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HUMORAL BETA-CELL AUTOIMMUNITY, AGER GENE POLYMORPHISM, AND CIRCULATING SOLUBLE RAGE IN PRE-CLINICAL AND CLINICAL TYPE 1 DIABETES

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ACADEMIC DISSERTATION

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ABSTRACT

Type 1 diabetes is a disease characterized by a preclinical phase with autoimmunity against beta cells of the pancreas and eventually hyperglycemia and dependence on external insulin treatment. Finland has the highest annual incidence of the disease worldwide. Incidence has been increasing over the decades at an accelerated rate, although the incidence rates from the past few years suggest that a peak may have been reached. The reason for the increase is unknown. Children and adolescents with significantly increased risk for type 1 diabetes can be recognized from the general population based on HLA-genotypes associated with increased risk for type 1 diabetes and using a panel of beta-cell autoantigen-specific antibody assays. However, the initial trigger for the autoimmune process remains obscure. Recently, a novel autoantibody specificity was discovered, the zinc transporter 8 antibodies (ZnT8A).

The advanced glycation end products (AGEs) are produced by a non-enzymatic glycation reaction between reducing sugars and proteins or lipids. AGEs and their interaction with the receptor molecule, receptor for advanced glycation end products (RAGE), have been suggested to play a role in the pathogenesis and complications of both type 1 and type 2 diabetes. The soluble form of RAGE (sRAGE) counteracts the effects of the proinflammatory membrane-bound RAGE. The circulating concentration of sRAGE is associated with various disease states. An initial low sRAGE might be a risk factor for inflammatory and metabolic disease, and a decrease in sRAGE has been observed in various studies on acute inflammation, both autoimmune and caused by other factors. In contrast, higher than normal sRAGE concentrations have also been observed in pathological states, especially in diabetic populations and subjects with compromised renal function. Polymorphisms of the gene encoding RAGE, *AGER*, are associated with the risk for diabetes and the complications of diabetes according to numerous studies.

The first study of this thesis focused on the role of ZnT8A positivity in the phenotype and the prevalence of ZnT8A in newly diagnosed patients with type 1 diabetes. In concordance with previous studies, 63% of newly diagnosed Finnish children and adolescents with type 1 diabetes had ZnT8A. Positivity for ZnT8A was related to age and metabolic state at diagnosis as well as to HLA genotype. ZnT8A-assay did not significantly improve the detection rate of beta-cell autoimmunity in the current study population when used in addition to previously available autoantibody analyses since only 0.3% of the subjects had ZnT8A as their single autoantibody.

The objective of Studies II, III, and IV was to identify possible roles for RAGE and AGEs in the clinical phenotype of newly diagnosed patients with type 1 diabetes and the emergence of humoral autoimmunity against islet cell antigens during the preclinical phase. In the second publication, we defined the relationship between humoral beta-cell autoimmunity, HLA-genotype, state of metabolic compensation, polymorphisms of the AGER gene, and concentrations of sRAGE in 2115 children with newly diagnosed type 1 diabetes. Children who did not have type 1 diabetes and tested negative for beta-cell autoantibodies were used as controls. In the third part of the thesis, we analyzed serum concentrations and sRAGE from 114 children who progressed to type 1 diabetes during prospective observation in the Diabetes Prediction and Prevention study (DIPP-study). Concentrations of an abundant AGE, carboxymethyllysine (CML), were analyzed as well. We assessed the dynamics of sRAGE during the disease process leading to type 1 diabetes also in the fourth publication by measuring sRAGE before and after the appearance of beta-cell-specific autoantibodies and annually after the seroconversion, until the diagnosis or end of follow-up. In Study IV, the study population included 211 children with permanent positivity for at least two autoantibodies.

The results of this thesis suggest that AGEs and RAGE seem to have a role in the pathogenesis of type 1 diabetes. Concentrations of soluble RAGE are positively associated with age in children with newly diagnosed type 1 diabetes but not in the control population. The aggressiveness of humoral beta-cell autoimmunity does not correlate with sRAGE concentrations, but the state of metabolic decompensation at diagnosis and the HLA-genotype associated with the highest risk of type 1 diabetes, the DR3/DR4 heterozygosity, were related to lower sRAGE concentrations. Interestingly, two polymorphic variants of the *AGER* gene associated with increased risk for type 1 diabetes were associated with lower levels of sRAGE as well.

