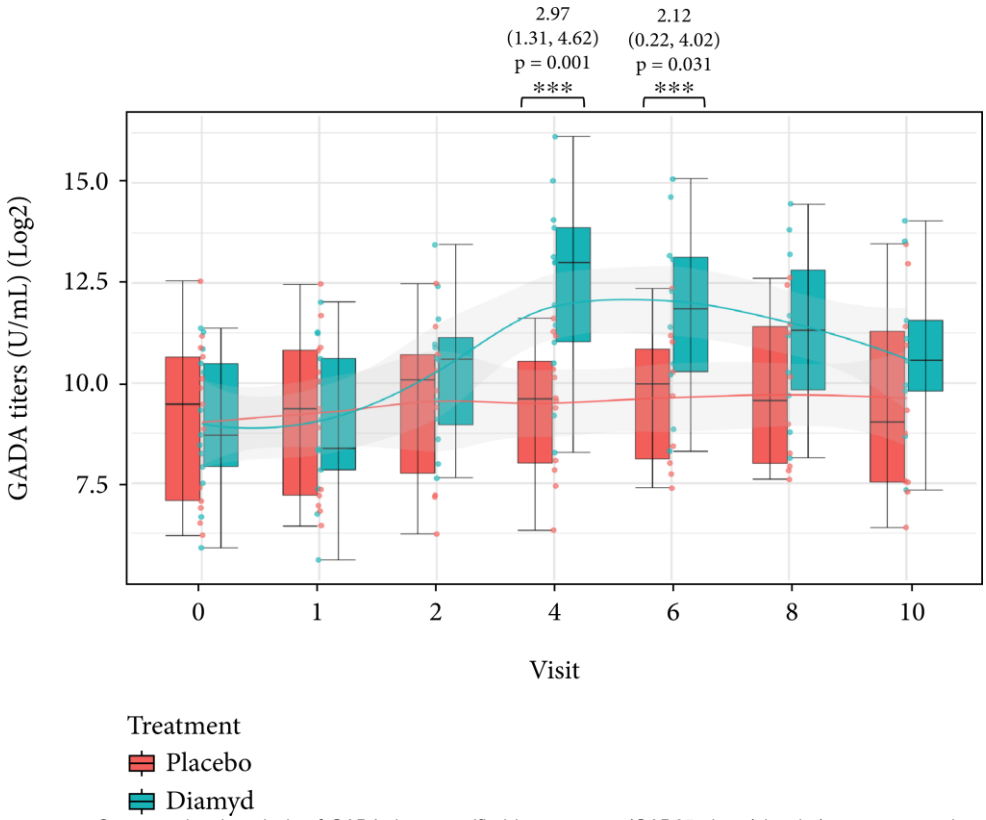

Figure 12. Cross-sectional analysis of GADA titers stratified by treatment (GAD65-alum /placebo) was presented as boxplots at study follow-up visits 0, 1, 2, 4, 6, 8, and 10.

Discussion

The studies presented in this thesis have provided a new understanding of the association between islet beta-cell autoantibodies and peripheral blood cells with red blood cell parameters, HbA1c, glucose metabolism, and the long-term effect of GAD-alum on T-cells in asymptomatic children at increased risk for T1D. Covering different knowledge gaps in the pathogenesis of T1D, will increase the understanding of disease mechanisms, improve heterogeneity typing, and refine the different stages of T1D, which all lead to the goal of personalized medicine therapies and addressing necessary steps in screening for T1D in the general population targeting standard clinical practice for prediction. Currently, screening of T1D in children in screening and surveillance programs, such as TEDDY, has reduced the frequency of DKA, leading to reduced diabetic complications. However, these screenings are currently only achieved for research efforts due to the heterogeneity in the disease progression and the substantial variation in time between T1D stages.

The complete blood count is not well studied in children at genetic risk for T1D. Therefore, we investigated the correlation between autoimmunity and CBC. We found a reduction in neutrophil counts in boys and children with HLA-DR3/DR4 and a reduction in red blood cell counts, hemoglobin, and hematocrit in girls and children with HLA-DR3/DR4 associated with the number of autoantibodies. This association and the result that none of the CBC parameters were associated with seroconversion describe the achieved associations in paper I due to autoimmunity. Following earlier cross-sectional, relatively smaller studies with FDRs (205, 206), a reduction of neutrophils was found in autoantibody-positive children. However, our study is longitudinal, including a substantial number of participants showing that neutrophil reduction is associated with increased autoantibodies, predominantly in boys and children with HLA DR3/DR4. Nevertheless, the reduction in red blood cells and their parameters are, to our knowledge, a novel finding.

The strength of this study was the longitudinal follow-up of 448 children for 3 years with multiple CBC measurements. Another strength is that CBC in this study is prospective from healthy children with increased genetic risk, making our results more reliable and useful than pediatric reference intervals based on retrospectively collected data from hospitalized patients.

One limitation of this study is that CBC was only performed in Sweden and not in the other three TEDDY sites (USA, Germany, and Finland). Another weakness is

that the transition from one autoantibody status to another could not be investigated due to a lack of change in autoantibody status during follow-up, which prohibited the investigation of CBC change before and after seroconversion.

Neutrophils and a diminished number of white blood cells have been linked with several autoimmune diseases (206-210). Moreover, viral infections are linked with mild neutropenia (211). A neutrophil reduction could be due to impaired bone marrow hematopoiesis, peripheral damage consumption, apoptosis, pooling to other organs, such as the pancreas, and tissue detention (212, 213). Previous studies have suggested an accumulation of neutrophils in the pancreas that may be linked to insulitis (205, 206, 211, 214). Thus, the reduced neutrophil counts associated with three autoantibodies may promote accelerated pathogenesis by directing the autoimmune attack on beta-cells by increasing the risk for infections. Viral infections or toxins may alter the CBC leading to impaired hematopoiesis in these autoantibody positive children (211, 215, 216). The reduction of red blood cells, hemoglobin, and hematocrit in girls and children with HLA-DR3/DR4 with multiple autoantibodies could be due to perturbed hematopoiesis, infections, or peripheral destruction.

In paper II, we wanted to investigate whether these alterations in CBC associated with multiple autoantibodies were associated with glucose metabolism measurements or HbA1c reflecting the beta-cell function. Children from paper I and additional children who seroconverted during the 5 years of the follow-up period were included in paper II. The major findings of this paper are the inverse correlation between increased HBA1c and MCV, as well as MCH, HBA1c, red blood cells, hemoglobin, hematocrit, and fasting glucose in seroconverted children, all signaling a disorder of the red blood cell homeostasis and function associated with impaired glucose metabolism. On the other hand, a positive correlation between fasting insulin and red blood cells, hemoglobin, hematocrit, and MCH and a negative correlation with RDW was presented, reflecting normal blood cell homeostasis, and a normal beta-cell function. The strength of this study is the 5-year prospective longitudinal follow-up of 89 seroconverted healthy children with CBC, OGTT, and HbA1c.

Low red blood cells, hemoglobin hematocrit, MCH, and MCV may suggest an impaired hematopoietic cell production in the bone marrow related to the pathogenesis of T1D due to loss of beta-cell function, destruction, or both. It may also reflect deficient iron storage due to deficiency or low ferritin levels (217). Erythropoietin is a glycoprotein hormone that has a key role in the regulating of erythropoiesis and has also been linked to glucose metabolism by increasing glucose uptake in adipocytes (218). It could be speculated that innate deterioration of beta-cell function leading to lower insulin levels is a general regulator of protein synthesis and may affect hemoglobin synthesis, erythropoietin, or other proteins involved in the erythropoiesis by an unknown mechanism. Another speculation is that conspicuous loss of beta-cell function leading to increased blood glucose may

affect the erythropoiesis in the bone marrow leading to reduced red blood cells and red blood cell parameters.

We also presented trajectories of HbA1c, showing an increase of HbA1c in children with two or more autoantibodies compared with children with one single autoantibody. In addition to autoantibodies, age also affected the HbA1c increase over time.

Due to a shortage of resources, CBC was only measured in Sweden and not in the other TEDDY sites, which resulted in a smaller cohort. Another weakness in this study is the lack of HbA1c measurements in autoantibody-negative children who did not develop autoantibodies.

Reduced neutrophil counts associated with the number of autoantibodies were presented in paper I and confirmed in paper II; however, this was not affected by glucose metabolism measures. In contrast, increased white blood cells, mainly lymphocytes but also neutrophils and basophils, were associated with increased 120 min glucose (OGTT) in children positive for multiple autoantibodies. This agrees with earlier studies reporting an increase in white blood cells associated with impaired glucose tolerance in healthy and diabetic subjects (219, 220).

In paper III, we investigated if levels of HbA1c could predict the progression from the first autoantibody to the second, third, fourth, or T1D diagnosis. The main findings of this study were the association between increased HbA1c and T1D regardless of prior autoantibody status or combination, more rapid rate of increase in HbA1c with increasing autoantibody number and increasing rate of increase in HbA1c over time due to proximity to T1D onset. The rise of HbA1c in progressors could be predicted as early as 5 years before T1D onset. Another important novel finding is the transition to IA-2A as the second or fourth autoantibody associated with a decrease in HbA1c. This finding is important and has to be further investigated as it may reflect a more aggressive autoimmune attack on beta-cells leading to autoantibody spreading or insulin leakage into the bloodstream, given that IA-2A appearance is associated with a rapid progression risk of T1D (193, 221, 222).

The strength of this study is represented by the entire TEDDY cohort in Europe and the US with a large number of autoantibody positive children from the general population followed from birth until 15 years of age following the same protocol.

T1D disease progression is very heterogeneous among individuals affected by age, genetics, BMI, and gender (223). More accurate biomarkers predicting the time between each of the T1D stages are limited and thus needed to complement the autoantibody screening in prospective surveillance programs for improved prediction. Considering that the risk of multiple autoantibodies is age-related and declines exponentially with age emphasizes the need for more predictive age-related biomarkers (224).

The joint statistical model designed for this study could be further developed to be used as a tool with HbA1c to predict the time more accurately to T1D diagnosis, improving clinical trial designs and, in turn, improving personalized medicine.

The inability to analyze all combinations of autoantibodies is one limitation in this study due to the limited number of children having the combination. The HbA1c analysis in TEDDY started 4 years after the study, causing missed HbA1c information for the children in question. The HbA1c measurements were done from the 9-month visit by the earliest, as fetal hemoglobin may otherwise interfere with the outcome.

In agreement with earlier studies, HbA1c is a perfect biomarker for predicting T1D (188, 225). In this study, we present an advanced statistical prediction model that could be further used to predict the time to T1D with HbA1c.

The increased HbA1c levels associated with T1D onset are detected as early as 5 years from T1D diagnosis. These HbA1c values still within reference intervals may add to the literature better reference intervals corrected for children as the currently available reference intervals are derived from adults.

The effect of GAD-alum immune tolerance on T-cells in a longitudinal follow-up was investigated in paper IV, and the main finding was the long-term effect of GAD-alum associated with lower levels of T-cells, T-helper cells (CD3 + CD4+), and cytotoxic T-cells (CD3 + CD8+) together with other subgroups of both naïve and effector memory cells detected 18 and 24 months after treatment with GAD-alum in nondiabetic children positive for multiple beta-cell autoantibodies. These low levels of T-cells would be a sign of tolerance lasting at least 2 years post-treatment. The treatment of GAD-alum is safe and well-tolerated in children; however, the efficacy of the treatment in preventing or delaying T1D is still to be debated. To preserve beta-cell function or prevent the T-cell mediated autoimmunity, it is important in future studies to also intervene with GAD-alum immune tolerance treatment before seroconversion or positivity for GADA as a single autoantibody in children at genetic risk. Hence, the immunomodulatory effect of GAD-alum on T-cells is important to understand and improve clinical intervention studies with GAD-alum. This is to our knowledge the first study in which the immunomodulatory effects of GAD-alum were investigated in nondiabetic children with multiple autoantibodies. However, immunomodulatory effects of GAD-alum have been investigated mostly in children with T1D upon in vitro stimulation with GAD (226, 227). Recent studies with intralymphatic administered GAD-alum have shown preserved C-peptide up to 15 months, stronger immune responses, and reduced GAD-65 stimulated cytotoxic CD8+ and CD4+ T-helper central memory cells in patients with recent onset of T1D carrying the HLA DR3-DQ2 haplotype (228, 229). Together with our results, these results emphasize further studies on GAD-alum with different formulations and modes of administration in participants at stage 1, 2 or 3 of T1D or just at genetic risk before seroconversion.

The strength of this study is that all included participants are non-diabetic children positive for multiple autoantibodies, and to our knowledge, this is the first study dissecting the long-term effect of GAD-alum on T-cells in non-diabetic subjects. A major weakness is the limited number of study participants.

Future aspects

The cellular and molecular mechanisms behind the gender and HLA-related alterations in CBC with an increasing number of autoantibodies need to be further explored. In addition, as both the neutrophils and the red blood cells were reduced in children with multiple autoantibodies, the hematopoiesis and erythropoiesis need to be investigated to understand their possible role in the pathogenesis of T1D and to later refine the stages in the progression to T1D onset. The reduction in neutrophils by the number of autoantibodies may contribute to the pathogenesis of T1D and has thus to be further investigated. The decrease in red blood cells, hemoglobin, hematocrit, and red blood cell indices MCV and MCH associated with increased HbA1c and fasting glucose, reflecting an impaired glucose metabolism and thereby a diminished beta-cell function, may suggest these CBC measurements as predictive markers for T1D risk. The reason behind the reduction in red blood cells and red blood cell parameters needs to be elucidated, e.g., by measuring the ferritin level in children at increased genetic risk for T1D. The predictive dynamic statistical model presented in paper III has been developed to accurately predict time to T1D with mean HbA1c levels. A manuscript is in the process of presenting this predictive model as a tool to predict time to T1D with data from autoantibody-positive subjects in TEDDY. The transition to IA-2A positivity following GADA or GADA, IAA, and ZnT8A associated with a decrease in HbA1c is a novel finding that needs further investigation in children with multiple autoantibodies. The elevated levels of HbA1c in children still in normal ranges detected years before T1D is a warning signal that may suggest better normal reference intervals for HbA1c in children. The existing HbA1c reference interval for children needs to be refined for different ages, e.g., 42 mmol/mol as an upper limit for the normal range is slightly high for a 5-year-old but maybe not for a 13-year-old. The results in paper IV show a long-term effect of GAD-alum on T-cells which in turn emphasizes further intervention studies with different formulations and modes of administration in participants within stage 1, 2, or 3 or, only at genetic risk for T1D to preserve beta-cell function or prevent autoimmunity.

Conclusions

- The gender and HLA genotype-related reductions in CBC associated with the number of beta-cell autoantibodies may be a consequence of islet beta-cell autoimmunity.
- Reduction in neutrophils, primarily in boys and children with HLA-DR3/DR4 was inversely associated with the number of autoantibodies.
- Reduction in red blood cells, hemoglobin, and hematocrit was associated with multiple autoantibodies, primarily in girls and children with HLA DR3/DR4.
- The negative associations between red blood cell counts, hemoglobin, hematocrit, and the red blood cell indices, MCV and MCH, with the β-cell function in seroconverted children suggest that these CBC measurements may be used as predictive markers in the prediction of T1D.
- Subtle and insidious changes in glucose levels may affect the hematopoiesis in the bone marrow resulting in changes in the red blood cell counts and the levels of its parameters.
- The reduction in neutrophil counts associated with the number of autoantibodies was not associated with glucose metabolism measures.
- HbA1c is a useful predictive biomarker that can predict T1D several years before onset.
- The statistical dynamic prediction model with HbA1c, the predictive biomarker, could further be developed to predict the time for T1D onset.
- A rapid rate of increase of HbA1c over time is associated with three autoantibodies.
- The appearance of IA-2A as the second autoantibody following GADA or as the fourth following GADA, IAA, and ZnT8A is associated with a decrease in HbA1c and needs further investigation.
- Immune tolerance treatment with GAD-alum has a long-term impact on different subtypes of T-cells suggesting a persistent effect that lasts for at least 2 years. This warrants further investigation to improve the efficacy of GAD-alum.

Populärvetenskaplig sammanfattning

Markörer i blodet som förutsäger typ 1 diabetes

Autoimmuna sjukdomar är ett samlingsnamn på sjukdomar där det egna immunförsvaret attackerar kroppsegen vävnad, typ 1 diabetes (T1D) är en sådan livslång sjukdom. T1D uppstår till följd av att immunförsvaret i kroppen angriper och förstör de insulinproducerande kroppsegna cellerna, de så kallade Langerhanska cellöarna i bukspottskörteln vilket leder till total förlust av insulinproduktionen. Hormonet insulin hjälper kroppen att tillgodogöra sig energi i form av glukos från maten vilket därmed reglerar sockernivån i blodet. Utan insulin kan inte glukosen tas upp av cellerna och ansamlas därmed i blodet vilket leder till högt blodsocker som är både farligt och skadligt för kroppen. Första tecknen på T1D hos barn är stora mängder urin, ökad törst, trötthet och viktnedgång. Obehandlat högt blodsocker kan leda till ett livshotande tillstånd, kallat ketoacidos som kan leda till medvetslöshet, koma och död. Sjukdomen uppträder vanligtvis under barndomen eller tonåren men kan också uppträda i vuxen ålder. Förekomsten av T1D har ökat under de senaste decennierna bland barn i hela världen. Idag finns inget botemedel och drabbade barn och vuxna behandlas flera gånger dagligen med insulininjektioner för att ersätta det kroppsegna insulinet och kunna överleva. Behandlingen är idag riktad på att hålla blodsockret på rätt nivå med hjälp av insulin, kost och en hälsosam livsstil för att förhindra komplikationerna orsakade av högt eller lågt blodsocker.

Den högsta förekomsten av T1D finns i Finland följt av Sverige. Detta beror på att folk i Norden har en viss medfödd benägenhet genom specifika arvsanlag vilket medför risk för att utveckla T1D. Den ökade risken kan härledas till vissa transplantationsantigener, så kallade HLA-gener vilka bidrar till omkring 50% av den genetiska risken och kodar för proteiner på immuncellers yta som presenterar fragment (antigen) till specifika vita blodkroppar och därmed aktiverar T-cellerna vilka utgör en viktig del i immunförsvaret mot virus. I Sverige bär 20% av befolkningen på riskanlagen. Dock insjuknar endast 1,5% av dessa individer med ökad risk vilket tyder på andra hittills okända miljöfaktorer som tillsammans med ärftlig risk kan leda till sjukdomen. Virusinfektioner, rubbningar i mag- och tarmkanalen och kost är miljöfaktorer som i vissa studier kunnat kopplas till risken för att drabbas. Den autoimmuna processen som förstör de insulinproducerande beta-cellerna börjar månader till år före diagnos och karaktäriseras av symtom som

uppträder först då 90 % av beta-cellerna är förlorade och en omedelbar behandling med insulin krävs. Orsaken till sjukdomen är således okänd. Vår forskning går ut på att försöka lösa gåtan kring de hittills okända faktorerna som leder till T1D, samt att hitta markörer för att bättre förutsäga risk för sjukdom och förstå sjukdomsförloppet. Resultaten kan i framtiden användas till att förhindra den autoimmuna attacken eller bevara den kvarvarande funktionen av de insulinproducerande cellerna hos barn med förhöjd risk att drabbas.

T1D föregås av autoantikroppar som kan mätas genom ett enkelt blodprov. Dessa är antikroppar som kroppen bildar mot de insulinproducerande cellerna och utgör markörer för immunförsvarets attack mot de insulinproducerande cellerna. Det finns fyra sorters T1D autoantikroppar: IAA, GADA, IA-2A och ZnT8A. Förekomst av autoantikroppar markerar ökad risk för T1D och ju fler som utvecklas desto större risk har man. Tiden från första autoantikroppen till sjukdomsdebut variera mellan månader och flera år där vuxna har den längsta fördröjningen medan barn insjuknar tidigare. Barn som har två eller fler autoantikroppar har en hög risk för att drabbas inom 5–10 år. Förloppet före T1D debut brukar delas in i tre steg för att bäst kunna förutsäga sjukdom och möjlighet att utföra fördröjande eller förebyggande kliniska studier. Första steget kännetecknas av utvecklingen av två eller fler autoantikroppar, steg 2 kännetecknas av en nedsatt glukostolerans. Nedsatt glukosintolerans innebär en försämrad funktion hos de insulinproducerande cellerna att producera insulin och hantera blodsockret. Den nedsatta glukostoleransen kan mätas med en sockerbelastning och utvärderas genom mått på glukos, insulin och C-peptid (biprodukt till insulin). En nedsatt glukostolerans innebär en stor förlust av de insulinproducerande cellerna. T1D debuterar vid steg 3 med klassiska T1D symtom och ca 90 procent av de insulinproducerande cellerna har förstörts. Tiden mellan de olika stegen varierar mycket mellan individer och kan ta dagar till år innan T1D diagnos.

Barnen som har studerats i denna avhandling är friska med förhöjd ärftlig risk för T1D i åldern 0–15 år och har deltagit i en longitudinell uppföljning i antingen The Diabetes Determinants of Diabetes in the Young (TEDDY) studien eller i Diabetes Prevention - Immune Tolerance 2 (DiAPREV-IT 2) studien.

TEDDY studien är en stor internationell forskningsstudie som genomförs i Finland, Sverige, Tyskland och USA. Målet med studien är att följa upp friska barn från födseln och tills de fyllt 15 år för att ta reda på olika miljöfaktorer som kan utlösa en autoimmunitet som i slutändan leder till diagnos av T1D. Nyfödda barn screenades med hjälp av navelsträngsblod för arvsanlag för T1D. Totalt 8676 barn med förhöjd ärftlig risk inkluderades i studien mellan 2004–2010. Barnen följs upp var tredje månad från födseln fram tills de fyllt 4 år därefter följs autoantikroppsnegativa barn var sjätte månad medan autoantikroppspositiva barn fortsätter att följas upp var tredje månad. Prover och information som samlas in vid besöken omfattar blodprover (för analys av antikroppar, olika celler, glukosmetabolism, arvsanlag och olika proteiner), urin, tånaglar, saliv, avföring,

nässekret, vatten från hushållet, allmän hälsoundersökning, tillväxt och pubertetstecken, matdagböcker, information om fysisk aktivitet, medicinering samt viktiga livshändelser så som sjukdom.