Prediabetic children seem to have higher circulating concentrations of sRAGE than autoantibody-negative controls. A reduction in the circulating sRAGE concentrations coincides with the appearance of diabetes-predictive autoantibodies in children progressing to overt type 1 diabetes, but not in healthy controls. We could not see a similar drop in sRAGE in children seroconverting to autoantibody positivity later in childhood. After the seroconversion, the sRAGE concentrations remained stable in both groups. The CML concentrations were similar between cases and controls in the samples taken before the appearance of autoantibodies against beta cells in the prediabetic children, but the RAGE/AGE ratio was higher in the cases than in the controls.

To conclude, sRAGE, which has been considered cytoprotective in previous studies, is positively associated with older age at disease onset,

protection from metabolic decompensation at diagnosis, and *AGER* genotypes with a lower risk for type 1 diabetes. Children who seroconvert to humoral islet cell autoimmunity early in childhood experience a drop in sRAGE concentration coinciding with the appearance of the first autoantibodies. They have higher sRAGE concentrations and sRAGE/AGE ratio than the controls before seroconversion. These observed associations might be a result of an intrinsic protective mechanism that fails at seroconversion.

Keywords: Type 1 diabetes, prediabetes, AGEs, RAGE, ZnT8A

TIIVISTELMÄ

Tyypin 1 diabetes on immuunivälitteinen sairaus, jossa haiman insuliinia tuottavien solujen tuhoutuminen johtaa kohonneeseen veren sokeripitoisuuteen ja elinikäiseen insuliinihoidon tarpeeseen. Suomessa tyypin 1 diabeteksen ilmaantuvuus on maailman korkeinta. Ilmaantuvuus on lisääntynyt viime vuosikymmeninä kiihtyvällä nopeudella, vaikkakin aivan viime vuosina uusien tapausten määrän kasvu suhteessa väestöön näyttää saavuttaneen huippunsa. Syytä sairauden ilmaantuvuuden lisääntymiselle ei tiedetä. Merkittävä osa lapsista ja nuorista, joilla on lisääntynyt riski sairastua tyypin 1 diabetekseen, voidaan tunnistaa tutkimalla heidän genotyyppinsä sairastumisriskiin liittyvien HLA-geenien suhteen ja määrittämällä insuliinia tuottaviin soluihin kohdistuvien autovasta-aineiden pitoisuudet. Sairastumisen laukaiseva tekijä ei kuitenkaan ole tiedossa. Aiemmin tunnistettujen vasta-aineiden lisäksi muutamia vuosia sitten löydettiin uusi, sairastumisriskiin liittyvä vasta-ainetyyppi, ZnT8-vasta-aineet (ZnT8A).

Glykosylaation kehittyneet lopputuotteet (englanniksi: advanced glycation end product, AGE) syntyvät sokereiden liittyessä valkuaisaineisiin ja rasvoihin glykaatioreaktiolla, jossa ei ole osallisena entsyymejä. AGEt ja niiden vuorovaikutus etenkin yhden reseptorimolekyylinsä, receptor for advanced glycation end products (RAGE), kanssa on ollut aktiivisen tutkimuksen kohteena mahdollisesta osallisuudestaan sekä tyypin 1 että tyypin 2 diabeteksen patogeneesiin ja komplikaatioihin. RAGEn liukoinen muoto, soluble RAGE (sRAGE) toimii tulehdusreaktiota edistävän solukalvoon sitoutuneen RAGEn vastavaikuttajana. Kiertävän sRAGEn pitoisuudet ovat yhteydessä useisiin sairaustiloihin. Ilmeisesti matala sRAGE-pitoisuus saattaa olla riskitekijä tulehduksellisille ja aineenvaihdunnallisille sairauksille. Aiemmissa tutkimuksissa on havaittu sRAGE-pitoisuuden laskua liittyen sekä autoimmuunivälitteiseen että muista syistä johtuvaan akuuttiin tulehdukseen. Myös keskimääräistä korkeampia sRAGE-pitoisuuksia on kuvattu sairauteen liittyen, etenkin diabetekseen ja munuaissairauksiin. Pistemutaatiot RAGEa koodittavassa geenissä ovat useiden tutkimusten perusteella yhteydessä diabetesriskiin ja sairauden komplikaatioihin.

Tämän väitöskirjan ensimmäisessä osatyössä selvitettiin ZnT8-vasta-aineiden esiintymistä 2115 vastasairastuneella lapsella ja nuorella, joilla on todettu tyypin 1 diabetes. ZnT8A esiintyvyys aineistossa oli 63%. ZnT8A-positiivisuus oli yhteydessä sairastumisikään, diabeettisen ketoasidoosin esiintymiseen diagnoosihetkellä ja HLA-genotyyppiin. ZnT8A-määritys ei lisännyt insuliinia tuottaviin soluihin kohdistuvan autoimmuniteetin

toteamisherkkyyttä, sillä vain 7 lapsella (0.3%) ZnT8A oli ainoa todettu vasta-ainetyppi.

Toisen, kolmannen ja neljännen osatyön tavoitteena oli selvittää RAGEn ja AGE:jen mahdollista yhteyttä vastasairastuneiden diabeetikoiden taudinkuvaan ja autovasta-aineiden ilmaantumiseen sairastumisprosessin aikana. Toisessa osatyössä tutkittiin RAGEa koodittavan *AGER*-geenin geneettisen polymorfismin ja liukoisen RAGEn pitoisuuden suhdetta insuliinia tuottaviin beetasoluihin kohdistuvan vasta-ainevälitteisen immuunivasteen ja diagnoosivaiheen taudinkuvan välillä 2115 lapsella ja nuorella, joilla on hiljattain todettu tyypin 1 diabetes. Tuloksia verrattiin 317 terveen kontrollin aineistoon.

Väitöskirjan kolmannessa osatyössä määritettiin liukoisen RAGEn ja keskeisen AGEn, carboxymethyllysine:n (CML), pitoisuudet 114 syntymästä alkaneen seurannan aikana diabetekseen sairastuneelta ja 114 iän, sukupuolen ja HLA-genotyypin suhteen kaltaistetulta, myös seurannassa mukana olleelta kontrollilapselta, joille ei ollut ilmaantunut autovasta-aineita. Neljännessä osatyössä selvitettiin myös liukoisen RAGEn pitoisuusvaihtelua esidiabeteksen aikana 211 vasta-ainepositiivisella lapsella, joista 168 oli sairastunut tyypin 1 diabetekseen seurannan aikana.

Väitöstutkimuksen tulosten perusteella AGE:t ja RAGE näyttäisivät olevan osallisina tyypin 1 diabeteksen patogeneesissa. Liukoisen RAGEn pitoisuudella oli positiivinen yhteys sairastumisikään, eli nuorimpina sairastuneilla oli matalimmat pitoisuudet. Myös ketoasidoosi diagnoosivaiheessa ja korkeaan diabetesriskiin liittyvä HLA-genotyyppi ennustivat matalaa liukoisen RAGEn pitoisuutta. Lisäksi kaksi RAGEa koodittavan *AGER*-geenin pistemutaatiota, joiden on todettu liittyvän lisääntyneeseen tyypin 1 diabetesriskiin, olivat yhteydessä alhaiseen liukoisen RAGEn pitoisuuteen.

Myöhemmin diabetekseen sairastuvilla lapsilla oli korkeammat liukoisen RAGEn pitoisuudet kuin autovasta-ainenegatiivisilla kontrollilapsilla, ennen diabetekseen liittyvien vasta-aineiden ilmaantumista. Liukoisen RAGEn pitoisuudessa todettiin laskua vasta-aineiden ilmaantumisen aikaan lapsilla, jotka sairastuivat tyypin 1 diabetekseen. Terveillä kontrolleilla ja lapsilla, joilla ensimmäiset autovasta-aineet todettiin varhaislapsuuden jälkeen, ei vastaavaa laskua todettu. Autovasta-aineiden ilmaantumisen jälkeen liukoisen RAGEn pitoisuudet pysyivät vakaina. CML-pitoisuuksissa ei ollut eroa sairastuneiden ja kontrollien välillä, mutta diabetekseen myöhemmin sairastuvilla lapsilla RAGE/AGE-suhde oli korkeampi ennen autovasta-aineiden ilmaantumista.

Yhteenvetona voidaan todeta, että korkea suojaavan liukoisen RAGEn pitoisuus oli yhteydessä myöhäisempään sairastumisikään tyypin 1 diabetekseen, vähäisempään ketoasidoosin esiintymiseen diagnoosivaiheessa ja niihin *AGER*-geenin alleeleihin, joihin ei liity lisääntynyttä riskiä sairastua tyypin 1 diabetekseen. Lapsilla, jotka sairastuivat tyypin 1 diabetekseen syntymästä alkaneen seurannan aikana, havaittiin laskua liukoisen RAGEn pitoisuudessa autovasta-aineiden ilmaantumisen aikoihin. Diabetekseen sairastuvilla lapsilla on korkeammat liukoisen RAGEn pitoisuudet ja RAGE/AGE-suhde kuin terveillä kontrolleilla ennen autovasta-aineiden ilmaantumista. Tämä saattaa olla merkki elimistön kompensatorisesta suojamekanismista, joka pettää autovasta-aineiden ilmaantuessa.