DiAPREV-IT 2 är en klinisk studie med målet att förhindra T1D eller fördröja insjuknandet i T1D med hjälp av GAD-alum behandling som ska vänja kroppen vid GAD-antigenet genom att introducera immuntolerans. Studien planerades att inkludera 80 barn där hälften behandlades med GAD-alum injektion och ett dagligt tillskott av D-vitamin för att förstärka immunsystemet och hälften behandlades med en injektion av en overksam substans tillsammans med ett dagligt D-vitamintillskott för att jämföra effekterna av behandlingen. Endast 26 barn hann inkluderas i studien innan resultat från en tidigare studie med samma GAD-alum behandling visade att behandlingen inte kunde fördröja eller stoppa insjuknandet och därför stoppades rekrytering till studien. Dessa 26 barn följdes i två år med regelbundna besök var tredje månad med olika prover däribland blodprover för att följa autoantikroppsutvecklingen och glukosmetabolismen.

Målet med denna avhandling var att hitta olika markörer i blodet som bättre förutsäger olika steg i sjukdomsprocessen samt öka kunskapen kring olika cellulära sjukdomsmekanismer vid autoimmunitet. Syftet med första studien var att undersöka om det fanns en relation mellan ändringar i blodbilden dvs antalet vita och röda blodceller i blodet och positivitet för autoantikroppar eller antalet autoantikroppar hos svenska barn som deltar i TEDDY studien med eller utan autoantikroppar. Resultaten visade att barn som bär HLA gener med högst risk (HLA-DR3/DR4) och pojkar hade låga nivåer av neutrofiler (en viss typ av vita blodkroppar) jämfört med barn som inte hade utvecklat autoantikroppar. Medan barn med HLA-DR3/DR4 och flickor hade lägre antal av röda blodceller tillsammans med lägre röda blodcells parametrar (hemoglobin och hematokrit) som beskriver egenskaper hos cellen. Ju fler antikroppar desto lägre nivåer av neutrophiler och röda blodceller hos barnen.

Syftet med andra studien var att undersöka om den förändrade blodbilden som påvisades i första studien påverkas av nedsatt funktion av de insulinproducerande cellerna som återspeglas i tester för långtidssockret i blodet så kallat HbA1c och sockerbelastningar. HbA1c är ett test som anger medelsockernivån i blodet över de senaste tre månaderna. Resultaten visade att en ökad HbA1c nivå över tid som återspeglar en nedsatt förmåga för kroppen att hantera blodsockret vilket leder till högt blodsocker är associerad med en lägre genomsnittlig hemoglobinmassa i blodcellen och en mindre storlek av den röda blodcellen (MCH och MCV) vilket tyder på lägre järndepåer i blodet. Lägre nivåer av röda blodceller, hemoglobin och hematokrit var associerade med en ökad blodsockernivå vid fasta. Dessa resultat tyder på nedsatt förmåga i kroppen för glukosmetabolism. Högre insulinnivåer vid fasta återspeglar en normal beta-cells funktion och är associerad med högre nivå av röda blodceller, hemoglobin, hematokrit, och MCH.

I den tredje studien ville vi undersöka om en ökning av HbA1c kunde förutsäga utvecklingen av en andra, tredje, fjärde antikropp eller T1D debut. Resultaten visade att en högre HbA1c nivå gav en högre risk för utveckling av T1D som kunde förutspås redan fem år innan T1D debut. HbA1c hade en snabbare stegring då antalet autoantikroppar ökade från en till tre. En lägre HbA1c nivå kunde förutsäga utvecklingen av autoantikroppstypen IA-2A som en andra eller fjärde autoantikropp. Ett intressant fynd är att utvecklingen av IA-2A är associerad med en mer aggressiv autoimmun attack mot de insulinproducerande cellerna vilket kan leda till läckage av insulin från attackerade celler som i sin tur kan leda till lägre blodsocker och lägre HbA1c.

Syftet med studie fyra var att undersöka hur behandlingen med GAD-alum påverkar en typ av immunceller, T-cellerna. Resultaten visade en långtidspåverkan av GAD-alum på olika typer av T-celler som varade i två år efter behandling med GAD-alum. Barn som behandlats med GAD-alum hade lägre T-cells nivåer jämfört med barnen som fick en overksam behandling. Resultatet kan tyda på en ökad immuntolerans som medför en lägre autoimmunitet.

Sammanfattning

- Ändring i blodbilden hos barn med T1D autoantikroppar relaterade till kön och högrisk HLA gener kan ha framkallats av den autoimmuna processen i bukspottskörteln.
- Antalet neutrofiler sjunker med antalet autoantikroppar främst hos pojkar och barn som bär på den högsta HLA riskgenen.
- Lägre nivåer av röda blodceller, hemoglobin och hematokrit var associerade med flera autoantikroppar, främst hos flickor och barn med den högsta HLA riskgenen.
- Lägre nivåer av röda blodceller och hemoglobin är associerade med ökande autoantikroppsantal och en nedsatt funktion hos de insulinproducerande beta-cellerna. Nivåer av dessa kan därför optimeras för användning som markörer vid sidan av autoantikropparna för att förutsäga progressionen av sjukdomsförloppet.
- En förklaring till lägre nivåer av röda blodceller och neutrofiler med ökande antal autoantikroppar kan ha orsakats av subtila variationer i blodsockernivån i blodet som på ett okänt sätt påverkar blodbildningsprocessen (hematopoesen) i benmärgen.
- En ökad HbA1c nivå över tid kan användas för att förutsäga tiden för T1D debut.
- En snabb ökning av HbA1c uppträder vid övergången från en autoantikropp till tre autoantikroppar.

- Uppkomsten av IA-2A som den andra eller den fjärde autoantikroppen är associerad med en minskning av HbA1c.
- Behandlingen med GAD-alum har en långtidseffekt på olika typer av T-celler, vilket tyder på en ihållande effekt eller immuntolerans som varar i minst två år.

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Paper I

Reduction in White Blood Cell, Neutrophil, and Red Blood Cell Counts Related to Sex, HLA, and Islet Autoantibodies in Swedish TEDDY Children at Increased Risk for Type 1 Diabetes

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Islet autoantibodies (IAs) precede the clinical onset of type 1 diabetes (T1D); however, the knowledge is limited about whether the prodrome affects complete blood counts (CBCs) in 4- to 12-year-old children with increased genetic risk for T1D. This study tested whether CBCs were altered in 4- to 12-year-old children without (*n* = 376) or with one or several IAs against insulin, GAD65, or IA-2 (*n* = 72). CBC was analyzed during longitudinal follow-up in 448 Swedish children enrolled in The Environmental Determinants of Diabetes in the Young (TEDDY) study. A linear mixed-effects model was used to assess potential association between IA and CBC measurements over time. The white blood cell and neutrophil counts were reduced in children with IAs, primarily in boys. In contrast, girls had lower levels of hemoglobin and hematocrit. Positivity for multiple IAs showed the lowest counts in white blood cells and neutrophils in boys and red blood cells, hemoglobin, and hematocrit in girls. These associations were primarily observed in children with the HLA-DR3-DQ2/DR4-DQ8 genotype. We conclude that the reduction in neutrophils and red blood cells in children with multiple IAs and HLA-DR3-DQ2/DR4-DQ8 genotype may signal a sex-dependent islet autoimmunity detected in longitudinal CBCs.

Autoantibodies against the β-cell autoantigens insulin (IAA), GAD antibody (GADA), or protein tyrosine phosphatase-like (IA-2A) precede the clinical onset of autoimmune type 1 diabetes (T1D). Children at genetic risk for T1D were monitored from birth in The Environmental Determinants of Diabetes in the Young (TEDDY) study for a first appearing islet autoantibody (IA), be it IAA or GADA (1–3). The IAA incidence rate was highest in the 1st year of life (18 months in Sweden) and declined over the following 5 years, whereas the incidence rate of GADA increased during the first 2 years (2.5 years in Sweden) of life and remained seemingly constant until the age of 6 years (1–3). The appearance of IAA or GADA as the first IA was related to the HLA-DR/DQ genotype (1,2). After an initial event that triggers autoimmunity reflected by a first appearing IAA or GADA, the pathogenesis progresses toward the clinical onset of disease more rapidly with an increasing number of IAs (4,5), which may be critical because islet β-cells are thought to be destroyed by autoreactive T cells, not by autoantibodies (6,7). So far, β-cell–specific autoantibodies are the best predictors of an ongoing autoimmune process resulting in clinical onset (8). Notwithstanding the critical role of the adaptive immune response in the etiology and pathogenesis of T1D (reviewed in [9–12]), there is growing evidence that the innate immune response also contributes to the pathogenesis (12–14). Neutrophils are thought to contribute to the etiological triggering and the pathogenesis of T1D in mouse models (reviewed in [15]). A reduction in peripheral blood neutrophils has recently been reported in healthy IA-positive children all having a first-degree relative with the disease (16). Similarly, compared with healthy children,

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white blood cells, neutrophils, and lymphocytes were reduced in IA-positive children with a family history of T1D (17), and there is an unknown effect of the peripheral immune cell counts on the pathogenesis of T1D (18).

We have studied children in Sweden who are enrolled in the TEDDY study (19,20) and monitored from birth to determine the first appearing IA (1) as well as progression to multiple autoantibodies and clinical onset of diabetes (4,5). We specifically questioned whether complete blood count (CBC) was associated with the IA status in 4- to 12-year-old Swedish TEDDY children and whether the association differed by sex and HLA DQ-DR genotype.

RESEARCH DESIGN AND METHODS

TEDDY Design

The TEDDY study is a prospective cohort study funded by the U.S. National Institutes of Health with the primary goal to identify environmental causes of T1D. TEDDY includes six clinical research centers—three in the U.S.: Colorado, Georgia/Florida, and Washington, and three in Europe: Finland, Germany, and Sweden. Detailed study design and methods have been previously published (19,21). Written informed consents were obtained for all study participants from a parent or primary caretaker, separately, for genetic screening and participation in prospective follow-up. The current study was approved by the Regional Ethics Review Board in Lund and is monitored by an External Advisory Board formed by the National Institutes of Health.

CBC Measurements in TEDDY Children

The study cohort consists of 448 children, 4–12 years old, from the TEDDY clinic in Malmö, Sweden (Table 1). The CBC measurements were initiated in June 2014 and completed in February 2017. The samples were analyzed at their scheduled visits and within 8 h after sample draw. The TEDDY protocol requires visits every 3 months for children who were IA positive and every 6 months for children who remained autoantibody negative.

CBC

CBC was determined in a multiparameter automated hematology analyzer (CELL-Dyn Ruby; Abbott Laboratories, Diagnostic Division, Abbott Park, IL) (22). The instrument was operated according to the instructions by the manufacturer, including a daily calibration. Counts (cells $\times 10^9$/L) of white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils, platelets, red blood cells (cells $\times 10^{12}$/L) and red blood cell parameters; hemoglobin (g/L), hematocrit (L/L), mean corpuscular volume (fL), mean corpuscular hemoglobin (MCH) (pg), MCH concentration (MCHC) (g/L), and red cell distribution width (% coefficient of variation) were obtained.

HLA-DR-DQ Typing

Genotype screening (20) was conducted using a dried blood spot punch or a small volume whole-blood lysate specimen format, as previously published (23). Infants from the general population were eligible for the study if they had any one of the following HLA genotypes (excluding those with DR4 subtype DRB1*04:03):

1. DR4-DQA1*03:0X-DQB1*03:02/DR3-DQA1*05:01-DQB1*02:01
2. DR4-DQA1*03:0X-DQB1*03:02/DR4-DQA1*03:0X-DQB1*03:02[1]
3. DR4-DQA1*03:0X-DQB1*03:02/DR8-DQA1*04:01-DQB1*04:02
4. DR3-DQA1*05:01-DQB1*02:01/DR3-DQA1*05:01-DQB1*02:01

Note: [1]Acceptable alleles in this haplotype include both DQB1*03:02 and *03:04.

Infants with a first-degree relative with T1D were eligible for enrollment if they had any of the following HLA genotypes:

1. DR4-DQA1*03:0X-DQB1*03:02[1]/DR3-DQA1*05:01-DQB1*02:01
2. DR4-DQA1*03:0X-DQB1*03:02[1]/DR4-DQA1*03:0X-DQB1*03:02[1]
3. DR4-DQA1*030X-DQB1*0302[1]/DR8-DQA1*04:01-DQB1*04:02
4. DR3-DQA1*05:01-DQB1*02:0[1]/DR3-DQA1*05:01-DQB1*02:01 (24)

Table 1—Characteristics of TEDDY children (n = 448) investigated for CBC when negative or positive for one or several IAs

	IA negative (n = 376)	IA positive (n = 72)
Children, n (%)	376 (84)	72 (16)
Sex, n		
Girls	182	30
Boys	194	42
Age at first CBC (months), median (min–max)	91 (52–145)	101.5 (59–139) n.s.
Girls	91 (53–144)	103 (59–139)
Boys	94 (52–145)	99 (59–137)
CBC measures per child, min–max	1–6	1–9
Months of follow-up, min–max	1–30	1–30
IAs, n		
1	0	25
2	0	16
3	0	31
Change in IA status	None	None
HLA DR-DQ, n (%)		
DR3/4-DQ2/8	151 (40.2)	39 (54.1)
DR4/4-DQ 8/8	87 (23.1)	12 (16.7)
DR4/8-DQ 8/4	40 (10.6)	12 (16.7)
DR3/3-DQ 2/2	91 (24.2)	9 (12.5)
DR4/1-DQ 8/5	4 (1.1)	0
DR4/13-DQ 8/6	2 (0.5)	0
HLA ineligible	1 (0.3)	0
Total	376 (100)	72 (100)

5. DR4-DQA1*03:0X-DQB1*03:02[1]/DR4-DQA1*03:0X-DQB1*02:0X
6. DR4-DQA1*03:0X-DQB1*03:02[1]/DR12-DQA1*0101-DQB1*05:01[2]
7. DR4-DQA1*030X-DQB1*03:02[1]/DR13-DQA1*01:02-DQB1*06:04
8. DR4-DQA1*03:0X-DQB1*03:02/DR4-DQA1*03:0X-DQB1*03:04
9. DR4-DQA1*03:0X-DQB1*03:02[1]/DR9-DQA1*03:0X-DQB1*03:03 (23)
10. DR3-DQA1*05:01-DQB1*02:01/DR9-DQA1*03:0X-DQB1*03:03

Notes: [1]Acceptable alleles in this haplotype included both DQB1*03:02 and *03:04. [2]In this DQB1*05:01 haplotype, DR10 was excluded. Only DR1 was eligible.

Autoantibody Measurements

IAA, GADA, or IA-2A were measured in two laboratories by radiobinding assays (25,26). In Europe, all sera were assayed at the University of Bristol, Bristol, U.K. All samples positive for IA and 5% of negative samples were retested at the Barbara Davis Center for Childhood Diabetes at the University of Colorado, Denver, and deemed confirmed if concordant. Both laboratories have previously shown high sensitivity and specificity as well as concordance (27).

IA Analyses

Persistent IA positivity was defined as confirmed positive IAA, GADA, or IA-2A on at least two consecutive study visits. The first appearance of persistent confirmed IA in the follow-up was considered and counted.

Statistical Analysis

Considering correlations between measures from the same subject (within-subject correlation), a linear mixed-effects model was used to assess the association of islet autoimmunity on each CBC measurement. We first examined whether the association between the status of IA and each CBC measurement was different by sampling age, but no significant difference was noted in all CBC analyses. Hence, the model included random intercept and random slope as well as sampling age and the status of IAs as fixed effects. Unstructured within-subject correlation was assumed for the random error. The regression coefficient for the status of IAs was assessed to determine whether the associations of islet autoimmunity were ignorable or not. The association between age and each CBC measurement was assessed among IA-negative children. Age of initial measurement was compared using the Wilcoxon rank sum test, and the proportions of girls and HLA-DR3-DQ2/DR4-DQ8 subjects were compared using the Fisher exact test. Age-dependent effects were observed in most CBC parameters and later corrected for in the linear mixed-effects model. Due to this procedure, the observed effects of an increasing number of IAs on neutrophils and red blood cell parameters were all corrected for age.

Table 2—Effects of age (years) on each CBC measurement in IA-negative subjects (n = 376)

CBC	Estimate	SE	P value
White blood cells (10^9 cells/L)	**−0.123**	**0.044**	**0.005**
Neutrophils (10^9 cells/L)	−0.060	0.031	0.053
Lymphocytes (10^9 cells/L)	**−0.036**	**0.013**	**0.006**
Monocytes (10^9 cells/L)	**−0.010**	**0.004**	**0.015**
Eosinophils (10^9 cells/L)	−0.002	0.008	0.766
Basophils (10^9 cells/L)	−0.001	0.001	0.306
Red blood cells (10^{12} cells/L)	**0.040**	**0.015**	**0.010**
Hemoglobin (g/L)	**0.167**	**0.040**	**<0.0001**

Values in boldface type are statistically significant ($P < 0.05$).

Two-sided P values of less than 0.05 were considered for statistical significance. All analyses were performed using SAS 9.4 software (SAS Institute, Cary, NC).

RESULTS

CBC in Relation to Age in IA Negative Children

CBC was examined in 448 children participating in the longitudinal TEDDY study. Children were examined at one to six visits if negative (84%) or one to nine visits (16%) if positive for any IA. The number of CBC measurements was therefore higher in the IA-positive subjects (n = 72; median, 3) than in the IA-negative subjects (n = 376; median, 2), but the significant difference in the age when the CBC measurements started was not significant (Table 1). We examined whether CBC varied with age in the IA-negative children (Table 2). In agreement with earlier studies, older age was significantly associated with decreasing cell counts in the different white blood cell types, except for a non-significant association in eosinophils and basophils (Table 2). In contrast to the nucleated cells, red blood cell counts and hemoglobin levels increase with age, which was also confirmed in our study (28,29).

CBC in Children With and Without IAs

In the next step, differences in the peripheral blood cell counts were examined between children with or without IAs (Table 3). Children with IAs had reduced white blood cell counts (P = 0.046) as a result from a reduction in neutrophil cell counts (P = 0.017). The two red blood cell parameters, hemoglobin (P = 0.026) and hematocrit (P = 0.031), were also reduced in children with IA. The neutrophil-to-lymphocyte ratio did not differ between the two groups.

CBC in Relation to Sex in Children With and Without IAs

Stratified analyses by sex were performed to evaluate sex differences (Table 3). Reduction of white blood cell counts (P = 0.02) caused by a reduction of neutrophil (P = 0.012) and basophil (P = 0.029) counts showed a significant difference between children with and without IAs in boys, whereas a reduction of hemoglobin (P = 0.012) and hematocrit (P = 0.047) was found in girls with IA.

Table 3—The association between IA and each CBC measurement and association to sex

CBC	Estimate	SE	*P* value
White blood cells (10^9 cells/L)			
All subjects	**−0.315**	**0.157**	**0.046**
Girls	−0.025	0.212	0.906
Boys	**−0.540**	**0.229**	**0.019**
Neutrophils (10^9 cells/L)			
All subjects	**−0.239**	**0.099**	**0.017**
Girls	−0.031	0.128	0.807
Boys	**−0.386**	**0.151**	**0.012**
Lymphocytes (10^9 cells/L)			
All subjects	−0.034	0.057	0.553
NLR (all)	−0.115	0.067	0.090
Girls	0.048	0.082	0.555
Boys	−0.084	0.080	0.295
Monocytes (10^9 cells/L)			
All subjects	−0.024	0.015	0.108
Girls	−0.024	0.022	0.281
Boys	−0.024	0.021	0.248
Eosinophils (10^9 cells/L)			
All subjects	−0.023	0.030	0.437
Girls	−0.031	0.045	0.490
Boys	−0.028	0.040	0.486
Basophils (10^9 cells/L)			
All subjects	−0.004	0.002	0.064
Girls	−0.001	0.003	0.702
Boys	**−0.007**	**0.003**	**0.029**
Platelets (10^9 cells/L)			
All subjects	−8.190	6.519	0.210
Girls	0.995	8.731	0.909
Boys	−16.260	9.542	0.090
Red blood cells (10^{12} cells/L)			
All subjects	−0.089	0.050	0.077
Girls	−0.134	0.077	0.084
Boys	−0.065	0.065	0.322
Hemoglobin (g/L)			
All subjects	**−0.297**	**0.133**	**0.026**
Girls	**−0.491**	**0.192**	**0.012**
Boys	−0.180	0.185	0.337
Hematocrit (L/L)			
All subjects	**−0.800**	**0.366**	**0.031**
Girls	**−1.170**	**0.581**	**0.047**
Boys	−0.518	0.521	0.328
Mean corpuscular volume (fL)			
All subjects	0.042	0.374	0.911
Girls	−0.089	0.544	0.870
Boys	0.330	0.500	0.510
Red cell distribution width (%CV)			
All subjects	0.109	0.075	0.146
Girls	0.094	0.121	0.440
Boys	0.130	0.095	0.171

Values in boldface type are statistically significant ($P < 0.05$). CV, coefficient of variation; NLR, neutrophil-to-lymphocyte ratio.

CBC in Relation to the Number of IAs and HLA Genotype

We investigated whether the number of IAs was associated with CBC by comparing the different cell counts (Table 4). The number of IAs was counted as the appearance of any of the following three IAs—IAA, GADA, or IA-2A—in the follow-up. Of the 72 children with IA, all 3 IAs appeared in 31 children, 2 IAs appeared in 16 children, and 1 IA appeared in 25 children. The number of IAs was inversely correlated with the number of white blood cells, neutrophils, and lymphocytes. Sex differences were also identified in this analysis. Children with three IAs and therefore at the greatest risk for T1D showed a reduction in white blood cell counts ($P = 0.007$), primarily in boys ($P = 0.019$) and in children with HLA-DR3-DQ2/DR4-DQ8 ($P = 0.045$). The reduction of white blood cells was mainly caused by the reduction in neutrophil counts ($P = 0.003$), primarily in boys ($P = 0.004$) and in children with HLA-DR3-DQ2/DR4-DQ8 ($P = 0.010$), but also the reduction of lymphocyte counts ($P = 0.038$) in all children.

Red cell parameters were also associated with the number of IA, especially when all three IAs appeared. The red blood cell count was reduced in all children with two IAs ($P = 0.026$), particularly in girls ($P = 0.002$) and in children with the HLA-DR3/4-DQ2/8 genotype and three IAs ($P = 0.006$) (Table 4). These reductions in red blood cell counts were reflected in reduced concentrations of both hemoglobin and hematocrit in the presence of two or more IAs in girls and in children with the HLA-DR3-DQ2/DR4-DQ8 genotype (Table 4). Furthermore, in children with the HLA-DR3-DQ2/DR4-DQ8 genotype and one IA, the MCH was also decreased ($P = 0.042$). In contrast, boys with one positive IA had increased mean corpuscular volume levels ($P = 0.044$), particularly in children not carrying the HLA-DR3-DQ2/DR4-DQ8 genotype ($P = 0.008$).

DISCUSSION

The major findings of CBC in TEDDY children were lower numbers of white blood cells, primarily of neutrophils in boys and in children with HLA-DR3-DQ2/DR4-DQ8 with an increasing number of IAs. Also, an increasing number of IAs was related to lower numbers of hemoglobin and hematocrit in girls and in children with HLA-DR3-DQ2/DR4-DQ8. These findings may be related to an interaction between HLA risk and development of chronic islet autoimmunity. In TEDDY, chronic islet autoimmunity is defined as the length of being persistent confirmed IA positive after seroconversion and also in relation to the number of different IAs (30,31). The major finding that levels of neutrophils, primarily in boys, as well as in children with HLA-DR3-DQ2/DR4-DQ8, decreased with an increasing number of IAs is a novel finding, which to our knowledge has not been reported before. Our data are otherwise consistent with a reduction in peripheral blood neutrophils as recently reported in healthy IA-positive children all with a first degree relative with the disease (16,17). However, in contrast to one of these reports, a reduction in platelet counts in children positive for one or several IAs was not detected. The difference may be explained by the fact that our study was longitudinal, including a relatively larger number of children. Our results take these previous reports to a next step, indicating that the larger the number of IA, the lower the number of neutrophils. Reduction of red blood cells, hemoglobin, and hematocrit in girls positive for two or more IAs has not been reported previously.

Table 4—Association between the number of IA and each CBC measurement in 72 subjects (boys *n* = 42)

CBC	*n* IAs	Estimate	SE	*P* value
White blood cells (10^9 cells/L)				
All subjects	1	0.142	0.257	0.582
	2	−0.379	0.293	0.197
	3	**−0.613**	**0.225**	**0.007**
Boys	1	−0.001	0.392	0.998
	2	−0.701	0.404	0.086
	3	**−0.789**	**0.332**	**0.019**
HLA-DR3-DQ2/DR4-DQ8	1	0.512	0.401	0.203
	2	−0.392	0.415	0.347
	3	**−0.729**	**0.360**	**0.045**
Neutrophils (10^9 cells/L)				
All subjects	1	0.081	0.161	0.616
	2	−0.328	0.178	0.068
	3	**−0.427**	**0.141**	**0.003**
Boys	1	0.033	0.251	0.897
	2	−0.479	0.259	0.068
	3	**−0.634**	**0.216**	**0.004**
HLA-DR3-DQ2/DR4-DQ8	1	0.270	0.245	0.275
	2	−0.420	0.246	0.091
	3	**−0.572**	**0.216**	**0.010**
Lymphocytes (10^9 cells/L)				
All subjects	1	0.092	0.091	0.315
	2	0.025	0.109	0.822
	3	**−0.175**	**0.0840**	**0.038**
Girls	1	0.080	0.124	0.519
	2	**0.368**	**0.175**	**0.038**
	3	−0.115	0.118	0.332
Monocytes (10^9 cells/L)				
All subjects	1	0.003	0.0244	0.885
	2	**−0.057**	**0.0281**	**0.044**
	3	−0.026	0.0214	0.231
Red blood cells (10^{12} cells/L)				
All subjects	1	0.019	0.080	0.813
	2	**−0.200**	**0.089**	**0.026**
	3	−0.112	0.073	0.125
Girls	1	0.060	0.119	0.616
	2	**−0.471**	**0.142**	**0.002**
	3	−0.109	0.104	0.295
HLA-DR3-DQ2/DR4-DQ8	1	0.029	0.129	0.826
	2	−0.211	0.136	0.125
	3	**−0.320**	**0.113**	**0.006**
Hemoglobin (g/L)				
All subjects	1	−0.031	0.210	0.882
	2	**−0.635**	**0.235**	**0.008**
	3	−0.282	0.193	0.147
Girls	1	−0.038	0.300	0.900
	2	**−1.248**	**0.335**	**0.0007**
	3	−0.407	0.255	0.116
HLA DR3/4-DQ2/8	1	−0.255	0.372	0.495
	2	−0.614	0.388	0.116
	3	**−0.710**	**0.326**	**0.031**
Hematocrit (L/L)				
All subjects	1	0.147	0.577	0.799
	2	**−1.801**	**0.640**	**0.006**
	3	**−1.034**	**0.525**	0.050
Girls	1	0.395	0.907	0.664
	2	**−3.635**	**0.990**	**0.0007**
	3	−1.011	0.776	0.200
HLA-DR3-DQ2/DR4-DQ8	1	−0.375	0.996	0.707
	2	−1.922	1.029	0.065
	3	**−2.514**	**0.853**	**0.004**

Continued on p. 5

Table 4—Continued

CBC	*n* IAs	Estimate	SE	*P* value
Mean corpuscular volume (fL)				
All subjects	1	0.854	0.597	0.153
	2	−0.481	0.734	0.513
	3	−0.351	0.544	0.519
Boys	**1**	**1.728**	**0.850**	**0.044**
	2	−0.505	0.900	0.576
	3	−0.118	0.716	0.869
Not HLA-DR3-DQ2/DR4-DQ8	**1**	**2.210**	**0.826**	**0.008**
	2	−0.605	1.246	0.628
	3	−0.192	0.722	0.791
MCH (pg)				
HLA-DR3-DQ2/DR4-DQ8	**1**	**−0.861**	**0.418**	**0.042**
	2	0.034	0.433	0.938
	3	0.562	0.354	0.116

Values in boldface type are statistically significant ($P < 0.05$).

The strength of the current study is that we have been able to determine CBC during almost 3 years in this TEDDY subset of 448 children who visit the Malmö clinic. They represent ~15% of all children in TEDDY. During this period of investigation, children with IAs underwent CBC measurements more than three times and children with no IA one to two times because they only visit every 6 months. Another strength is that the CBC was done at random because our TEDDY laboratory could only perform the CBC in a maximum of eight children per day with blood samples collected in the morning. During the current period of investigating these 4- to 12-year-olds, we did not expect that we would come across a child converting to IA because of small numbers of IA-positive children monitored in this study. Although TEDDY aims to identify the environmental factors behind seroconversion, the strength of the current study was to contribute to the second end point in TEDDY, which is to identify factors that predispose to T1D, to determine the pathogenic mechanisms that eventually result in clinical onset of T1D.

The weakness was that none of the children changed IA status during the follow–up, and therefore, we cannot test whether the observed changes in CBC may precede seroconversion. Therefore, our data underscore the importance to investigate CBC when children at increased genetic risk for T1D are monitored from birth in the future. Another limitation is the number of IA-positive children monitored for CBC. Therefore, we continue the CBC follow-up in children with and without IAs to understand underlying mechanisms of altered CBC. Validation of these results outside of the TEDDY cohort may be accomplished in studies monitoring newborns, such as in the recently initiated Primary Oral Insulin Trial (POInT) study (32).

Pediatric blood reference intervals are mainly based on retrospective data from hospitalized individuals (28,33). However, the data in this study are prospective from healthy children with a genetic risk for T1D. This premise makes our data more reliable and useful when IA-positive

children are compared with those negative for any IA. HLA risk eligibility for TEDDY in Sweden represented 7.5% of all newborns (20). Further CBC analyses are needed because there is an apparent lack of information of CBC neutrophil and red blood cell parameters in relation to HLA-DR-DQ risk not only for T1D but also celiac disease, thyroiditis, multiple sclerosis, and other HLA-associated organ-specific autoimmune disorders.

In agreement with numerous studies, white blood cell counts varied during the 1st years of life and decreased slightly thereafter. Moreover, the red blood cell count is known to increase by age in children, which was found also in this study (28,33). Furthermore, we examined the association between age and CBC in the IA-negative children considered as the general population with the above limitations. As revealed in Table 2, age-dependent effects were observed in most CBC parameters and later corrected for in the linear mixed-effects model. Due to this procedure, the observed associations between the increasing number of IAs and the reduction in neutrophils and red blood cell parameters were all corrected for age.

A diminished number of white blood cells was reported in several autoimmune diseases (17,18,34). Even though neutrophils are innate immune cells, they are involved in the activation and recruitment of both innate and adaptive immune cells (35,36). An impaired neutrophil response is thought to cause or initiate several autoimmune diseases, including systemic lupus erythematosus, vasculitis, and multiple sclerosis (35,37). Our results demonstrate a reduction in neutrophils in boys and in HLA-DR3-DQ2/DR4-DQ8 children positive for three IAs. Recent studies have also reported a reduction in circulating neutrophils in patients newly diagnosed with T1D (who did not have diabetic ketoacidosis at onset) and in IA-positive healthy individuals, suggesting β-cell specific autoimmunity (16,17,38). Neutrophils are thought to be recruited to infiltrate pancreatic islets by the physiological death of β-cells and by a signaling cross talk with other innate immune cells activating the autoreactive T cells (36). Recent studies have also suggested that the reduction of neutrophil numbers may be related to an accumulation of neutrophils in the pancreas (16,17), perhaps associated with insulitis (39). These data would be consistent with the observations that the larger the number of IAs, the higher the risk for insulitis (40,41). However, further studies need to dissect the neutrophil count reduction in relation to pathophysiological changes that occur in the pancreas before the clinical onset of T1D.

Mild neutropenia is a common finding in children with viral infections (42). The reduction in the neutrophil counts in healthy Swedish TEDDY boys and children with the HLA-DR3-DQ2/DR4-DQ8 genotype could be due to an impaired hematopoietic cell production in the bone marrow, impaired maturation, apoptosis, peripheral consumption or damage, pooling to other organs such as the pancreas, and perhaps tissue detention (43,44). The reduction in neutrophil counts associated with a presence of three IAs may contribute to an accelerated pathogenesis by potentiating the autoimmune attack on β-cells, by increasing the risk for infection, or by some other processes. However, it can also be a secondary phenomenon due to detention of neutrophils in the pancreas when the β-cell destruction is more pronounced. Alterations in the CBC among autoantibody-positive children may be caused by an impaired hematopoiesis caused by infections or toxins (42,45).

Under normal circumstances, no sex differences in neutrophil, lymphocyte, or basophil counts at the age of 4–12 years have been reported (28). Boys in this study had reduced basophil and neutrophil counts and increased MCHC counts associated with the status of being positive for three IAs. The MCHC count is normally decreasing by age (28). Viral and bacterial infections may alter neutrophil, basophil, and lymphocyte counts; for instance, whooping cough is known to decrease basophil numbers. Basophils have been shown to migrate to secondary lymphoid organs where they cross talk with T- and B-lymphocytes thereby linking the T-helper cell environment as a contributor to the development of autoimmune systemic lupus erythematosus, and as a consequence, the basophils in the circulation decrease (24). Girls with two IAs had increased levels of lymphocytes which could be associated with viral infections because many viruses, such as rotavirus, mumps virus, and others, have been associated with the pathogenesis of T1D (46,47).

The reduction of red blood cells, hemoglobin and hematocrit associated with two IAs in girls may be explained by a peripheral destruction of red blood cells, impaired hematopoiesis, or infections. Previous studies have associated rotavirus and enterovirus with islet β-cell autoimmunity and T1D (48,49). Hence, whether a reduction in CBC is caused by or contributes to the development of the second or third IA is unclear. Our results in TEDDY girls suggest that an alteration of CBC was observed with a second IA.

We concluded from the current study that deviations in CBC after seroconversion were common primarily in boys and in children with the HLA-DR3-DQ2/DR4-DQ8 genotype. Our statistical models indicate that the reduction of neutrophil counts in boys and HLA-DR3-DQ2/DR4-DQ8 children positive for three autoantibodies may be consequences of islet β-cell autoimmunity, because the reduction in neutrophil levels correlated with the number of IAs. Additional factors influencing the CBC in children at genetic risk for T1D and positive for one to three IAs were also affected by sex, HLA genotype, and number of IAs. The cellular and molecular mechanisms behind the aggravating CBC alterations different in boys or girls with an increasing number of IAs need to be further explored.

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SUPPLEMENTARY DATA

Supplementary Table 1. Complete blood count (CBC) (median (range)) at first visit in 448 TEDDY children without or with one or several islet autoantibodies (IA).

	IA negative	**IA positive**
n	**376**	**72**
White blood cells (WBC) (10E9 Cells/L)	4.87 (2.09-12.3)	4.54 (2.08-8.02)
Neutrophil (10E9 cells/L)	2.13 (0.59-9.42)	1.95 (0.85-5.05)
Lymphocyte (LYM) (10E9 cells/L)	1.79 (0.50-3.93)	1.81 (0.80-3.22)
Neutrophil Lymphocyte Ratio (NLR)	1.20 (0.28-7.73)	1.18 (0.42-4.45)
Monocyte (MONO) (10E9 cells/L)	0.37 (0.11-1.07)	0.37 (0.18-0.78)
Eosinophils (EOS) (10E9 cells/L)	0.23 (0.01-1.88)	0.21 (0-1.05)
Basophil (BASO) (10E9 cells/L)	0.05 (0.01-0.15)	0.05 (0.01-0.10)
Platelets (PLT) (10E9 cells/L)	255 (99.4-496)	235 (113-350)
Red blood cells (RBC) (10E12 cells/L)	3.99 (2.13-6.66)	3.92 (1.99-5.35)
Red blood cell parameters		
Hemoglobin (HGB) (g/L)	11.4 (6.66-19.7)	11.0 (5.79-14.9)
Hematocrit (HCT) (L/L)	31.1 (17.1-51.7)	30.1 (15-41)
Mean corpuscular volume (MCV) (fL)	77.5 (68.7-88.7)	77.6 (70.6-87.1)
Mean corpuscular hemoglobin (MCH) (pg)	28.7 (23.4-51.3)	28.4 (24.3-31.4)
Mean corpuscular hemoglobin concentration (MCHC) (g/L)	36.9 (33-65.7)	36.7 (34.3-41)
Red cell distribution width (RDW) (%CV)	11.8 (10.5-15.4)	11.8 (10.7-13.5)

SUPPLEMENTARY DATA

Supplementary Table 2. The Association between islet autoantibodies (IA) and each complete blood count (CBC) measurement by HLA-DR3-DQ2/DR4-DQ8.

HLA DR3/4-DQ2/8	CBC	Estimate	Standard error	P-value
Yes	White blood cells (10E9cells/L)	-0.240	0.247	0.333
No		-0.329	0.207	0.116
Yes	Neutrophils (10E9cells/L)	-0.275	0.155	0.078
No		-0.192	0.139	0.171
Yes	Lymphocytes (10E9cells/L)	0.040	0.086	0.640
No		-0.055	0.075	0.461
Yes	Monocytes (10E9cells/L)	-0.006	0.019	0.766
No		**-0.047**	**0.023**	**0.042**
Yes	Eosinophils (10E9cells/L)	-0.020	0.044	0.651
No	Model does not fit			
yes	Basophils (10E9cells/L)	-0.002	0.003	0.557
No		-0.006	0.003	0.071
Yes	Platelets	-4.171	10.201	0.683
No		-13.258	8.721	0.130
Yes	Red blood cells	**-0.177**	**0.080**	**0.030**
No		0.003	0.062	0.966
Yes	Hemoglobin	**-0.548**	**0.224**	**0.016**
No	Model does not fit			
Yes	Hematocrit	**-1.712**	**0.599**	**0.005**
No	Model does not fit			
Yes	Mean corpuscular volume	-0.463	0.539	0.392
No		0.614	0.524	0.242
Yes	Mean corpuscular hemoglobin	-0.010	0.255	0.969
No	Model does not fit			
Yes	Red cell distribution width	0.096	0.107	0.372
No		0.144	0.101	0.156

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Paper II

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ORIGINAL RESEARCH ARTICLE

Complete blood counts with red blood cell determinants associate with reduced beta-cell function in seroconverted Swedish TEDDY children

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Abstract

Objectives: To investigate whether changes in complete blood count (CBC) in islet autoantibody positive children with increased genetic risk for type 1 diabetes are associated with oral glucose tolerance tests (OGTT) and HbA1c over time.

Methods: The Environmental Determinants of Diabetes in the Young (TEDDY) study follows children with increased risk for type 1 diabetes in the United States, Germany, Sweden and Finland. In the current study, 89 Swedish TEDDY children (median age 8.8 years) positive for one or multiple islet autoantibodies were followed up to 5 (median 2.3) years for CBC, OGTT and HbA1c. A statistical mixed effect model was used to investigate the association between CBC and OGTT or HbA1c.

Results: HbA1c over time increased by the number of autoantibodies ($p < .001$). Reduction in mean corpuscular haemoglobin (MCH) and mean cell volume (MCV) was both associated with an increase in HbA1c ($p < .001$). A reduction in red blood cell (RBC) counts ($p = .003$), haemoglobin ($p = .002$) and haematocrit ($p = .006$) levels was associated with increased fasting glucose. Increased red blood cells, haemoglobin, haematocrit and MCH but decreased levels of red blood cell distribution widths (RDW) were all associated with increased fasting insulin.

Conclusions: The decrease in RBC indices with increasing HbA1c and the decrease in RBC and its parameters with increasing fasting glucose in seroconverted children may reflect an insidious deterioration in glucose metabolism associated with islet beta-cell autoimmunity.

KEYWORDS
autoimmunity, glucose metabolism, HbA1c, red blood cells, type 1 diabetes

The TEDDY study group is listed in the online supplemental appendix.

1 | INTRODUCTION

Autoimmune diabetes also known as type 1 diabetes is a chronic disease affecting children and young people. The incidence rate of autoimmune diabetes among children is increasing worldwide by 3%–4% a year, while the aetiology and pathogenesis are still not fully understood.[1,2] The disease is associated with a genetic predisposition of class II HLA-DR-DQ risk genotypes, accounting for 30%–50% of the genetic type 1 diabetes risk.[3,4] The HLA risk genotype together with an unknown environmental trigger(s) is causing islet autoimmunity marked by autoantibodies to pancreatic islet beta-cell proteins that are related to the loss of beta cells and progression to type 1 diabetes. The autoimmune attack is predicted by one or multiple autoantibodies against the beta-cell autoantigens glutamic acid decarboxylase (GAD), islet antigen-2 (IA-2), insulin and Zn transporter 8 (ZnT8).

The Environmental Determinants of Diabetes in the Young (TEDDY) is a multicentre prospective cohort study that aims to investigate environmental factors that trigger islet autoantibodies and type 1 diabetes.[5] Children at genetic risk for type 1 diabetes are followed from birth until 15 years of age to identify seroconversion to one or several islet autoantibodies (GADA, IAA or IA-2A) as the first primary end-point and diagnosis of type 1 diabetes as the second primary end-point.[6] The development of two or more islet autoantibodies is increasing the risk for type 1 diabetes above 70% during childhood or adolescence.[7,8] Previously, we have shown a change in complete blood count (CBC) primarily in counts of neutrophils and red blood cells or levels of haemoglobin or haematocrit in Swedish TEDDY children with multiple islet autoantibodies.[9] Multiple islet autoantibodies were associated with a reduction of neutrophil and red blood cell counts as well as reduced levels of haemoglobin and haematocrit.[9]

The possible roles of neutrophils, red blood cells, haemoglobin and haematocrit in the pathogenesis of type 1 diabetes are not understood. In a cross-sectional study, reduced neutrophil counts in islet autoantibody positive adults with increased genetic risk for type 1 diabetes were associated with a reduced beta-cell function estimated by both fasting and stimulated C-peptide.[10] In the present study, the first aim was to investigate whether changes in CBC over time in Swedish TEDDY children positive for one or more islet autoantibodies were associated with glucose metabolism measures (oral glucose tolerance test (OGTT) and haemoglobin A1c (HbA1c)). Second, as the children in this study are longitudinally followed for CBC and HbA1c over time, we tested whether trajectories of these parameters would distinguish single autoantibody from multiple autoantibody positive children. The third aim was to relate the islet autoantibody status at baseline to the change in CBC over time. The hypothesis was that changes in glucose metabolism (OGTT and HbA1c) tracked over time would affect CBC parameters including leukocytes and RBC measures. As CBC is clinical routine and cellular immune mechanisms in type 1 diabetes are not understood, CBC measurements may prove useful to monitor children at increased risk for type 1 diabetes.

2 | RESEARCH DESIGN AND METHODS

2.1 | TEDDY study design

The TEDDY study is a longitudinal prospective study composed of six clinical research centres: three in the United States (Colorado, Georgia and Washington) and three in Europe (Finland, Germany, and Sweden). The primary objective of TEDDY was the identification of environmental exposures that are associated with increased risk of type 1 diabetes in children with high-risk genetic HLA-DR-DQ. For all participants, written informed consents were obtained from a parent or a primary caretaker, separately, prior to genetic screening and if eligible prior to enrolment and participation in the TEDDY follow-up.[11] The genotype screening of newborns (0–3 months) from general population (90% eligible) and newborns with first degree relatives (10% eligible) was conducted using either a dried blood spot punch or a small volume whole blood lysate specimen format, as previously published.[12] Enrolled eligible newborns from the general population or with a first degree relative (FDR) had one of the high-risk HLA genotypes as presented in Table 1. Detailed study design, eligibility and methods have been previously published.[5,6,11,13] The current study has been approved by the Regional Ethics Review Board in Lund, Sweden and observed by an External Advisory Board formed by the National Institutes of Health, United States.