Avainsanat: tyypin 1 diabetes, esidiabetes, AGE, RAGE, ZnT8A

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Salonen KM, Ryhänen S, Härkönen T, Ilonen J, Knip M; Finnish Pediatric Diabetes Register. Autoantibodies against zinc transporter 8 are related to age, metabolic state and HLA DR genotype in children with newly diagnosed type 1 diabetes. *Diabetes/Metabolism Research and Reviews 29:646-54, 2013 Copyright © 2013 John Wiley & Sons, Ltd. This publication has been used with the permission of John Wiley & Sons, Ltd.*

II Salonen KM, Ryhänen SJ, Forbes JM, Härkönen T, Ilonen J, Laine AP, Groop PH, Knip M; Finnish Pediatric Diabetes Register. Circulating concentrations of soluble receptor for AGE are associated with age and *AGER* gene polymorphisms in children with newly diagnosed type 1 diabetes. *Diabetes Care 37:1975-81, 2014 American Diabetes Association, 2014. Copyright and all rights reserved. Material from this publication has been used with the permission of the American Diabetes Association.*

III X Salonen KM, Ryhänen SJ, Forbes JM, Borg DJ, Härkönen T, Ilonen J, Simell O, Veijola R, Groop PH, Knip M. Decrease in circulating concentrations of soluble receptors for advanced glycation end products at the time of seroconversion to autoantibody positivity in children with prediabetes. *Diabetes Care 38:665-70, 2015 American Diabetes Association, 2015. Copyright and all rights reserved. Material from this publication has been used with the permission of the American Diabetes Association.*

IV Salonen KM, Ryhänen SJ, Forbes JM, Härkönen T, Ilonen J, Simell O, Veijola R, Groop PH, Knip M. A drop in the circulating concentrations of soluble receptor for AGEs is associated with seroconversion to autoantibody positivity but not with subsequent progression to clinical disease in children *en route* to type 1 diabetes. *Submitted for publication.*

The publications are referred to in the text by their Roman numerals.

ABBREVIATIONS

aa	amino acid
AGE	advanced glycation end product
AGER	advanced glycation end product-specific receptor gene
CML	carboxymethyllysine
esRAGE	endogenous secretory receptor for advanced glycation end products
GADA	glutamic acid decarboxylase antibodies
HbA1c	glycated hemoglobin
HBMG1	high mobility group box 1
HLA	human leukocyte antigen
IA-2A	islet antigen 2 antibodies
IAA	insulin autoantibodies
ICA	islet cell antibodies
MG	methylglyoxal
mRNA	messenger ribonucleic acid
RAGE	receptor for advanced glycation end products
SNP	single-nucleotide polymorphism
sRAGE	soluble receptor for advanced glycation end products
ZnT8A	zinc transporter 8 antibodies

1 INTRODUCTION

Type 1 diabetes is an immune-mediated disease that usually manifests in childhood and adolescence in a substantial proportion of the affected patients. Finland is the country with the highest disease rate in the world, but the incidence has increased at an accelerated pace worldwide over the last decades (Patterson et al. 2014). In fact, the overall incidence rate of type 1 diabetes in Finnish children doubled between 1980 and 2005 (Harjutsalo, Sjöberg & Tuomilehto 2008). The exact reason for the increase in incidence and for the variation between populations with comparable known genetic risk factors is not known. Neither is there an undisputed explanation for the observed plateau in the incidence rate seen in Finland within the last few years. Around half of the genetic susceptibility to type 1 diabetes is linked to the HLA loci on the short arm of chromosome 6 (Concannon et al. 2005), while the other half is scattered to more than 50 non-HLA loci, the functions of which are not yet fully comprehended or described for all polymorphisms. Seroconversion to positivity for one or more diabetes-associated autoantibodies is perceived as an early biomarker of initiation of the disease process, but the duration of the preclinical asymptomatic phase ranges from a few months to more than 20 years (Knip et al. 2010a). However, recent research in subjects with prediabetes indicates that changes associated with the development of type 1 diabetes may be detectable even before the first autoantibodies are recognized (Mejia-Leon, Barca 2015). Environmental risk factors may potentially be operating already during the prenatal period (Atkinson, Eisenbarth & Michels 2014).