2.2 | Study population and complete blood count (CBC) measurement

The current study included 89 (37 girls and 52 boys), 4–15 years old, Swedish TEDDY children with at least one autoantibody (GADA, IAA, IA-2A) and if positive for one or more islet autoantibodies, the ZnT8 transporter autoantibody (ZnT8A) was also included (Table 2). The children were followed longitudinally with CBC measurements, analysed at their scheduled TEDDY protocol required follow-up visits, every third month.[5,11] The CBC follow-up was initiated in June 2014 and completed in April 2019. The flow chart in Figure 1 presents the number of islet autoantibody (IA) positive TEDDY children included in this study and for whom the different tests of CBC, HbA1c and OGTT data were obtained. A blood sample was drawn in 8 ml BD Vacutainer® CPT™ tubes supplemented with sodium citrate as anti-coagulant, from which 300 µl was taken for CBC analysis within 8 h after blood draw. CBC includes cell counts (cells $\times 10^9$/L) of white blood cells (WBC), neutrophils (Neu), lymphocytes (Lym), monocytes (Mono), eosinophils (EOS), basophils (Baso), platelets (PLT), counts (cells $\times 10^{12}$/L) of red blood cells (RBC) and red blood cell indices; haemoglobin (HGB) (g/L), haematocrit (HCT) (L/L), mean corpuscular haemoglobin (MCH) (pg), mean corpuscular haemoglobin concentration (MCHC) (g/L), mean corpuscular volume (MCV) (fL) and red cell distribution width (RDW) (% coefficient of variation). CBC was determined in a multiparameter automated haematology analyser (CELL-Dyn Ruby; Abbott Laboratories, Diagnostic Division)

TABLE 1 Eligible high-risk HLA genotypes for the enrolment in the TEDDY study

TEDDY Code	Full Genotype	Abbreviation	General population
A	DRB1*04-DQA1*03:01-DQB1*03:02/DRB1*03-DQA1*05:01-DQB1*02:01	DR3/DR4	Yes
B	DRB1*04-DQA1*03:01-DQB1*03:02/DRB1*04-DQA1*03:01-DQB1*03:02[1]	DR4/DR4	Yes
C	DRB1*04-DQA1*03:01-DQB1*03:02/DRB1*08-DQA1*04:01-DQB1*04:02	DR4/DR8	Yes
D	DRB1*03-DQA1*05:01-DQB1*02:01/DRB1*03-DQA1*05:01-DQB1*02:01	DR3/DR3	Yes
E	DRB1*04-DQA1*03:01-DQB1*03:02/DRB1*04-DQA1*03:01-DQB1*02:01	DR4/DR4	No
F	DRB1*04-DQA1*03:01-DQB1*03:02/DRB1#-DQA1*01:01-DQB1*05:01[2]	DR4/DR1	No
G	DRB1*04-DQA1*03:01-DQB1*03:02/DRB1*13-DQA1*01:02-DQB1*06:04	DR4/DR13	No
H	DRB4*01- DQA1*03:01-DQB1*03:02/ DRB1*04-DQA1*03:01-DQB1*03:04	DR4/DR4	No
I	DRB1*04-DQA1*03:01-DQB1*03:02/RB1*09-DQA1*03:01-DQB1*03:03	DR4/DR9	No
J	DRB1*03-DQA1*05:01-DQB1*02:01/DRB1*09-DQA1*03:01-DQB1*03:03	DR3/DR9	No

The DR4 subtypes DRB1*0403 were excluded.[1] Acceptable alleles in this haplotype include both DQB1*0302 and *0304.[2] In this DQB1*0501 haplotype, DR10 must be excluded. Only DR1 is eligible.

operated as previously described according to the manufacturer's manual of instructions.[9,14]

2.3 | Detection of islet autoantibodies

The three different islet autoantibodies (GADA, IAA and IA-2A) were analysed in two reference laboratories, in the United States at Barbara Davis Center for Childhood Diabetes at the University of Colorado Denver and in Europe at the University of Bristol, the U.K, by radio binding assays as previously described. These two laboratories have high sensitivity and high specificity as well as concordance.[15] If positive in one of the two reference laboratories, the sample was confirmed in the other laboratory. ZnT8A was analysed in one of the two reference laboratories and only in subjects positive for at least one of the other three autoantibodies. The islet autoantibody status was determined from three months of age and if positive for one or more islet autoantibodies each third month thereafter until either 15 years of age or the diagnosis of type 1 diabetes. Persistent autoimmunity was defined after confirmed in Denver and Bristol positive islet autoantibodies on at least two consecutive visits.

2.4 | Oral Glucose Tolerance Test (OGTT)

Once the TEDDY child developed a second islet autoantibody, an OGTT was performed twice a year on a scheduled TEDDY visit. Children with one or no islet autoantibodies were not subjected to OGTT. Based on observations in TrialNet, the Finnish DIPP and the German BABY DIAB studies, children with only one islet autoantibody rarely show a deterioration in the OGTT. This is in contrast to children with two or more autoantibodies which over time also develop Impaired Glucose Tolerance (IGT). TEDDY children with two or more autoantibodies were therefore asked to perform a complete OGTT. The amendment to the TEDDY protocol was also done in consideration of the large blood volume taken from the children when a complete OGTT was done. At the TEDDY clinics in Sweden, the

TABLE 2 Characteristics of all children in the study cohort with one or multiple persistent confirmed islet autoantibodies (IA)

	Persistent confirmed IA *n* = 89	Single IA *n* = 34	Multiple IA *n* = 55
Gender			
Girls	37 (42%)	15 (44%)	22 (40%)
Boys	52 (58%)	19 (56%)	33 (60%)
HLA-DR/DQ			
DR3-DQ2/DR4-DQ8	47 (53%)	17 (50%)	30 (55%)
DR4-DQ8/DR4-DQ8	17 (19%)	3 (8%)	14 (25%)
DR4-DQ8/DR8-DQ4	14 (16%)	7 (21%)	7 (13%)
DR3-DQ2/DR3-DQ2	11 (12%)	7 (21%)	4 (7%)
Number of IA at first CBC			
0	6 (7%)	4 (12%)	2 (4%)
1	35 (39%)	30 (88%)	5 (9%)
2	21 (24%)	0	21 (38%)
3	27 (30%)	0	27 (49%)
Age at first CBC (years)			
Median (SD)	8.8 (1.8)	9.3 (1.5)	8.1 (1.8)
Min-Max	5.0–12.0	5.2–12.0	5.0–11.4
CBC follow-up (years)			
Median (SD)	2.3 (1.7)	2.5 (1.8)	2.0 (1.6)
Min-Max	0.0–4.9	0.0–4.7	0.0–4.9

IA is islet autoantibodies, CBC is complete blood count and SD standard deviation.

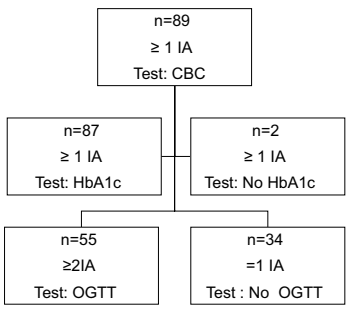

FIGURE 1 Flow chart of islet autoantibody (IA) positive TEDDY children included in this study and for whom the different tests of CBC, HbA1c and OGTT data were available

2-hour OGTT capillary blood glucose was determined by a glucometer (Hemocue® Glucose 201 system, HemoCue AB). The fasting plasma sample was used to determine glucose, insulin and C-peptide representing fasting glucose (glucose (0)), glucose (120 min, OGTT), fasting insulin (insulin (0)) and fasting C-peptide (C-peptide (0)). Glucose, C-peptide and insulin levels were analysed at The Northwest Lipid Metabolism and Diabetes Research Laboratories, University of Washington, Seattle, WA. Glucose levels were determined using the recognized hexokinase method.[16,17] Fasting insulin and C-peptide levels were determined using Tosoh reagents on TOSOH 2000 autoanalyzer (TOSOH, Biosciences, Inc.) as described.[18]

2.5 | Haemoglobin A1c (HbA1c)

Samples for HbA1c analysis were obtained at every scheduled visit in TEDDY children positive for at least one islet autoantibody. The HbA1c sample was processed and analysed at the Diabetes Diagnostic Laboratory (DDL), University of Missouri as described.[19,20]

3 | STATISTICAL METHODS

3.1 | HbA1c, CBC and number of islet autoantibodies

CBC data from subjects with at least one persistent confirmed autoantibody were analysed by mixed model repeated measures analysis similar to our previous report.[9] Specifically, a CBC end-point (e.g., white blood cell count) was the dependent variable and independent fixed variables for age, gender, HLA category and time dependent variables of number of positive islet autoantibodies and HbA1c. A random intercept and slope for age for each subject was assumed with unstructured covariance for the random effects. HbA1c was also analysed in a similar mixed model repeated measures analysis with the number of positive islet autoantibodies as the sole time dependent variable. Best linear unbiased predictions[21] for each subject were estimated for HbA1c and CBC's of interest from the mixed model analyses.

3.2 | CBC association with OGTT measures

Subjects with multiple persistent confirmed autoantibodies had their OGTT data summarized by four measures, glucose at time 0 (fasting), glucose at time 120 min and insulin at time 0. CBC data from subjects with multiple persistent confirmed autoantibodies were analysed with mixed model repeated measures analysis with each CBC endpoint as the dependent variable. Independent variables included fixed variables for age, gender and HLA category; the intercept and slope for age were assumed to be random. Because of the high correlation across the four OGTT measures, the best subset of OGTT measures with a cut-off level of 0.05 was used for analysis and reporting.

4 | RESULTS

4.1 | CBC association with HbA1c

The longitudinal HbA1c and CBC measurements taken during a median period of 2.3 (SD 1.7) years and a maximum period of 4.9 years from children with one or multiple persistent confirmed islet autoantibodies are summarized in Table S1. A total of 89 islet autoantibody positive children were followed quarterly with CBC and 87 of them with HbA1c. The association between CBC and HbA1c is shown by mixed model repeated measures results (Table 3). As known, counts of white blood cells, lymphocytes, eosinophils, basophils, platelets, red blood cells and levels of haemoglobin, haematocrit, MCHC and MCV are all age-dependent. Consistent with our earlier findings, white blood cell counts were significantly ($p < .001$) associated with the number of islet autoantibodies and this association was primarily due to the same significant ($p < .001$) association of reduced neutrophil counts. Counts of lymphocytes ($p = .018$) and monocytes ($p = .050$) also tended to be associated with the number of islet autoantibodies. HbA1c levels increased by decreasing levels of both MCH (Estimate (SE) = −1.51 (0.3)) ($p < .001$) and MCV (Estimate (SE) = −3.67 (0.57)) ($p < .001$). This association was affected by the four type 1 diabetes highest-risk HLA-DR-DQ genotypes (DR3-DQ2/DR4-DQ8, DR4-DQ8/DR4-DQ8, DR4-DQ8/DR8-DQ4 and DR3-DQ2/DR3-DQ2) and either IAA first or GADA first for both MCH ($p = .019$, $p < .001$) and MCV ($p < .001$, $p = .004$) (Figure S2).

TABLE 3 Association between complete blood count (CBC) and HbA1c, age or gender presented as mixed model regression parameter estimates for 87 islet beta-cell autoantibody positive children. P-values for the association between CBC and number of autoantibodies are also shown.

CBC	HbA1c estimate (SE)	Age estimate (SE)	Female estimate (SE)	Number of autoantibodies p-value
White blood cells	0.73 (0.32) p=0.249	−0.10 (0.04) **p=0.010**	0.38 (0.19) p=0.054	**<0.001**
Neutrophils	0.08 (0.22) p=0.711	0.00 (0.02) p=0.901	0.22 (0.11) p=0.057	**<0.001**
Lymphocytes	0.23 (0.12) p=0.055	−0.09 (0.01) **p<0.001**	0.17 (0.09) **p=0.049**	**0.018**
Monocytes	0.04 (0.03) p=0.249	−0.01 (0.00) p=0.132	−0.00 (0.02) p=0.915	**0.050**
Eosinophils	0.03 (0.05) p=0.587	−0.02 (0.00) **p=0.015**	−0.02 (0.04) p=0.552	0.871
Basophils	0.00 (0.00) p=0.133	−0.00 (0.00) **p=0.013**	−0.00 (0.00) p=0.877	0.689
Platelets	24.80 (12.60) **p=0.050**	−10.40 (1.50) **p<0.001**	11.70 (8.70) 0.181	0.248
Red blood cells	0.26 (0.19) 0.175	0.17 (0.02) **<0.001**	−0.13 (0.11) 0.249	0.452
Haemoglobin	−0.40 (7.10) p=0.952	5.00 (0.80) **p<0.001**	−3.00 (5.10) 9=0.564	0.369
Haematocrit	0.00 (0.01) p=0.938	0.01 (0.00) **p<0.001**	−0.00 (0.00) p=0.540	0.452
MCH	−1.51 (0.30) **p<0.001**	−0.02 (0.04) p=0.540	0.31 (0.26) p=0.228	0.074
MCHC	−5.40 (3.00) p=0.070	−0.90 (0.30) **p=0.007**	−1.40 (1.70) p=0.409	0.292
MCV	−3.67 (0.57) **p<0.001**	0.15 (0.07) **p=0.041**	1.71 (0.58) **p=0.002**	0.173
RDW	0.08 (0.17) p=0.633	0.02 (0.02) p=0.328	−0.05 (0.13) p=0.678	0.573

4.2 | CBC association with OGTT measures

The red blood cell counts, levels of haemoglobin, haematocrit, MCV, MCH and RDW in children with multiple islet autoantibodies were all associated with at least one of the glucose metabolism measures obtained from the OGTT (Table 4). Red blood cell counts, haematocrit and haemoglobin levels in 49 children positive for multiple islet autoantibodies decreased by the increasing levels of fasting glucose and increased by the increase of fasting insulin level. Similar to the RBC, haemoglobin and haematocrit, an increase in MCH was associated with an increase in fasting insulin. RDW was associated with an increase in fasting insulin. Increased white blood cell, lymphocyte, neutrophil and basophil counts were all associated with an increase in the glucose (120 min) measure.

4.3 | Trajectories of neutrophils and HbA1c

The mixed model analysis of HbA1c as the dependent variable indicated that the number of islet autoantibodies was associated with increased levels of HbA1c ($p < .001$). The increase was primarily due to the cohort with three islet autoantibodies when compared to the cohort with two islet autoantibodies, the estimated increase was 0.17 (SE = 0.04, $p < .001$). Since the study population was longitudinally followed for CBC and HbA1c, predicted trajectories over the 2–4.9 years of follow-up for each subject were estimated from the linear mixed model analysis. Each subject was categorized by their initial baseline autoantibody status (single or multiple ≥2 islet autoantibodies) to investigate any difference between children with single or multiple islet autoantibodies. No difference could be found in predicted trajectories for monocytes, lymphocytes, red blood cells, haemoglobin, MCH and MCV (Figure S1). A difference was identified in predicted trajectories for both neutrophils and HbA1c where the number of islet autoantibodies had a significant impact ($p < .001$) on the predicted values (Figure 2). Predicted trajectories for neutrophils resulted in a decrease of neutrophil counts in children with multiple islet autoantibodies compared to children with a single islet autoantibody. The subjects with multiple islet autoantibodies at baseline had lower predicted neutrophil counts than those with single islet autoantibody at baseline, and also, those single autoantibody positive subjects had large reductions in predicted values of neutrophils when the number of islet autoantibodies increased (Figure 2A). Age seemed to have little effect on the predicted neutrophil counts (linear age $p = 0.901$). In contrast, predicted values of HbA1c increased by both age ($p < .001$) and the number of islet autoantibodies ($p < .001$). Variability in the rate of increase of HbA1c, estimated by the slopes in Figure 2B, appears to be low across these children.

5 | DISCUSSION

CBC is one of the most important and commonly used clinical laboratory tests. It gives the differential of white blood cells, red blood cell counts, haemoglobin levels and red blood cell indices and thus provides information about the immune system, the production of all blood cells and identifies the oxygen-carrying capacity.[22] CBC is not well studied in children at increased genetic risk for type 1 diabetes and positive for one or multiple islet autoantibodies of GADA, IAA, IA-2A or ZnT8A. Consistent with our previous CBC study in Swedish TEDDY children, the number of autoantibodies was significantly associated with changes in neutrophil and lymphocyte counts.[9] This is expected since the 72 islet autoantibody positive children included in the previous study have also continued to be followed up in the

TABLE 4 Associations between different CBC with OGTT measures in children with multiple autoantibodies.

CBC	n	Glucose (0) estimate (SE)	Glucose (120) estimate (SE)	Insulin (0) estimate (SE)
Red blood cells	49	- 0.023 (0.008) p=0.003	-	0.13 (0.03) p<0.001
Haematocrit	49	−0.002 (0.0006) p=0.002	-	0.011 (0.002) p<0.001
Haemoglobin	49	−0.58 (0.20) p=0.006	-	3.9 (0.8) p<0.001
MCV	50	-	-	-
MCH	54	-	-	0.13 (0.05) p=0.021
RDW	54	-	-	−0.55 (0.024) p=0.021
White blood cells	50	-	0.007 (0.002) p=0.002	
Neutrophils	50		0.004 (0.002) p=0.016	
Lymphocytes	50		0.003 (0.001) p=0.008	
Basophils	50		0.00008 (0.00004) p=0.021	

Glucose (0); fasting glucose, Insulin (0); fasting insulin.

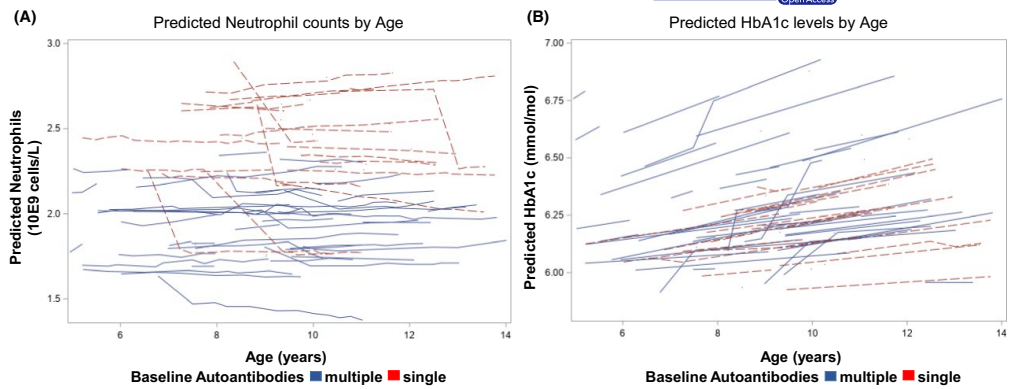

FIGURE 2 Predicted trajectories from the mixed models analysis for neutrophils (A) and HbA1c (B) stratified by the number of islet autoantibodies (single or multiple ≥2 islet autoantibodies) at initial observation. Each trajectory represents a single subject

current study for a longer period and account for 80% of the children included. Interestingly, results from the current study revealed that these changes in leukocyte counts were not associated with glucose metabolism. The major findings in this study were the significant associations in children with multiple islet autoantibodies between the red blood cell counts, parameters, and indices with glucose metabolism, estimated with fasting glucose, fasting insulin and HbA1c as products of beta-cell function.

The strength in our study is the longitudinal follow-up of 89 seroconverted children at increased risk for type 1 diabetes during almost five years of prospective CBC, OGTT and HbA1c measurements.

Some subjects with type 1 diabetes do have a peripheral insulin resistance that causes the plasma glucose levels to remain at increased levels for a longer time upon a glucose challenge.[23] The decrease of MCV and MCH with increased levels of HbA1c, the decrease of red blood cell counts, levels of haemoglobin and haematocrit with the increase in fasting glucose are all indicating a disorder of the red blood cell homeostasis and function associated with increased blood glucose levels or impaired glucose metabolism. The increase in red blood cell counts, levels of haemoglobin, haematocrit, MCH and the decrease of RDW reflecting a normal blood cell homeostasis was associated with an increase in fasting insulin reflecting a normal beta-cell function. However, increased fasting insulin in children with multiple autoantibodies is a sign of beta-cell destruction or leakage of insulin.[24] These new findings have to our knowledge not been presented before and may suggest an impaired haematopoietic cell production in the bone marrow related to the pathogenesis of type 1 diabetes due to loss of beta-cell function, destruction or both.

A potential weakness of the study is that only Swedish children from one of the three Swedish TEDDY clinics were followed up for CBC, which resulted in a smaller cohort. Lack of resources was one reason why CBC was not carried out at other TEDDY sites. For the same reason, ferritin levels have not been measured routinely in TEDDY.

While MCV defines the size, MCH defines the haemoglobin mass of the red blood cell and a decrease in those indicates the senescence of red blood cells.[25] Low levels of haemoglobin, MCV and MCH usually reflect a deficient storage of iron due to low intake from the diet or low levels of ferritin.[26] Further studies are needed to shed light on this issue in healthy islet autoantibody positive subjects. The significant inverse association between HbA1c with MCV and MCH as presented in this study is important for two reasons: the first is that a relative decrease in insulin may affect erythropoietin or other factors that are important to erythropoiesis, and the second is that MCV and MCH could be useful for better prediction of the time to type 1 diabetes in children with multiple islet autoantibodies. It is well known that insulin is a general regulator of protein synthesis; therefore, it might be speculated that innate deterioration of beta-cell function may be the reason for the relationships observed. Therefore, a primary decrement in the level of beta-cell function could be leading to lower levels of insulin secretion, which could be leading to a decrease in haemoglobin synthesis. Besides, experimental studies have found that erythropoietin decreases the circulating levels of glucose.[27] Therefore, it might be speculated that a diminished erythropoiesis due to low levels of erythropoietin may be an underlying factor for diminished glucose metabolism that cause increased levels of glucose and HbA1c.

The pathogenic process of type 1 diabetes is heterogeneous with differences in the initiation of islet autoimmunity by either IAA first or GADA first depending on HLA risk genotype and yet to be identified environmental triggers.[28-31] This data may explain the association between HLA-DR-DQ and the first appearing islet autoantibody (GADA or IAA) and an interaction with the inverse association between HbA1c and MCV or MCH. An association between diabetes

risk and MCV and MCH was also shown previously among young adults,[32] adults[33] and pre-menopausal women.[34] Furthermore, several studies have shown an increase in HbA1c levels with iron-deficiency that negatively affects the red blood cell indices.[35] This information together with our results of the inverse association between MCV and MCH with HbA1c represents a process that takes place already during an autoimmune attack on the beta cells and may remain until after diagnosis of type 1 diabetes. Intriguingly, data from non-diabetic pregnant women have suggested that there is a positive correlation between HbA1c and haemoglobin, haematocrit and MCHC.[36]

Because HbA1c is only tested in seroconverted children in TEDDY, future studies should determine whether this association occurs before or after seroconversion.

The beta-cell function was investigated through OGTT measurements. A decrease in the red blood cell counts, haematocrit and haemoglobin levels was associated with an increase of fasting blood glucose levels that reflects the progression to impaired glucose tolerance (stage 2 in type 1 diabetes staging).[37] In contrast, the increase in red blood cell counts, haemoglobin, haematocrit and MCH levels and the decrease in RDW were all associated with an increase of fasting insulin level, reflecting a normal beta-cell function and a normal red blood cell status. The increase of fasting insulin in children with multiple autoantibody may also reflect beta-cell dysfunction or damage leading to insulin leakage.[24]

Our hypothesis indicated above is that the conspicuous loss of beta-cell function leading to increased blood glucose levels would cause bone marrow failure affecting the erythropoiesis and thereby red blood cells and their parameters. Slowly rising HbA1c and a slowly deteriorating glucose metabolism over time within normal limit values may affect the erythropoiesis. The insidious reduction over time needs to be explained. It may be due to a slowly diminishing insulin production which may affect erythropoiesis. An alternative is that an insidious increase in HbA1c is detrimental also to early steps in the erythropoiesis.[38]

The increase in white blood cell counts primarily by the increase of lymphocytes followed by the neutrophils and basophils was associated with increased 120 minute time point glucose indicating impaired glucose tolerance. Earlier studies in both healthy and diabetic adults have shown elevated white blood cell counts associated with impaired glucose tolerance suggesting the white blood cell count as a measure of risk for impaired glucose metabolism.[39,40]

The novel finding presented by the predicted trajectories of neutrophil counts is that the counts are stable by age and as already shown in previous studies reduced by the number of autoantibodies.[9,41] The lower predicted neutrophil counts in children with multiple autoantibodies need further investigation as the reduced numbers of neutrophils may contribute to the disease pathogenesis.

As presented from the predicted trajectories, both age and number of autoantibodies are affecting the HbA1c predicted values. However, the effect of the numbers of autoantibodies was detected regardless of the age of the autoantibody positive child. HbA1c has been suggested to predict time to clinical onset of type 1 diabetes in children at risk.[42,43] Predicted trajectories of HbA1c could therefore be of great importance in the clinic to further develop a model to predict time to clinical onset of type 1 diabetes.

6 | CONCLUSION

There are negative associations between red blood cell counts, haemoglobin and haematocrit with increased fasting glucose and for the indices (MCH and MCV) also increasing HbA1c indicating a progression to a diminished glucose metabolism and a reduced beta-cell function in seroconverted children with increased risk for developing type 1 diabetes. In contrast, a positive correlation was found between red blood cell count, haemoglobin, haematocrit, MCH and a negative association for RDW with increasing fasting insulin may indicate a normal beta-cell production of insulin but also insulin leakage due to beta-cell damage. The significant negative associations suggest an unknown cellular mechanism that may originate from the early haematopoiesis in the bone marrow, triggered by a more aggressive autoimmune attack on the beta cells resulting in increased blood glucose levels and later a total beta-cell loss and type 1 diabetes. Subtle and insidious changes in glucose levels may also affect the bone marrow resulting in changes in the red blood cell counts and the levels of its parameters. Further investigation of erythrocytes and the erythropoiesis in children positive for islet autoantibodies may help to better define the stages of the pathogenesis prior to type 1 diabetes clinical diagnosis.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

F.S. was overseeing the CBC analyses, interpreted data and wrote the manuscript. R.T. performed statistical analyses, reviewed and edited the manuscript. H.E.L. and C.T. reviewed and edited the manuscript. Å.L. conceived the study, contributed to study design and reviewed and edited the manuscript. Å.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

DATA AVAILABILITY STATEMENT

All the generated and analysed data presented in this study will be made available in the National Institute of Diabetes and Digestive

and Kidney Diseases (NIDDK) Central Repository at https://www.niddkrepository.org/studies/teddy.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Paper III

HbA1c as a time predictive biomarker for an additional islet autoantibody and type 1 diabetes in seroconverted TEDDY children

Short running title: HbA1c as a predictive biomarker

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Conflict of interest

The authors have no conflict of interest to disclose.

Ethics approval

The study was approved by local regional ethics boards in each of the participating countries and was also monitored by an external committee established by the National Institute of Health (NIH).

Data availability statement

The generated and analysed data presented in this study will be made available in the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Central Repository at https://www.niddkrepository.org/studies/teddy.

Abstract

Background/Objectives

Increased level of glycated hemoglobin (HbA1c) is associated with type 1 diabetes onset that in turn is preceded by one to several autoantibodies against the pancreatic islet beta cell autoantigens; insulin (IA), glutamic acid decarboxylase (GAD), islet antigen-2 (IA-2) and zinc transporter 8 (ZnT8). The risk for type 1 diabetes diagnosis increases by autoantibody number. Biomarkers predicting the development of a second or a subsequent autoantibody and type 1 diabetes are needed to predict disease stages and improve secondary prevention trials. This study aimed to investigate whether HbA1c possibly predicts the progression from first to a subsequent autoantibody or type 1 diabetes in healthy children participating in the Environmental Determinants of Diabetes in the Young (TEDDY) study.

Methods

A joint model was designed to assess the association of longitudinal HbA1c levels with the development of first (insulin or GAD autoantibodies) to a second, second to third, third to fourth autoantibody or type 1 diabetes in healthy children prospectively followed from birth until 15 years of age.

Results

It was found that increased levels of HbA1c were associated with a higher risk of type 1 diabetes (HR 1.82, 95% CI [1.57-2.10], $p<0.001$) regardless of first appearing autoantibody, autoantibody number or type. A decrease in HbA1c levels was associated with the development of IA-2A as a second autoantibody following GADA (HR 0.85, 95% CI [0.75,0.97], $p=0.017$) and a fourth autoantibody following GADA, IAA and ZnT8A (HR 0.90, 95% CI [0.82,0.99], $p=0.036$). HbA1c trajectory analyses showed a significant increase of HbA1c over time

($p<0.001$) and that the increase is more rapid as the number of autoantibodies increased from one to three ($p<0.001$).

Conclusion

In conclusion, increased HbA1c is a reliable time predictive marker for type 1 diabetes onset. The increased rate of increase of HbA1c from first to third autoantibody and the decrease in HbA1c predicting the development of IA-2A are novel findings proving the link between HbA1c and the appearance of autoantibodies.

Introduction

Autoimmune type 1 diabetes (type 1 diabetes) is preceded by autoantibodies targeting islet beta cell autoantigens. The autoantibodies against glutamic acid decarboxylase (GADA), insulin (IAA), islet antigen-2 (IA-2A), and zinc transporter 8 (ZnT8A), in turn serve as the strongest predictors of type 1 diabetes clinical onset to date. The development of one or several islet beta cell autoantibodies is a hallmark of an ongoing autoimmune process conferring an increased risk of type 1 diabetes. It has been estimated that children with multiple beta cell autoantibodies have a 70% risk to develop type 1 diabetes in 10 years and a lifetime risk approaching 100% (1). Specific HLA-DR-DQ genotypes together with unknown exogenous factors are likely to trigger an autoimmune reaction against the beta cell autoantigens, predominantly GAD or insulin reflected by the first appearing autoantibody. In recent years, HLA associated endotypes have been identified, the first one is the predisposition of HLA-DR4-DQ8 haplotype associated with IAA as the first appearing autoantibody and the second is the predisposition of HLA-DR3-DQ2 associated with GADA as the first appearing autoantibody (2). Furthermore, three distinct stages of type 1 diabetes have been proposed to characterize disease progression starting with two or more autoantibodies and normoglycemia (stage 1), followed by dysglycaemia (stage 2), and lastly clinical onset of type 1 diabetes with hyperglycemia and symptoms as defined by ADA and WHO (stage 3) (3). However, the time between these stages varies from weeks to years and complicates the prediction of disease progression and the design of secondary prevention trials. Additional biomarkers to complement autoantibody analysis are therefore greatly warranted to predict time to an additional autoantibody or to type 1 diabetes clinical onset. The development of accurate time prediction tools would improve therapeutic interventions aiming to maintain beta cell function. Increase of the glycated hemoglobin A1c (HbA1c), the well-known dysglycaemia marker, has been evaluated in several studies as a biomarker for type 1 diabetes progression and suggested to be used as a tool for time to

diagnosis prediction in children at increased risk (4-6). The Environmental Determinants of Diabetes in the Young (TEDDY) study is a multi-site, multi-country (Finland, Germany, Sweden, and USA) prospective study aimed to study environmental factors triggering islet autoimmunity and to explore the progression of type 1 diabetes by following children at increased genetic risk for type 1 diabetes from birth until 15 years of age (7). The aim of the present study was to investigate the possible association between HbA1c and the progression to an additional autoantibody or to the diagnosis of type 1 diabetes in seroconverted TEDDY children during follow-up and if so to investigate whether there is a difference between the two endotypes of IAA or GADA as the first appearing autoantibody.

Materials and Methods

TEDDY is a prospective cohort study conducted in three clinical research centers in Europe (Finland, Germany, and Sweden) and three in the US (Colorado, Georgia/Florida, and Washington State) aiming primarily to identify environmental triggers of autoimmunity and progression to type 1 diabetes. The study design, eligibility and methods were previously reported (8). A total of 424,788 newborns were screened for high-risk HLA-DR-DQ genotypes associated with type 1 diabetes at the different TEDDY sites between September 2004 and February 2010. The eligible 8 556 children with consents were enrolled and 89% represented the general population while the remaining 11% had a first-degree relative with type 1 diabetes. Enrolled healthy children started the prospective clinical follow-up from three months of age and were monitored for development of islet autoantibodies every three month during the first 4 years and semiannually until 15 years of age. Once seroconverted, children with one or several islet autoantibodies continued the study follow-up each third month until 15 years of age or until they developed type 1 diabetes.

Study participants

The study participants included were all enrolled TEDDY children who reported a persistent confirmed positivity for islet autoantibody as of May 31, 2021. These subjects were divided into four subcohorts depending on their islet autoantibody combination (IAA, GADA, IA-2A, and ZnT8A) of the first, second, third, or fourth appearing islet autoantibody. The progression from the first autoantibody to the second or type 1 diabetes, the second to the third or type 1 diabetes, and the third to the fourth or type 1 diabetes are referred to as transition states in this study. The given starting state islet autoantibody combination at first visit with positivity for the respective autoantibodies in each subcohort is presented in Table 1, together with the possible types of islet autoantibodies that could subsequently appear.

HLA analysis

Cord-blood or heel stick capillary blood samples taken in the first months of life were used to identify high risk HLA DR-DQ genotypes meeting the eligibility criteria of the TEDDY protocol. Typing utilized PCR amplification, Sanger sequencing, oligonucleotide probe hybridization and/or denaturing gel electrophoresis (9). The HLA genotypes were then confirmed at 9-12 months of age using reverse line blot hybridization at a central HLA Reference Laboratory at Roche Molecular Systems, Oakland, CA (10).

Islet autoantibody analysis

Islet autoantibody surveillance for IAA, GADA and IA-2A started at three to four months of age. It was then repeated every third month until 4 years of age, and thereafter every 3 to 6 months until 15 years of age. In order to confirm the islet autoantibody positivity, IAA, GADA, and IA-2A were analyzed in two different reference laboratories, one in the United States at Barbara Davis Center for Childhood Diabetes at the University of Colorado Denver and the other in Europe at the University of Bristol in the U.K, by radiobinding assays as previously

described (11-13). Both reference laboratories have high sensitivity and high specificity as well as concordance for the islet autoantibody assays. A detected autoantibody was considered as persistent when confirmed by both reference laboratories in a sample drawn at a consecutive follow-up study visit. Persistent autoimmunity was defined by the presence of one or several persistent confirmed autoantibodies. The ZnT8A surveillance started once a child was positive but not confirmed for another primary islet autoantibody (IAA, GADA, IA-2A). ZnT8A were also analyzed at one of the two reference laboratories and considered persistent upon two consecutive positive samples in one of the reference labs (14).

HbA1c test

The HbA1c test was performed from the nine months TEDDY visit in islet autoantibody positive children and every third month thereafter until 15 years of age. This requirement for HbA1c measurement was added to the TEDDY protocol 4 years after the start of the study. HbA1c samples were analyzed using an ion-exchange HPLC method on a Tosoh G8 instrument at the Diabetes Diagnostic Laboratory (DDL) at the University of Missouri, standardized using the Diabetes Control and Complications Trial reference method (imprecision coefficient of variation < 1.3%) (15; 16).

Statistical analysis

The association between HbA1c and the transition from one islet beta cell autoantibody state to the subsequent autoantibody transition state was assessed using joint models of competing risk and longitudinal data. The joint models defined time as the time in years from the start of the initial state. The competing risks models studied the relationships between the covariates and the risks of transitioning from a given autoantibody state to the next autoantibody state or type 1 diabetes diagnosis. Four separate sets corresponding to the four different starting states, IAA first, GADA first, IAA + GADA, and IAA + GADA + ZnT8A were analysed. The

corresponding sub-cohorts were defined as the subjects going through the four starting states under consideration. HLA (DR3/DR4 yes or no), gender, country, BMI-z score, and HbA1c were included as covariates in the competing risks model and proportional hazards for competing events are assumed. Longitudinal models are used to model the change of HbA1c and BMI-z scores over time. The trajectories of HbA1c and BMI-z scores were modeled by longitudinal mixed effects models with constant, linear, and quadratic orthogonal polynomials. All HbA1c data from the initial autoantibody visit were included and HbA1c data after the time of type 1 diabetes diagnosis were excluded. Due to the multiplicity of subcohorts and events, a consecutive $p<0.01$ was considered statistically significant. Technical details of the statistical models are described in the supplemented Technical Appendix (Figure 1A-D).

Results

Demographics, number of HbA1c measures and number of type 1 diabetes diagnoses for the next autoantibody state, for the four subcohorts at the first visit with a single autoantibody (IAA first and GADA first), the first visit with two autoantibodies (IAA and GADA), and the first visit with three autoantibodies (IAA, GADA, and ZnT8A) are presented in Table 2. The number of subjects in each of the four subcohorts at the first visit with the given starting state autoantibody was 300 IAA first appearing, 361 GADA first appearing, 257 IAA and GADA double positive, and 115 IAA, GADA and ZnT8A triple positive. The subcohorts are not mutually exclusive, thus one child could be included in one or several subcohorts, for example if the child transitioned from the one autoantibody subcohort to the two autoantibody subcohort by developing an additional autoantibody.

Complete results from the joint model analyses for all four subcohorts with all proportional hazard ratios of covariates (HLA (DR3/DR4 yes or no), gender, country, BMI-z score, and

HbA1c) for each of the autoantibody transition states or type 1 diabetes (referred as events) are presented in Supplementary Table 1 A-D and presented below for each of the four subcohorts. The joint model analysis results yield estimated HbA1c trajectory curves for every subject in each event type within each subcohort. These results are visualized by plotting the mean estimated HbA1c for every subject in each event within each subcohort, in retrospective landmark plots going back five years in time from each event or transition into a subsequent islet beta cell autoantibody (Figure 1. A-D).

IAA single islet autoantibody subcohort and the transition to the next event (GADA, IA-2A, ZnT8A, >1 autoantibodies or type 1 diabetes).

Increased levels of HbA1c were associated with a higher risk of developing type 1 diabetes (HR 1.27, 95% CI [1.16, 1.39], p<0.001). The landmark plot shows the increase of HbA1c in IAA positive children from five years back from type 1 diabetes onset (Figure 1A). No statistically significant association between HbA1c and the transition from IAA as the single autoantibody to any subsequent second autoantibody (GADA, IA-2A, or ZnT8A) or multiple subsequent autoantibodies was found (Supplementary Table 1. A). In IAA positive children, HLA DR3/DR4 heterozygosity was associated with GADA as the second autoantibody (HR 1.89, 95% CI [1.27,2.800], p=0.002). Being an FDR with IAA was associated with two or more autoantibodies (HR 3.707, 95% CI [1.754,7.834], p<0.001). The HbA1c trajectory analysis in this subcohort showed a roughly linear increase of HbA1c over time (estimated covariate 0.34 SE 0.06, p<0.001).

GADA single islet autoantibody subcohort and the transition to the next event (IAA, IA-2A, ZnT8A, >1 autoantibodies or type 1 diabetes).

Comparable to the IAA only subcohort, increased HbA1c was only significantly associated with type 1 diabetes as the subsequent transition event after GADA as a single autoantibody

(HR 1.82, 95% CI [1.59,2.07], p<0.001). The landmark plot (Figure 1B) illustrates the linear increase of HbA1c during the five years prior to the type 1 diabetes event in GADA only positive children. HbA1c was not associated with the risk of any second islet autoantibody in GADA only positive children. However, lower HbA1c levels were significantly associated with IA-2A (HR 0.85, 95% CI [0.75,0.97], p=0.017) as a second autoantibody following GADA (Supplementary Table 1B). HLA DR3/DR4 heterozygosity was associated with IAA as the second autoantibody following GADA as the first (HR 2.16, 95% CI [1.43,3.26], p=0.001). The trajectory analysis of HbA1c present also for this GADA only subcohort a roughly linear increase of HbA1c over time (estimated covariate 0.64, SE 0.05, p<0.001).

IAA + GADA subcohort and the transition to the next event (ZnT8A, IA-2A, ZnT8A + IA-2A or type 1 diabetes)

Increased HbA1c levels were associated with type 1 diabetes (HR 1.82, 95% CI [1.58,2.10], p<0.001) in children positive for both IAA and GADA. The linear increase of HbA1c five years before type 1 diabetes clinical onset is presented in the landmark plot in Figure 1C. Increased HbA1c was not associated with any third autoantibody (Supplementary Table 1C). Similar to the two previously mentioned single autoantibody subcohorts, trajectories of HbA1c increased over time (estimated covariate 0.65, SE (0.06), p<0.001) in this subcohort of children with two autoantibodies. Female gender was associated with IA-2A as the third autoantibody preceded by GADA and IAA (HR 1.81, 95% CI [1.17,2.79], p=0.007).

IAA, GADA, and ZnT8A subcohort and the transition to the next event (IA-2A or type 1 diabetes)

In this subcohort with three autoantibodies, increased HbA1c levels were associated with type 1 diabetes clinical onset (HR 2.12, [1.79,2.51], p<0.001). The increase of HbA1c was linear (slope estimate 1.37, SE (0.148), p<0.001) with increasing rate (quadratic estimate 0.47, SE

(0.092), p <0.001) over time as proximity to clinical onset of type 1 diabetes increases. The increased trajectories of HbA1c five years back from the development of type 1 diabetes in the subgroup with three autoantibodies is illustrated in Figure 1D. IA-2A as the fourth autoantibody was not associated with higher levels of HbA1c, but possibly suggested lower HbA1c levels (HR 0.90, 95% CI [0.82,0.99], p=0.036) (Supplementary Table 1D).

Discussion

The main result of this study is the association of increasing HbA1c levels over time with significantly higher hazard ratios for type 1 diabetes, indicating a higher risk for the type 1 diabetes event, regardless of prior islet beta cell autoantibody number or combination. There was no association between increasing HbA1c and the transition to positivity for the second, third, or fourth islet beta cell autoantibody. However, the HbA1c trajectory analysis revealed a linear increase of HbA1c, in progression to type 1 diabetes, irrespective of the number and combinations of autoantibodies, larger HbA1c rate of increase with increasing autoantibody number from one to three autoantibodies, and finally increasing rate of HbA1c over time as proximity to type 1 diabetes diagnosis increases. The landmark plots presented a rise of HbA1c starting as early as five years prior to type 1 diabetes clinical onset. Nevertheless, the autoantibody transition from GADA or IAA, GADA and ZnT8A to IA-2A as the second or fourth autoantibody associated with lower levels of HbA1c is a novel finding emphasizing further investigation of autoantibodies and HbA1c together as biomarkers in the prediction of type 1 diabetes. To our knowledge, this is the first study evaluating the association between HbA1c and the progression to an additional autoantibody of specific combination in general population children who carried increased HLA-conferred risk of type 1 diabetes, had

seroconverted positive for at least one islet autoantibody and were younger than 15 years of age.

The autoantibody positive TEDDY cohort represents the strength of this study with a relatively large number of autoantibody positive children from the general population, followed from birth until 15 years of age in an accurate islet beta cell autoantibody surveillance program for various numbers and combinations of islet beta cell autoantibodies. The heterogeneity in the TEDDY cohort and the relatively large number of autoantibody positive children made it possible to distribute the children in different subcohorts with different combinations and numbers of autoantibodies.

The benefit of the statistical joint model used in this study that combined longitudinal and survival models is that the estimates of factors such as HbA1c were comparable across the different autoantibody categories since the underlying hazard function was the same (17).

One limitation of this study was the inability to analyze all combinations of islet beta cell autoantibodies (IA-2A first and ZnT8A first or both without any of IAA or GADA) due to limited number of children or progression to type 1 diabetes diagnosis in less than three months. Another limitation was the HbA1c not analyzed in TEDDY until four years after the study had started, therefore some children had limited HbA1c information.

Consistent with our results, it was reported in the population-based prospective Finnish Diabetes Prediction and Prevention (DIPP) study that a 10% increase of HbA1c during 3-12 months in children with multiple islet autoantibodies predicted type 1 diabetes diagnosis after a median time of 1.1 years. Moreover, mean HbA1c levels remained stable in autoantibody positive children who did not progress to type 1 diabetes (4). Similar results were recently reported in an international study showing that an increase of HbA1c of 20% to 30% from a previous sample predicted type 1 diabetes onset and appearance of first autoantibody but not

any multiple autoantibodies (18). Our study adds to these two findings by evaluating whether increased HbA1c is associated with a specific autoantibody type or combination as well as the risk of developing a subsequent autoantibody or type 1 diabetes in seroconverted children with high HLA risk.

The disease pathway to type 1 diabetes is heterogenous and associated with many factors including HLA genotype, age, age at the first appearing islet autoantibody, type of first appearing autoantibody, gender, and BMI giving rise to different endotypes (2; 19). Considering this, the TEDDY cohort was analyzed in two subcohorts with IAA or GADA as the first appearing autoantibody. The present analysis showed, however, that lower levels of HbA1c in GADA single positive TEDDY children was associated with the risk to develop IA-2A as the second autoantibody.

Irrespective of blood glucose levels reflecting beta cell function, a decrease in hemoglobin or iron can increase the HbA1c level (20-22). The HbA1c trajectories in the current study show an increase in HbA1c over time in autoantibody positive children driven by those who progress to type 1 diabetes. This HbA1c increase is still within normoglycemic ranges detected up to years before onset. We have previously shown in autoantibody positive TEDDY children an inverse association of mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) with HbA1c (23), indicating a decrease in iron levels generally caused by an aberrant erythropoiesis or a reduction in iron intake or absorption (24; 25). Thus, further research is required to clarify whether there are common factors associated with erythropoiesis and development of type 1 diabetes.

Unexpectedly, increased risk for developing IA-2A in GADA positive children and those with IAA, GADA, and ZnT8A was associated with lower levels of HbA1c. This may be explained by a more aggressive autoimmune attack on the beta cells leading to autoantibody spreading

or insulin leakage into the bloodstream, as IA-2A positivity is known to confer a rapid progression risk of type 1 diabetes (14; 26; 27).

Type 1 diabetes disease process is extremely heterogenous and varies with age, genetics, BMI, and sex (2; 28). The prediction of disease progression in pre-symptomatic type 1 diabetes children at stage 1 or 2 is currently made by autoantibody surveillance programs together with regular OGTTs and HbA1c monitoring. Within these follow-up programs, diabetic ketoacidosis can effectively be prevented, but close follow-up is costly and limits public health implementation (29; 30). However, biomarkers predicting the progression from one stage of type 1 diabetes to the next are limited, and more accurate predictive biomarkers are needed to complement the autoantibody screening. Given that the risk of multiple autoantibodies is age-related and declines exponentially by age (31), there is a need for additional studies evaluating age-related biomarkers. An ability to predict time more accurately to type 1 diabetes progression would improve clinical trial designs and move us closer towards personalized medicine. This study shows the high impact of HbA1c as a time predictive biomarker for type 1 diabetes onset. Thus, the joint model analysis designed in this study could be further developed with HbA1c as a tool predicting time to type 1 diabetes diagnosis.

Conclusion

In conclusion, rising HbA1c reflects deteriorating beta cell function several years before clinical onset of type 1 diabetes. While HbA1c increase was not associated with the development of a subsequent additional autoantibody, the association between increased HbA1c over time and the development of type 1 diabetes makes HbA1c a useful time predictive marker for type 1 diabetes onset. Lower levels of HbA1c associated with IA-2A as a second autoantibody following GADA or as the fourth autoantibody following GADA, IAA and ZnT8A need further investigation.

Author Contributions

F.S proposed the analysis, interpreted the findings, wrote, and edited the manuscript. R.N.T and L.Y designed the statistical model, performed the statistical analysis, reviewed, and edited the manuscript. C.T. proposed the analyses, reviewed, and edited the manuscript. H.E.L., M.L., R.V., M.J.H., reviewed and edited the manuscript. Å.L., J.K., A.-G.Z., J.T., M.R., J.-X.S., W.H., B.A., designed the study and reviewed and edited the manuscript. Å.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1. Islet autoantibody combinations in the four subcohorts observed in this study.

Subcohorts	**Autoantibody Transition state, or type 1 diabetes onset**	**Possible subsequent autoantibodies**
IAA (1st autoantibody)	1st to 2nd or type 1 diabetes	GADA IA-2A ZnT8A >1 autoantibody[1]
GADA (1st autoantibody)	1st to 2nd or type 1 diabetes	IAA IA-2A ZnT8A >1 autoantibody[2]
IAA + GADA	2nd to 3rd or type 1 diabetes	IA-2A ZnT8A IA-2A + ZnT8A
IAA + GADA + ZnT8A	3rd to 4th or type 1 diabetes	IA-2A

[1]> 1 autoantibodies; refers to any combination of GADA, IA-2A, or ZnT8A islet autoantibodies becoming positive at the same time.
[2]>1 autoantibody; refers to any combination of IAA, IA-2A, or ZnT8A islet autoantibodies becoming positive at the same time.

Table 2. Demographics, number of HbA1c measurements and number of type 1 diabetes diagnosis for next autoantibody state are presented for the subjects in the four subcohorts. Single autoantibody positive (IAA first and GADA first) at first autoantibody positive visit. IAA + GADA at first visit with two positive autoantibodies. IAA + GADA + ZnT8A at first visit with three autoantibodies.

Subcohorts **Autoantibody**	**IAA first n=300**	**GADA first n=361**	**IAA+GADA n=257**	**IAA+GADA+ZnT8A n=115**
Country, n (%)				
United States	98 (33)	133 (37)	80 (31)	43 (38)
Finland	95 (32)	71 (20)	65 (25)	33 (29)
Germany	18 (6)	19 (5)	25 (10)	5 (4)
Sweden	89 (30)	138 (38)	87 (34)	34 (30)
Gender, n (%)				
Female	139 (46)	168 (47)	116 (45)	54 (47)
Male	161 (54)	193 (53)	141 (55)	61 (53)
First degree relative, n (%)				
Yes	66 (22)	62 (17)	57 (22)	23 (20)
No	234 (78)	299 (83)	200 (78)	92 (80)
HLA genotype, n (%)				
DR4/DR3	144 (48)	177 (49)	161 (63)	66 (57)
DR4/DR4	54 (18)	56 (16)	39 (15)	20 (17)
DR4/DR8	62 (21)	43 (12)	28 (11)	14 (12)
DR3/DR3	26 (9)	79 (22)	15 (6)	8 (7)
Other	14 (5)	6 (2)	14 (5)	7 (6)
Baseline Age, median (min-max)	2.0 (0.3–13.7)	5.0 (0.3–14.0)	2.8 (0.5–14.6)	5.2 (1.2–13.8)
Number HbA1c* , median (min-max)	9 (1-43)	8 (1-34)	3 (1–40)	5 (1-43)
Number of type 1 diabetes**	14	11	28	15

*number of HbA1c measures until next state

**number of type 1 diabetes diagnoses for next state

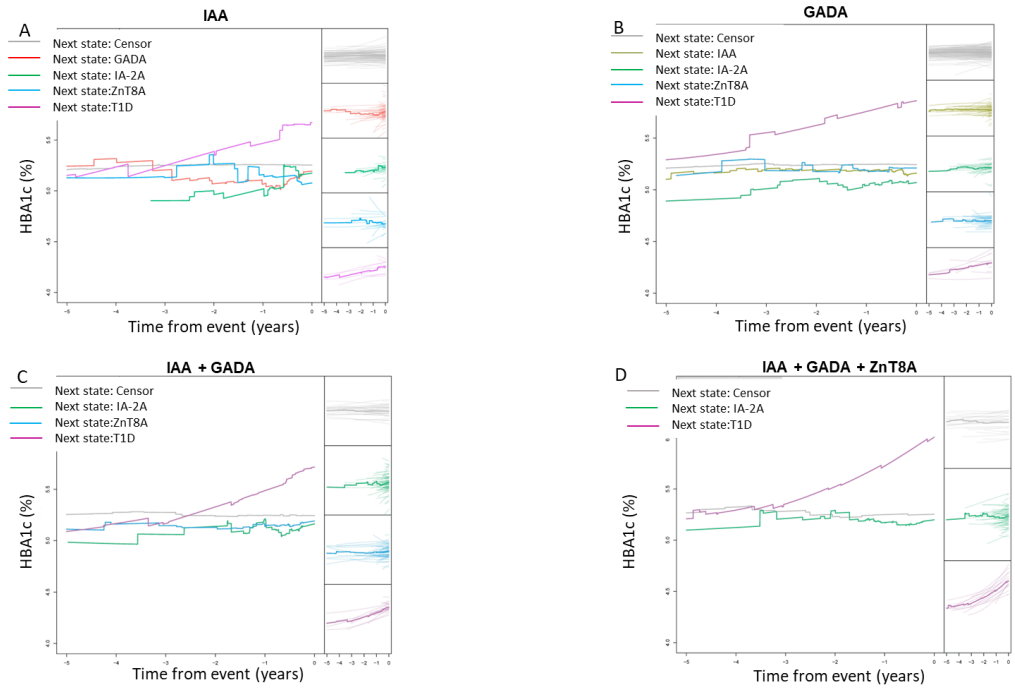

Figure 1. Retrospective landmark plots of HbA1c going back five years from each event. The left-hand panels show mean curves, and the right-hand panel shows individual curves for each event. The diagrams present each subcohort; **A)** IAA single autoantibody positives, **B)** GADA single autoantibody positives, **C)** IAA as well as GADA positives, and **D)** IAA, GADA together with ZnT8A positives. The left panels present mean curves of HbA1c going five years back in time, and the right panel presents individual subject curves for each event. The development of type 1 diabetes is associated with increased HbA1c in all four subcohorts. The slope of the increase also increases from one to two to three autoantibodies. Multiple autoantibody events are excluded from these Landmark plots. Censored grey lines present subjects that have lost follow-up before the transition into the next event of autoantibody development or type 1 diabetes.

Paper IV

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Research Article

Long-Term GAD-alum Treatment Effect on Different T-Cell Subpopulations in Healthy Children Positive for Multiple Beta Cell Autoantibodies

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Objective. The objective of this study was to explore whether recombinant GAD65 conjugated hydroxide (GAD-alum) treatment affected peripheral blood T-cell subpopulations in healthy children with multiple beta cell autoantibodies. *Method.* The Diabetes Prevention–Immune Tolerance 2 (DiAPREV-IT 2) clinical trial enrolled 26 children between 4 and 13 years of age, positive for glutamic acid decarboxylase autoantibody (GADA) and at least one other autoantibody (insulin, insulinoma antigen-2, or zinc transporter 8 autoantibody (IAA, IA-2A, or ZnT8A)) at baseline. The children were randomized to two doses of subcutaneously administered GAD-alum treatment or placebo, 30 days apart. Complete blood count (CBC) and immunophenotyping of T-cell subpopulations by flow cytometry were performed regularly during the 24 months of follow-up posttreatment. Cross-sectional analyses were performed comparing lymphocyte and T-cell subpopulations between GAD-alum and placebo-treated subjects. *Results.* GAD-alum-treated children had lower levels of lymphocytes (10^9 cells/L) ($p = 0.006$), T-cells (10^3 cells/μL) ($p = 0.008$), T-helper cells (10^3 cells/μL) ($p = 0.014$), and cytotoxic T-cells (10^3 cells/μL) ($p = 0.023$) compared to the placebo-treated children 18 months from first GAD-alum injection. This difference remained 24 months after the first treatment for lymphocytes ($p = 0.027$), T-cells ($p = 0.022$), T-helper cells ($p = 0.048$), and cytotoxic T-cells ($p = 0.018$). *Conclusion*. Our findings suggest that levels of total T-cells and T-cell subpopulations declined 18 and 24 months after GAD-alum treatment in healthy children with multiple beta-cell autoantibodies including GADA.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease that progresses in three distinct presymptomatic stages prior to clinical diagnosis, resulting in destruction of the pancreatic beta cells caused by autoreactive cytotoxic T-cells leading to insulin deficiency. The first and second stages are presymptomatic, defined by the detection of at least two beta-cell autoantibodies. Additionally, dysglycemia occurs at stage 2, and lastly, symptomatic T1D at stage 3 [1, 2]. Antigen-specific immunotherapies are currently tested in T1D prevention and intervention studies to induce immunologic tolerance to beta cell autoantigens and to preserve beta cell function in subjects at stages 2 and 3. Glutamic acid decarboxylase 65 (GAD65) is one of the most common autoantigens associated with T1D [3]. Recombinant human GAD65 formulated with aluminum hydroxide (GAD-alum) [4] subcutaneously injected or intralymphatically injected as a prime and boost regimen has been evaluated in several placebo-controlled, randomized trials, in individuals either

Table 1: Baseline characteristics.

	GAD-alum $n = 13$	Placebo $n = 13$
Gender, *n* (%)		
Female	7 (53.8)	4 (30.8)
Male	6 (46.2)	9 (69.2)
First degree relatives, *n* (%)		
Yes	4 (30.8)	2 (15.4)
No	9 (69.2)	11 (84.6)
High risk HLA haplotypes, *n* (%)		
DR3-DQ2	5 (38.4)	7 (53.8)
DR4-DQ8	13 (100)	12 (92.3)
Positive[1] beta cell autoantibodies, *n* (%)		
2	1 (7.7)	2 (15.4)
3	3 (23.1)	5 (38.5)
4	4 (30.8)	3 (23.1)
5	4 (30.8)	1 (7.7)
6	1 (7.7)	2 (15.4)
[2]GADA titers, mean (SD), (min-max) (U/mL)	916 (916), (58-2645)	1198 (1626), (72-6006)
Age, mean (SD) (min-max)	9.0 (2.9), (4.6-13.8)	9.4 (2.7), (4.6-13.0)

[1]Beta cell autoantibodies: GADA, IAA, IA-2A, ZnT8RA, ZnT8QA, and ZnT8WA. [2]The thresholds for GADA to be positive were GADA > 34 U/mL.

positive for islet autoantibodies (stage 1 and 2) or recently diagnosed with T1D (stage 3) [5–7]. The safety of GAD-alum has been proven through the treatment but neither prevented diabetes nor affected residual beta-cell function [8, 9]. Several mechanistic studies conducted in parallel with the GAD-alum clinical trials with newly diagnosed T1D patients have demonstrated both humoral and cellular immunomodulatory effects. GAD-alum was found to increase the GAD65 autoantibody titers and to be involved in CD4 T-lymphocyte activation inducing not only a T-helper 2 (Th2) anti-inflammatory response but also a T-helper 1 (Th1) proinflammatory response [10–13]. However, the immunomodulatory cellular effects of GAD-alum in treated healthy children positive for multiple pancreatic beta cell autoantibodies who are at stages 1-2 have still not been demonstrated. To improve the clinical efficacy of the treatment with GAD-alum, which is safe and simple to administer, it is of great importance to increase the understanding of the immunomodulatory effect of antigen-specific immunotherapy in order to preserve beta cell function or even halt the progression to stage 3, i.e., T1D onset. In this study, we aim to investigate whether two injections with GAD-alum in children positive for GADA and at least one more autoantibody participating in the Diabetes Prevention–Immune Tolerance 2 (DiAPREV-IT 2) clinical trial affect different T-lymphocyte subpopulations during two years of follow-up after treatment.

2. Research Design and Methods

DiAPREV-IT 2 was a randomized, double-blind, placebo-controlled clinical prevention study designed to determine the safety and efficacy of two doses of 20 μg GAD-alum in combination with high dose vitamin D treatment on the progression to T1D in children with multiple islet beta cell autoantibodies. The study was conducted in Sweden at the Skåne University Hospital as a sequel to DiAPREV-IT 1 (NCT01122446) [9] but with vitamin D supplement combined with the GAD-alum treatment. The study was designed to enroll 80 children but only 26 children were included during the period of April 2015 to May 2017 before the inconclusive results from the first study were presented indicating that GAD-alum did not affect the progression to T1D [9]. By these results, further subject enrollment and treatment with GAD-alum were stopped in DiAPREV-IT 2, and the study protocol was amended to only follow the 26 already included children for 24 months after the first vaccination dose. At each visit, levels of beta cell autoantibodies against GAD65 (GADA), insulin (IAA), insulinoma antigen-2 (IA-2A), and zinc transporter 8 (ZnT8A) were measured using in-house radiobinding assays [14]. Enrolled children recruited from the three different studies, DiPiS, TEDDY, and TrialNet were all HLA class II genotyped in the separate studies [15, 16, 17].

2.1. Study Population. The study participants in DiAPREV-IT 2 were all recruited from three population-based longitudinal follow-up studies: The Environmental Determinants of Diabetes in the Young (TEDDY) study, Diabetes Prediction in Skåne (DiPiS) study, and TrialNet, as healthy participants. The included children were between 4 and 17.99 years old and had to be positive for GADA and at least one more beta cell autoantibody (IAA, IA-2A, ZnT8RA, ZnT8QA, or ZnT8WA) at stage 1 or 2 in T1D progression, with or without impaired glucose metabolism. Baseline characteristics are described in Table 1.

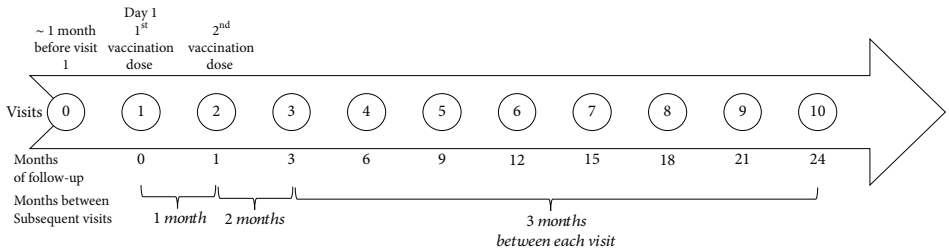


Figure 1: Time line presenting follow-up study visits.

Children were assessed for eligibility, informed about the study, and together with their parents given informed consents to sign at the baseline visit (visit 0) when they also started with a high daily dose of vitamin D (2000 IU) during the two years of follow-up. The 26 enrolled children were randomized for treatment with two doses of subcutaneously administered 20 μg GAD-alum (Diamyd®, Diamyd Medical, Stockholm, Sweden) or placebo (Alhydrogel®) one month after the baseline visit (visit 1). The second GAD-alum/placebo booster dose was administered one month after the first prime dose. The follow-up schedule is illustrated in Figure 1. The consort diagram shows the retention and drop-out of participating children during follow-up (Figure 2). DiAPREV-IT 2 children who were diagnosed with T1D during follow-up had a postdiagnosis follow-up intervention visit. However, in the present study, only healthy children are included and thus excluded from the study once diagnosed with T1D. During follow-up, three children were diagnosed with T1D, one in the GAD-alum-treated group (after 9 months of follow-up) and two in the placebo-treated group (after baseline visit and 6 months of follow-up, respectively). Two children in the GAD-alum-treated group withdrew during the 24 months of follow-up, one after 6 months, and the other after 9 months, resulting in 10 (76.9%) children completing their 24 months of follow-up in the GAD-alum-treated group and 11 (84.6%) children in the placebo-treated group.

2.2. Analysis of Islet Beta Cell Autoantibodies. Each of the six beta cell autoantibodies GADA, IAA, and IA-2A and the three amino acid variants of ZnT8 (R/W/Q) were analyzed at baseline, once a month for three months and every third month thereafter. Radioligand binding assay (RBA) was used to determine the six different beta cell autoantibodies as previously described [18, 19].

2.3. Flow Cytometric Phenotyping of T-Lymphocytes. Blood samples for cellular analysis were collected at scheduled follow-up visits, before the first and the second GAD-alum or placebo treatment, and every 6 months thereafter (at visit 0 or 1, 2, 4, 6, 8, and 10). The samples were drawn in EDTA tubes and prior to any treatment or test during the clinical visit. If the blood sample was for any reason missed at visit 0, a blood sample could be collected at visit 1 instead; hence, either visit 0 or 1 was considered as baseline in the current study. The peripheral blood lymphocyte count (10^9 cells/L) was determined within 4 h after blood draw by complete blood count (CBC) analysis in a multiparameter automated hematology analyzer (CELL-Dyn Ruby; Abbott Laboratories, Diagnostic Division) as previously described [20]. Whole blood samples were processed with BD FACS™ Lysing Solution (BD Biosciences) according to the manufacturer's instructions for the lysing of the red blood cells following immunostaining with monoclonal antibodies conjugated to fluorochromes, fixing, and washing of cells for the flow cytometric analysis. Processed, immunostained, and fixed peripheral blood leukocytes were stored in 1% formaldehyde in phosphate-buffered saline (PBS) at 2°-8°C in dark up to two days prior to the flowcytometric analysis. The samples were analyzed on two different flow cytometers, on BD FACSCalibur at the beginning of the study and later replaced by a CytoFLEX (Beckman Coulter Inc., Brea, CA, U.S.A). Both devices were compared at the Lund University Diabetes Centre Flow Cytometry Laboratory, showing statistically correlated data with no fixed or proportional bias between the sample acquisitions (Deming regression ($r = 0.98$, 95%CI = 0.91 – 0.99) and Passing and Bablok ($r = 0.95$, 95%CI = 0.82 – 1.13)). For the analysis on BD FACSCalibur, the samples were immunostained with CD [4/IgG2a/IgG1] fluorescein isothiocyanate (FITC), IgG1 isotype phycoerythrin (PE)/peridinin-chlorophyll-protein (PerCP)/allophycocyanin (APC) (clone MOPC-21), CD [3/16+56] FITC/PE (clone X39, X40, SK3), CD3 FITC (clone UCH1), CD4 PerCP (clone SK3), CD8 PerCP (clone SK1), CD45RA PE (clone HI100), CD45RO APC (clone UCHL1), and CD62L APC (clone DREG-56) all purchased from BD Biosciences.

For the CytoFLEX, cells were immunostained with CD3 PE-Cyanine 7 (PE-CY 7) (clone SK7), CD4 APC-R700 (clone RPA-T4), CD8 APC-H7 (clone SK1), CD45RA Brilliant™ Blue 515 (BB515) (clone HI100), CD45RO PerCP-CY5.5 (clone UCHL1), CD62L APC (clone DREG56), CD56 PE (clone B159), and CD16 APC (clone B73.1) all purchased from BD Biosciences. VersaComp Antibody Capture Kit (Beckman Coulter) was used according to the manufacturer's manual instructions for the compensation to correct the spectra overlap on the CytoFLEX. Unstained samples were used as negative controls in the acquisition plots and fluorescence minus one control (FMO) for each marker for accurate gating of the positive populations that were used when the monoclonal antibody T-lymphocyte panel was set [21].

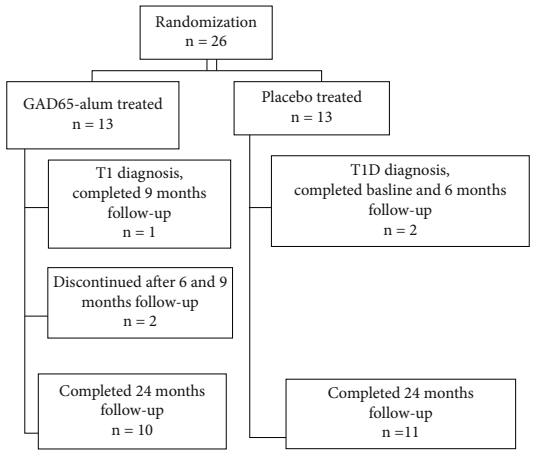


Figure 2: Flow diagram of DiAPREV-IT 2 study participants.

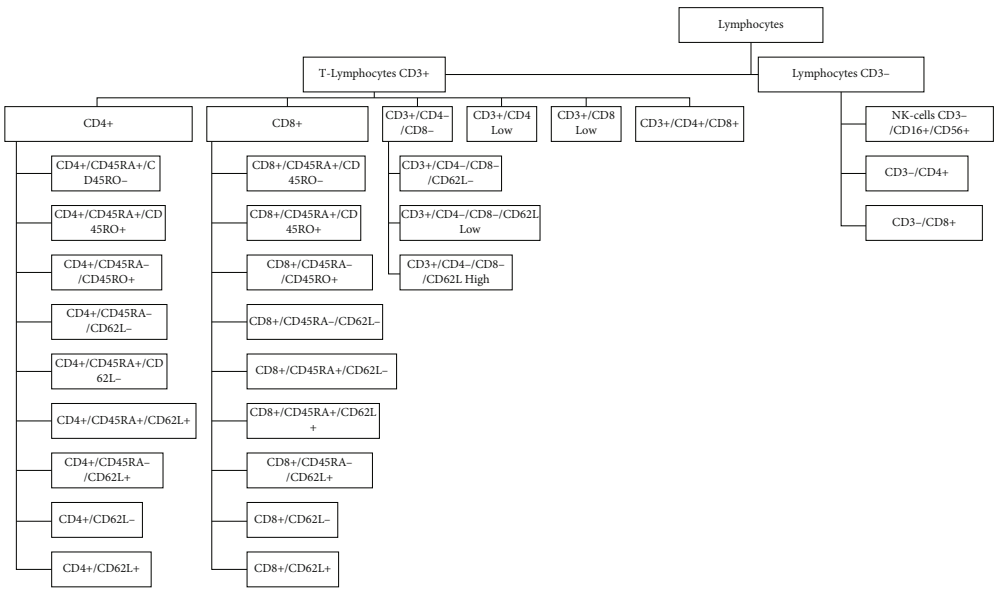
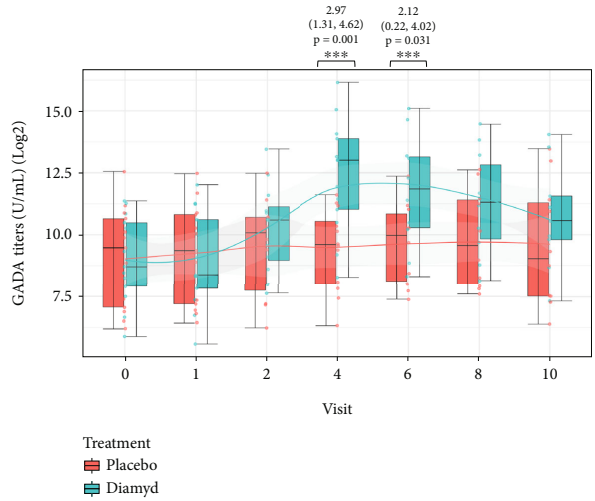


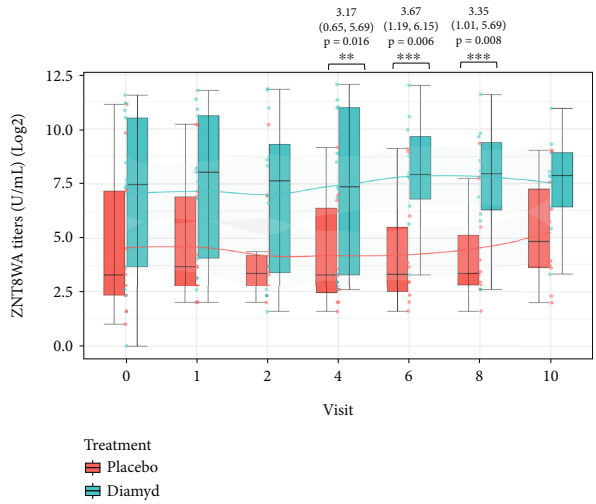
Figure 3: All the immunophenotyped lymphocyte and T-lymphocyte subpopulations.

Doublets were discriminated from singlets by plotting FSC Area against FSC Height in the acquisition analysis plot.

Acquired samples with FACSCalibur were analyzed using the CellQuest software program (Becton-Dickinson, Stockholm, Sweden), and the FACS data were later analyzed with Kaluza Analysis Software 1.5a (Beckman Coulter). For the FACS analysis by the CytoFLEX, the CytExpert software (version 2.3) (Beckman Coulter) was used for acquiring cells and for analyzing the FACS data. All T-cell subtypes analyzed in the current study are presented in Figure 3. Gating strategies for certain sought different phenotyped T-lymphocyte subpopulations are presented in supplemental Figure 1. The flowcytometric analyzed data given in percent for each of the T-cell subpopulations are presented as



(a)



(b)

Figure 4: Cross-sectional analysis of (a) GADA titers and (b) ZNT8WA titers stratified by treatment (GAD65-alum (Diamyd)/placebo) presented as boxplots at study follow-up visits 0, 1, 2, 4, 6, 8, and 10. (a) GADA titers were higher in the GAD65-alum-treated group compared to placebo at visits 4 and 6. (b) ZnT8WA titers were higher in in the GAD65-alum-treated group compared to placebo at visits 4, 6, and 8. The estimates, 95% confidence intervals, and p values for those visits are provided in the figure.

absolute counts counted from the lymphocyte absolute count obtained from the CBC test to reflect the physiological counts in 10^3 cells/μl.

2.4. Statistical Analysis. To evaluate longitudinal trends in cell counts of each cell type in each treatment group, we plotted the observed CBC levels at each visit using boxplots and plotted a smoothed trend line through the mean CBC level for each treatment at each visit. To estimate the association between the treatment with GAD-alum and beta cell function as measured by OGTT (levels of glucose, C-peptide, and insulin) and IvGTT (FPIR and k value), as well as lymphocyte counts and counts of each of the T-cell subpopulations and autoantibody titers (GADA, IAA, IA2A,ZnT8(R/W/Q)A), we used a t-test to compare the levels of each of those measures in the group treated with GAD-alum with

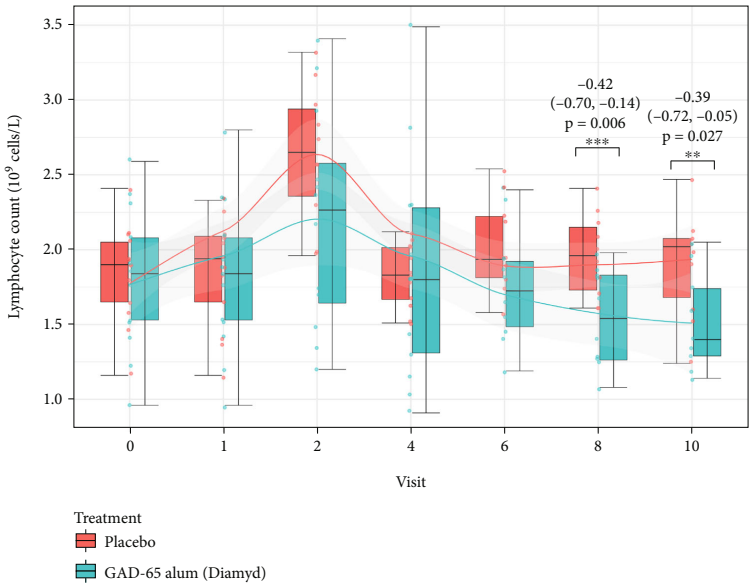


FIGURE 5: Cross-sectional analysis of blood lymphocyte counts stratified by treatment (GAD65-alum (Diamyd)/placebo) presented as boxplots at study follow-up visits 0, 1, 2, 4, 6, 8, and 10. Lymphocyte counts were lower in the GAD65-alum-treated group compared to placebo at visits 8 and 10. The estimates, 95% confidence intervals, and p values for those visits are provided in the figure, and complete results are presented in the Supplementary Table 1.

the placebo group at each visit (0 or 1, 2, 4, 6, 8, 10). Data were tested for normality prior to analysis. The reported p values are nominal and not adjusted for multiple comparisons. A p value <0.05 was considered marginally significant, and a p value <0.01 was considered significant. All statistical analyses were performed in *R* version 4.0.5 (R Core Team (2021). *R*: language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org.).

3. Results

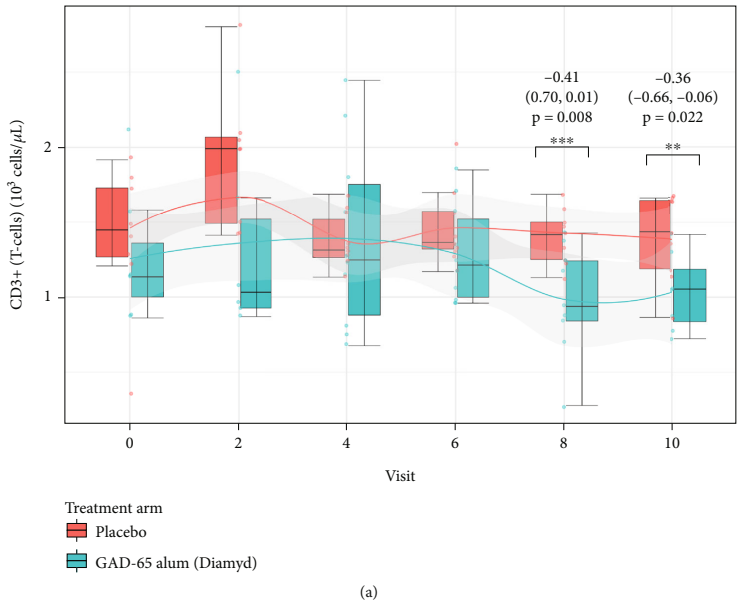
3.1. Higher GADA and ZnT8WA Titers Post-GAD-alum Treatment. The cross-sectional analysis revealed a significant increase of GADA titers in the GAD-alum-treated compared with the placebo-treated children at 6 (estimate = 2.97; 95% CI = 1.31, 4.62; $p = 0.001$) and 12 (estimate = 2.12; 95%CI = 0.22, 4.02, $p = 0.031$) months following the two doses of GAD-alum injections (Figure 4(a)).

Higher ZnT8WA titers were also found in the GAD-alum treated group 6 (estimate = 3.17; 95%CI = 0.65, 5.69; $p = 0.016$), 12 (estimate = 3.67; 95%CI = 1.19, 6.15; $p = 0.006$), and 18 (estimate = 3.35; 95%CI = 1.01, 5.69; $p = 0.008$) months (visit 4, 6, and 8) posttreatment (Figure 4(b)).

Comparable titers of each of IAA, IA2A, ZnT8RA, and ZnT8QA were found between the GAD-alum and the placebo-treated children throughout the 24 months of follow-up (data not presented).

3.2. Lower Lymphocyte Counts in the GAD-alum-Treated Group. Lymphocyte counts estimated from the CBC analysis were lower in the GAD-alum treated group compared with the placebo-treated group after 18 (visit 8) and 24 (visit 10) months of follow-up, (10^9 cells/L) (estimate = −0.42; 95 %CI = −0.70, -0.14; $p = 0.006$, estimate = −0.39; 95%CI = −0.72, -0.05; $p = 0.027$, respectively) (Figure 5, complete results are presented in Supplementary Table 1). We did not find any difference in the cell counts of leukocytes, neutrophils, monocytes, eosinophils, and basophils between the GAD-alum and placebo-treated groups (data not shown).

3.3. Lower Levels of CD3+ T-Cells and T-Cell Subpopulations in the GAD-alum-Treated Group. The frequencies of CD3+ T-cells and different T-cell subpopulations in peripheral whole blood were calculated from the lymphocyte count to peripheral blood counts (10^3 cells/μL). The cross-sectional analysis stratified by type of treatment (GAD-alum or placebo), done in order to evaluate whether GAD-alum treatment was associated with different T-cell subpopulation levels at visits 0, 1, 2, 4, 6, 8, and 10 during 24 months of follow-up, resulted in lower levels of T-cells and specific T-cell subpopulations in the GAD-alum-treated group during the last 18 and 24 months of follow-up compared to the placebo-treated group (Figures 6 and 7) (complete results



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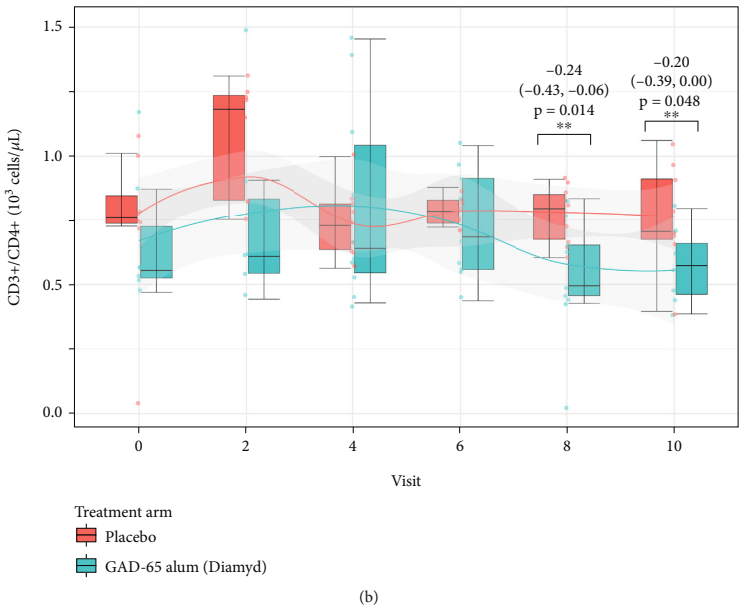

(b)

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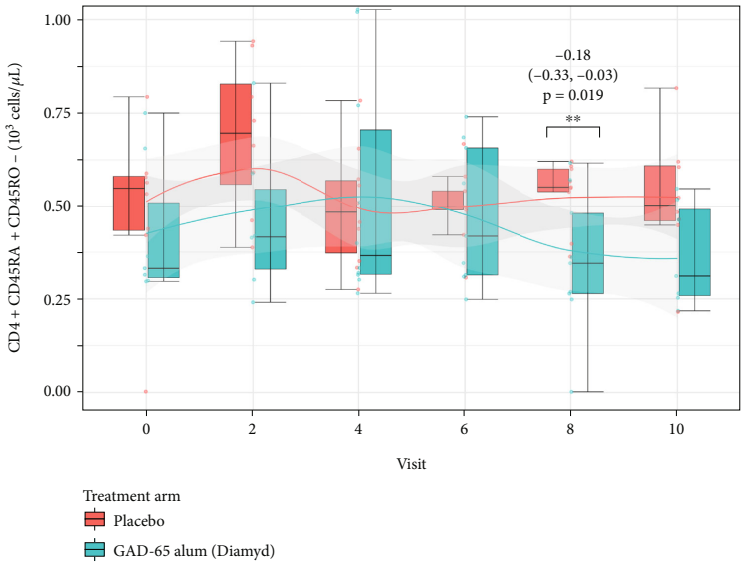

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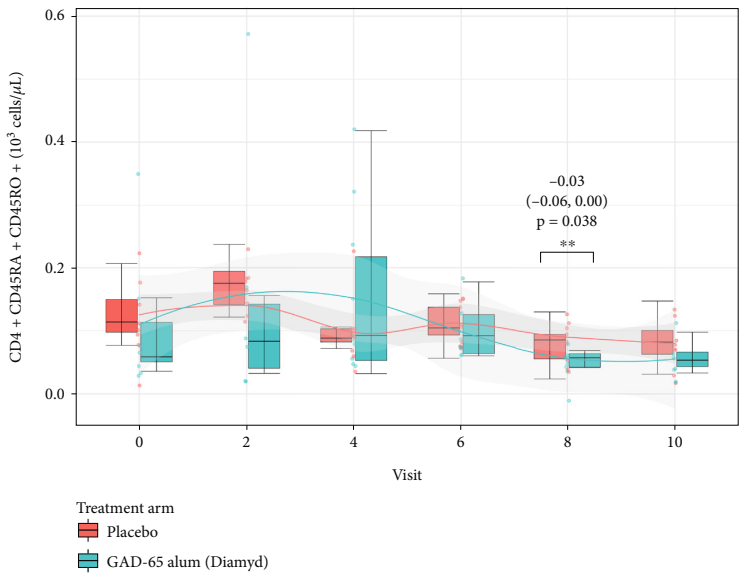

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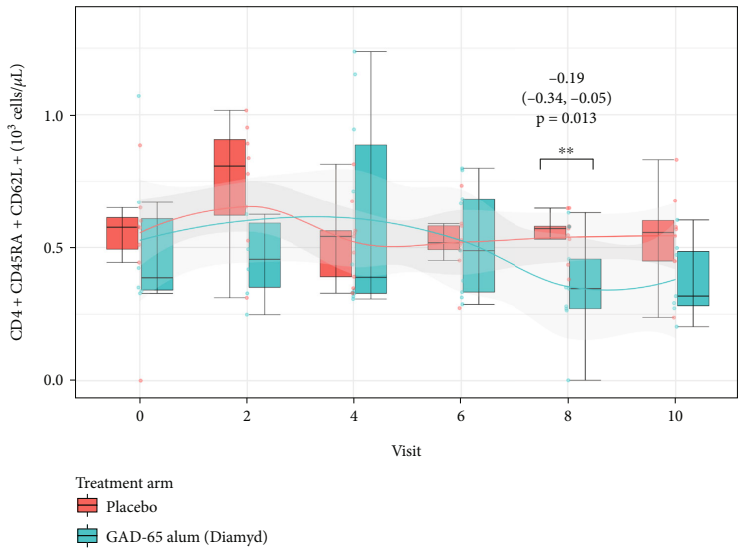

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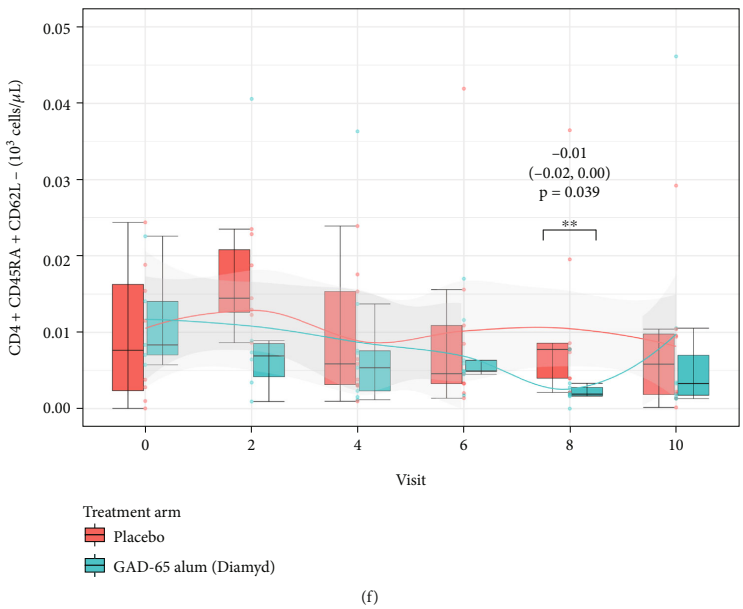

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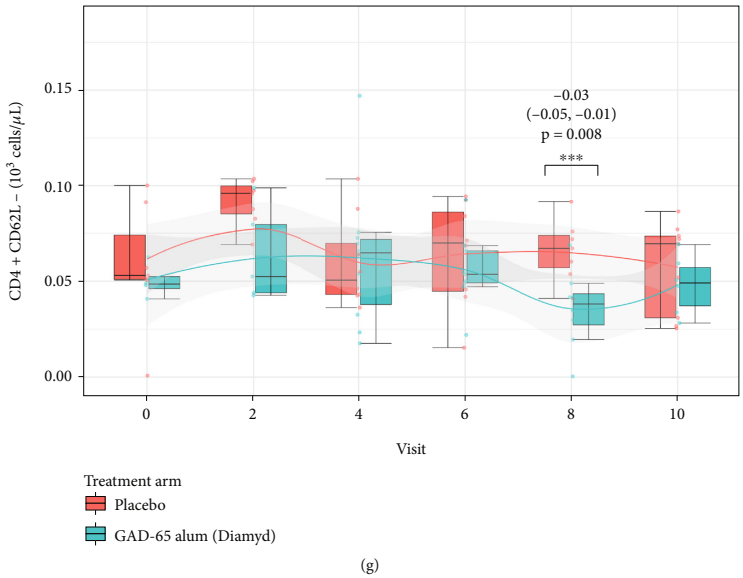

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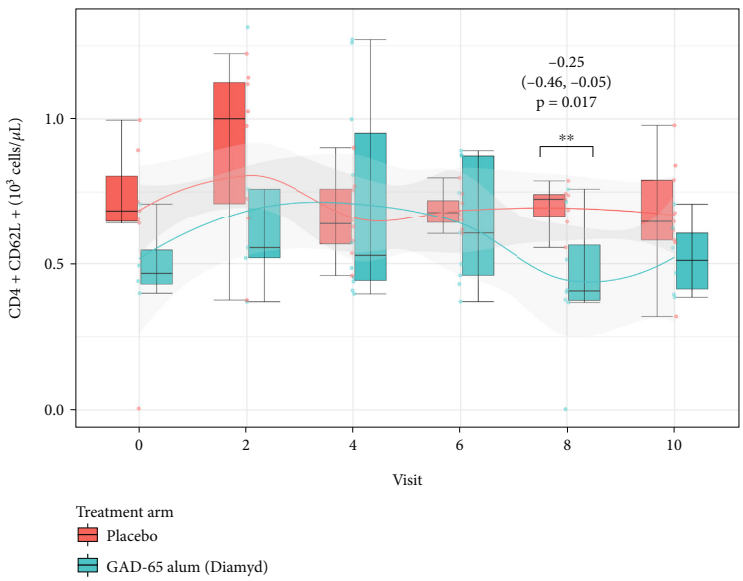
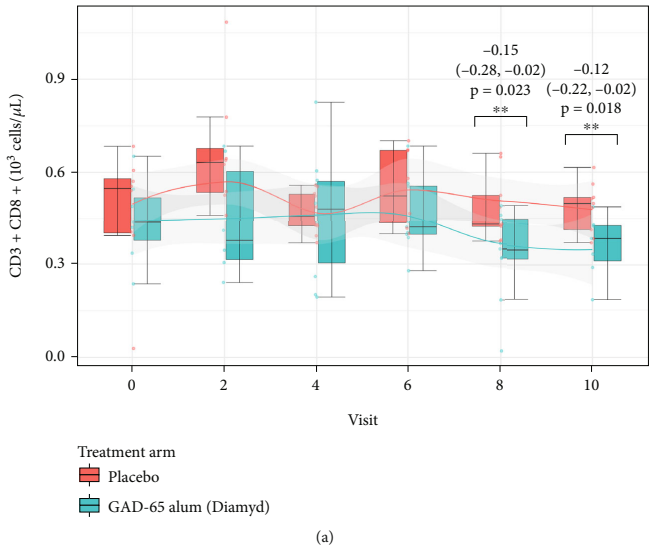
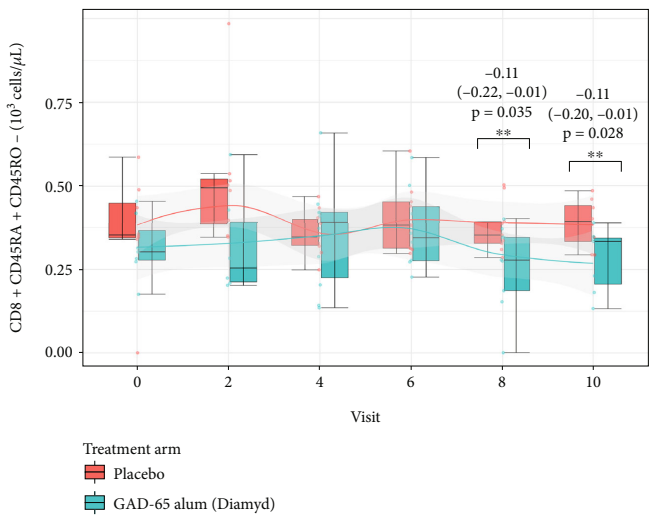


(h)

Figure 6: Cross-sectional analysis of T-cell counts and counts of seven subpopulations of CD4+ T-cells stratified by treatment (GAD65-alum (Diamyd)/placebo) presented as boxplots at study follow-up visits 0, 2, 4, 6, 8, and 10. Children treated with GAD65-alum had statistically significantly lower cell counts of (a) CD3+ T-cells at visits 8 and 10, (b) CD3 + CD4+ T-helper cells at visits 8 and 10, (c) CD4 + CD45RA + CD45RO-naïve T-helper cells at visit 8, (d) CD4+ CD45RA+ CD45RO+ double positive intermediate T-helper cells at visit 8, (e) CD4 + CD45RA + CD62L+ naïve T-helper cells at visit 8, (f) CD4 + CD45RA + CD62L-terminally differentiated effector memory T-helper cells at visit 8, (g) CD4 + CD62L-effector memory T-helper cells at visit 8, and (h) CD4 + CD62L+ naïve T-helper cells at visit 8 compared to the placebo group. The estimates, 95% confidence intervals, and p values for these results are provided in the figure, and complete results are presented in the Supplementary Table 2.



(a)



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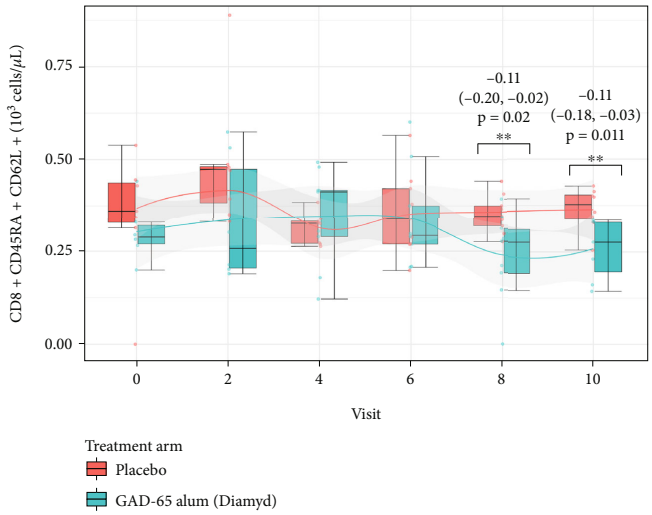

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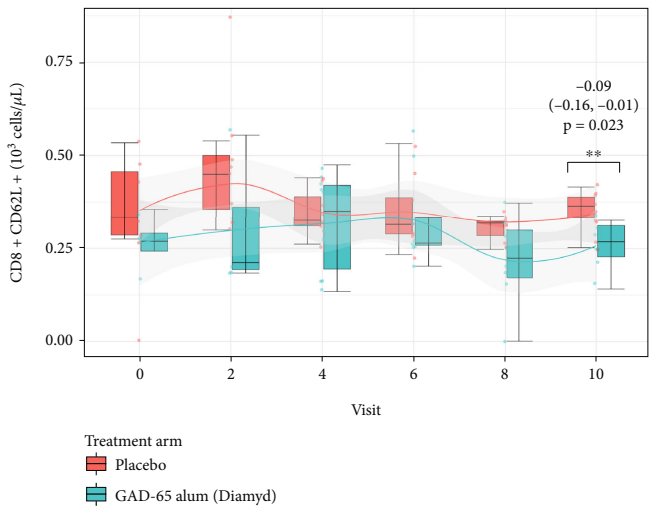

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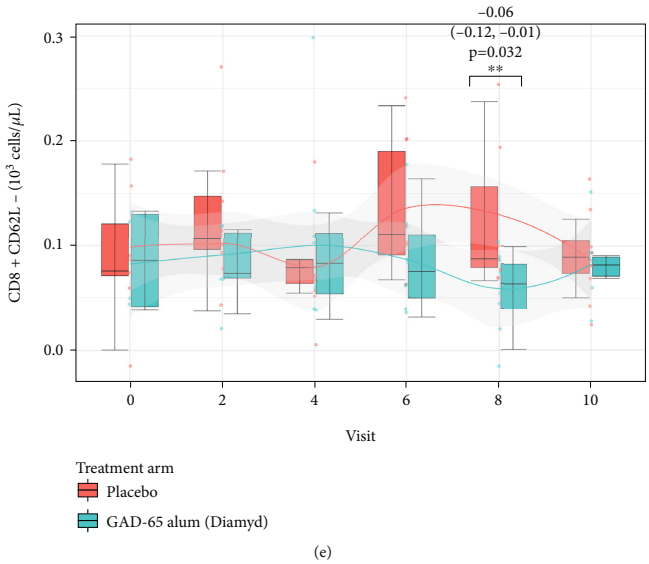

(e)

Figure 7: Cross-sectional analysis of counts of five subpopulations of CD8+ cytotoxic T-cell stratified by treatment (GAD65-alum (Diamyd)/placebo) presented using boxplots at study follow-up visits 0, 2, 4, 6, 8, and 10. Children treated with GAD65-alum had statistically significantly lower cell counts of (a) CD3 + CD8+ cytotoxic T-cells at visits 8 and 10, (b) CD8 + CD45RA + CD45RO-naïve cytotoxic T-cells at visits 8 and 10, (c) CD8 + CD45RA + CD62L+ naïve cytotoxic T-cells at visits 8 and 10, (d) CD8 + CD62L+ naïve cytotoxic T-cells at visit 10, and (e) CD8 + CD62L-effector memory cytotoxic T-cells at visit 8 compared to the placebo group. The estimates, 95% confidence intervals, and p values for these results are provided in the figure, and complete results are presented in the Supplementary Table 2.

presented in supplementary Table 2). The significant association between GAD-alum treatment and lower counts of different T-cell subpopulations compared to placebo treatment is summarized in Table 2. Treatment with GAD-alum was associated with lower levels of T-cells (CD3+) at 18 and 24 months after first dose of treatment, due to lower levels of T-helper cells (CD3+/CD4+), cytotoxic T-cells (CD3+/CD8+), naïve cytotoxic T-cells (CD8+ CD45RA+ CD45RO, CD8 + CD45RA + CD62L+) 18 and 24 months after first dose of treatment, naïve T-helper cells (CD4 + CD45RA + CD45RO-, CD4 + CD62L+), CD4+ CD45RA+ CD45RO+ double positive T-helper cells, central memory T-helper cells (CD4 + CD45RA + CD62L+), terminally differentiated effector memory T-cells (CD4 + CD45RA + CD62L-), effector memory T-helper cells (CD4 + CD62L-), effector memory cytotoxic T-cell (CD8 + CD62L-) 18 months after first dose of treatment, and naïve cytotoxic T-cells (CD8 + CD62L+) 24 months after first dose of treatment.

No statistically significant association between GAD-alum treatment and measurements reflecting beta cell function from OGTT or IVGTT was found (data not shown).

4. Discussion

The main result in this study is the long-term effect of GAD-alum associated with lower levels of T-cells, T-helper cells (CD3 + CD4+), and cytotoxic T-cells (CD3 + CD8+) together with other subgroups of both naïve and effector memory cells detected 18 and 24 months after subcutaneous prime and boost GAD-alum treatment in nondiabetic children positive for multiple beta cell autoantibodies. The rationale of an intravenous GAD-alum vaccine combined was to restore both central and peripheral immune tolerance towards GAD as a self-antigen. The reduced numbers of T-cells are likely to be related to the presence of GAD65 mixed with alum and not alum itself as it was given to the placebo group. Aluminum hydroxides enhance the adaptive immune response by the activation of innate immune cells. However, the exact mechanism by which aluminum hydroxide enhance the immune response remains poorly understood [22].

In accordance with previous studies, GADA titers increased upon GAD-alum treatment at visits 6 and 12 as a result of immunization to decrease thereafter. However, the increased ZnT8WA titers that lasted for several months after immunization may be deceptive since there was difference in the titers between the GAD-alum and the placebo groups already at baseline.

Antigen specific immune therapy with GAD-alum is simple, proved to be well-tolerated and safe among children with or without T1D. Recent results from a multicenter placebo-controlled study with intralymphatic GAD-alum treatment have shown preserved C-peptide up to 15 months

Table 2: Cross-sectional analysis stratified by treatment (GAD-alum/placebo) evaluating whether GAD-alum treatment is associated with different T-cell subpopulations during study follow-up.

Phenotyped T-cell populations	[1]Visit	[2]Estimate	95% CI	p value
T-cells CD3+	8	-0.41	-0.7, -0.12	0.008
	10	-0.36	-0.66, -0.06	0.022
CD3 + CD4+	8	-0.24	-0.43, -0.06	0.014
	10	-0.20	-0.39, 0	0.048
CD4+ CD45RA+ CD45RO-	8	-0.18	-0.33, -0.03	0.019
CD4+ CD45RA+ CD45RO+	8	-0.03	-0.06, 0	0.038
CD4 + CD45RA + CD62L+	8	-0.19	-0.34, -0.05	0.013
CD4 + CD45RA + CD62L-	8	-0.01	-0.02, 0	0.039
CD4 + CD62L+	8	-0.25	-0.46, -0.05	0.017
CD4 + CD62L-	8	-0.03	-0.05, -0.01	0.008
CD3 + CD8+	8	-0.15	-0.28, -0.02	0.023
	10	-0.12	-0.22, -0.02	0.018
CD8+ CD45RA+ CD45RO-	8	-0.11	-0.22, -0.01	0.035
	10	-0.11	-0.20, -0.01	0.028
CD8 + CD45RA + CD62L+	8	-0.11	-0.2, -0.02	0.020
	10	-0.11	-0.18, -0.03	0.011
CD8 + CD62L+	10	-0.09	-0.16, -0.01	0.023
CD8 + CD62L-	8	-0.06	-0.12, -0.01	0.032

[1]Visits 8 and 10 equal 18, respectively, 24 months of follow-up after first treatment dose. [2]Estimated difference between GAD-alum-treated children and placebo-treated children. 95% CI: 95% confidence interval.

in 12-24 years old patients with recent onset of T1D carrying the HLA DR3-DQ2 haplotype [23]. However, the treatment efficacy remains to be debated. The aluminum hydroxide adjuvant in the GAD-alum vaccine was chosen to skew the immune cells against a Th2 anti-inflammatory response upon costimulation with GAD avoiding a Th1 proinflammatory autoreactive response [24]. However, it was reported recently that GAD-alum was capable of inducing both a Th1 and a Th2 response [13]. Considering that only a fraction of beta cells would be preserved at T1D onset makes it important to intervene with GAD-alum immune tolerance treatment before T1D diagnosis at stages 1 and 2 in T1D progression to preserve beta cell function or delay onset by halting the T-cell mediated autoimmune process. To understand and further improve future studies with GAD-alum treatment, it is of great importance to increase the knowledge about the immunomodulatory effects of GAD-alum on T-cells [25]. Hence, the current study was aimed at investigating whether GAD-alum treatment in nondiabetic children positive for GADA and at least one more autoantibody at stage 1 or 2 was associated with T-lymphocyte and different T-cell subgroup levels during the follow-up of the DiAPREV-IT 2 study. Consistent with other studies, a long-lasting effect of GAD-alum on T-cells has previously been reported in GAD-alum-treated children, all diagnosed with T1D [26]. To our knowledge, no investigator has so far studied the immunomodulatory effects of GAD-alum in nondiabetic children positive for multiple beta cell autoantibodies. Nevertheless, immunomodulatory effects of GAD-alum have been frequently studied in children with T1D upon in vitro stimulation with GAD [10, 11]. A recent, relatively limited study indicated that the immune response differs between intralymphatic administration and subcutaneous administration of GAD-alum in individuals with recent onset of type 1 diabetes 15 months after the administration. The intralymphatic administration of smaller amounts of GAD-alum had better preservation of C-peptide, better increment of GADA, stronger immune responses, and reduced GAD-65 stimulated cytotoxic CD8+ and CD4+ T-helper central memory cells, which could be a sign of tolerance [27].

The main strength of this study is that all subjects included were nondiabetic children with multiple beta cell autoantibodies. Thus, these participants are candidates to benefit from a potential prevention treatment aimed at retaining remaining beta cell function and delaying the onset of T1D. Importantly, this is the first study investigating the long-term association between a treatment with GAD-alum and levels of T-cells in nondiabetic children positive for multiple beta cell autoantibodies. The major limitation on this study is the limited number of study participants.

Due to the inconclusive preventive efficacy of GAD-alum in DiAPREV-IT 1 [9] reported during the enrolment process in DiAPREV-IT 2, only the 26 already enrolled children out of 80 specified in the original study design were followed until the planned end of the study (24 months). During the enrollment and before the result of DiAPREV-IT 1 was revealed, a new flow cytometer was installed to replace the older device, and a new antibody panel was constructed with more cell subtypes and with some antibodies that differed from the first panel. Due to the difference between the panels, only results from the two flow cytometers with identical monoclonal antibodies could be used.

Even though efficacy assessment of GAD-alum failed, one explanation for the lower levels of T-cells, both CD4+ T-helper, and CD8+ cytotoxic T-cells presented in this study could be due to a minor T-cell exhaustion caused by the high GAD antigen exposure that in turn could emphasize a peripheral tolerance [28, 29]. Another explanation may indicate a hitherto unknown immunosuppressive effect of GAD-alum which needs to be further investigated.

5. Conclusion

In summary, an immune tolerance treatment with GAD-alum was associated with lower levels of lymphocytes, T-cells, T-helper (CD3 + CD4+), cytotoxic T-cells (CD3 + CD8+), and several T-cell subgroups of naïve and effector memory cells, at 18-24 months after receiving the 1st dose of treatment. We consider our results as hypothesis-generating and in need of further studies due to the small number of enrolled children. The long-term impact of GAD-alum on T-cells suggests a persistent effect, at least over a 2-year period, that warrants further investigation to improve the efficacy and safety of GAD-alum as a potential treatment for delaying, and possibly preventing, the onset of T1D.

Abbreviations

GADA:	Glutamic acid decarboxylase autoantibody
IA:	Insulin autoantibody
IA-2A:	Insulinoma antigen-2 autoantibody
ZnT8A:	Zinc transporter 8 autoantibody
GAD65:	Glutamic acid decarboxylase 65
GAD-alum:	Recombinant GAD65 conjugated to aluminum hydroxide
T1D:	Type 1 diabetes
HLA:	Human leukocyte antigen
Th1:	T-helper 1
Th2:	T-helper 2
DiAPREV-IT 2:	Diabetes Prevention–Immune Tolerance 2
OGTT:	Oral glucose tolerance test
IVGTT:	Intravenous glucose tolerance test
FPIR:	First-phase insulin response
TEDDY:	The Environmental Determinants of Diabetes in the Young
DiPiS:	Diabetes Prediction in Skåne
FDR:	First degree relatives
RBA:	Radioligand binding assay
CBC:	Complete blood count
FITC:	Fluorescein isothiocyanate
PE:	Phycoerythrin
PerCP:	Peridinin-chlorophyll-protein
APC:	Allophycocyanin
PE-CY 7:	PE-Cyanine 7
BB515:	Brilliant™ Blue 515
FMO:	Fluorescence minus one.

Data Availability

All data in this manuscript are available from the authors upon request.

Ethical Approval

The Diabetes Prevention–Immune Tolerance 2 (DiAPREV-IT 2) study is registered with the trial number NCT02387164. This study was approved by the Regional Ethical Review Board in Lund and the Swedish Medical Product Agency and carried out in accordance with the Declaration of Helsinki. The investigator obtained a signed informed consent from all subjects (and/or their parents/caregivers when applicable) prior to any study procedure was undertaken.

Conflicts of Interest

No potential conflicts of interest relevant to this article were reported. The study drug was supplied free of charge by Diamyd Medical AB.

Authors' Contributions

All authors have read and approved the final manuscript. F. S constructed and performed experiments, analyzed, and interpreted data and wrote the manuscript. L.S. performed statistical analyses. M.M. performed statistical analyses, reviewed, and edited the manuscript. M.L. contributed to the study design. C.B. and R.B. performed experiments and reviewed the manuscript. C.T. reviewed and edited the manuscript. M.H. contributed to the Method section. H.E.L. designed the study, reviewed, and edited the manuscript. H.E.L. is the guarantor of this study and as such had access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Supplementary Materials

Supplementary Table 1: cross-sectional analysis stratified by treatment (GAD-alum/placebo) evaluating whether GAD-alum treatment is associated with lymphocyte complete count during study follow-up. Supplementary Table 2: cross-sectional analysis stratified by treatment (GAD-alum/placebo) evaluating whether GAD-alum treatment is associated with different T-cell subpopulations during study follow-up. Supplementary Figure 1: the gating strategy is presented for the following T-cell populations, lymphocytes, T-lymphocytes, singlets of cells, T-cells (CD3+), T-helper cells (CD3 + CD4+), cytotoxic T-cells (CD3 + CD8+), CD4 + CD62L-, CD4 + CD62L+, CD8 + CD62L-, CD8 + CD62L+, CD4 + CD45RA + CD45RO-, CD4 + CD45RA + CD45RO+, CD4+ CD45RO + CD45RA-, CD8 + CD45RA + CD45RO-, CD8 + CD45RA + CD45RO+,

CD8 CD45RO + CD45RA-, CD4 + CD45RA + CD62L-, CD4 + CD45RA + CD62L+, CD4 + CD45RA-CD62L+, CD8 + CD45RA + CD62L-, CD8 + CD45RA + CD62L+, and CD8 + CD45RA-CD62L. *(Supplementary Materials)*

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Falastin Salami, MSc in molecular biology from Lund University, has worked in both national (DiAPREV-IT 2 and DiPiS) and international (TEDDY, TEFA, and GPPAD) prospective longitudinal studies to follow children at increased risk for type 1 diabetes. The aim was to study factors that predict or prevent type 1 diabetes onset. This thesis focuses on cellular biomarkers that may predict either autoimmunity or the progression to type 1 diabetes onset in children at increased genetic risk and the longitudinally measured effect of antigen-specific immune therapy with GAD-alum on peripheral blood T-cells.

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