Viral infections represent an environmental factor with the most extensive evidence for a role in the pathogenesis of type 1 diabetes (Knip, Simell 2012). Nutrition is an obvious candidate for being an element modifying the disease risk as well (Virtanen, Knip 2003). A noteworthy new candidate for a dietary element contributing to the autoimmune attack against beta cells are the advanced glycation end products (AGEs) and their interaction with the receptor for AGEs (RAGE) (Yap et al. 2012). AGEs are involved in the risk for type 1 and type 2 diabetes and the complications of these diseases based on accumulating data (Goldin et al. 2006, Beyan et al. 2012). Genetics of RAGE and the dynamics of RAGE are also linked in vivo to inflammation and diabetes (Niu et al. 2012, Prasad 2014).

This thesis project was started to document the characteristics of autoimmunity against a novel islet cell autoantigen, the zinc transporter 8 (ZnT8), in a large series of newly diagnosed Finnish children and adolescents. The objective was to analyze how ZnT8 autoimmunity is connected to the phenotype at the onset of clinical disease. We also assessed

the relationship between humoral beta-cell autoimmunity, HLA-derived genetic risk for type 1 diabetes, demographic and phenotype data, and the RAGE gene polymorphisms as well as soluble RAGE concentrations in children with newly diagnosed type 1 diabetes. In addition, we studied the changes in soluble RAGE and AGE concentrations in the preclinical phase of diabetes from the time before emergence of signs of humoral autoimmunity to the diagnosis of diabetes.

2 REVIEW OF THE LITERATURE

2.1 INCIDENCE, ETIOLOGY, AND PATHOGENESIS OF TYPE 1 DIABETES

2.1.1 INCIDENCE OF TYPE 1 DIABETES

In international medical vocabulary, type 1 diabetes is designated as an immune-mediated disease characterized by selective destruction of the beta cells in the pancreatic islets of Langerhans. This process is influenced by both genetic and environmental factors, many of which remain unknown (Knip, Siljander 2008).

The incidence of type 1 diabetes has been on the rise in all Western countries and among Finnish children, in particular, during the past decades (Harjutsalo, Sjöberg & Tuomilehto 2008), and until recently the rate of the increase has been accelerating (Harjutsalo et al. 2013). The first nationwide study in Finland showed an incidence of 12/100 000 person-years in 1953, and in 1965, when the drug treatment for type 1 diabetes became free of charge in Finland, the incidence was just below 20/100 000 in children 14 years or younger (Tuomilehto et al. 1999).

Globally, there is a 100-fold variation in the incidence of type 1 diabetes (Karvonen et al. 2000). Finland has the highest annual incidence of type 1 diabetes in the world. More than 60/100 000 children under the age of 15 years are diagnosed with type 1 diabetes every year in Finland, and the highest incidence so far, 64.2/100 000 person-years, was reported in 2005 (Harjutsalo et al. 2013). The distribution of known genotypes associated with high risk for type 1 diabetes explains a relatively small part of the variation between populations (Rønningen et al. 2001). Both the frequency of the high-risk genotype and the risk of seroconversion to diabetes-associated autoantibody positivity show geographical variation even between regions in Finland (Kukko et al. 2004). The difference in seroconversion rate is not explained by the difference in genetic disease susceptibility, implying that the impact of other factors, such as environmental determinants, may differ between regions (Kukko et al. 2004). There are striking differences in the type 1 diabetes incidence between geographically proximal areas like Finland and Russian Karelia, which are not explained by differences in the HLA genotype distribution (Kondrashova et al. 2013). On the other hand, children born in high-incidence countries to parents who have immigrated from low-incidence countries have a higher risk for type 1 diabetes than children in the

country of origin of their parents or children immigrating to a high-incidence country during childhood (Söderström, Aman & Hjern 2012).

2.1.2 ETIOLOGY AND PATHOGENESIS OF TYPE 1 DIABETES

In the field of genetics, studies on the human leukocyte antigen (HLA) genes have provided the most comprehensive evidence for involvement in susceptibility to type 1 diabetes. The HLA region is a cluster of genes located within the major histocompatibility complex on chromosome 6p21. HLA class II gene alleles HLADQA1, DQB1, and DRB1 (Pociot, McDermott 2002) and the HLA class I allele HLA-B*39 (Nejentsev et al. 2007) confer predisposition to type 1 diabetes.

Approximately 40% of the genetic predisposition to type 1 diabetes can be attributed to allelic variation of HLA loci (Concannon et al. 2005). In a large Finnish study on twins, less than one-third of monozygotic twins were concordant for type 1 diabetes (Hyttinen et al. 2003), indicating that environmental factors also play an important role in the risk of developing the disease. Several environmental agents have been studied for their potential role in the etiology of type 1 diabetes. Distinct seasonal patterns of type 1 diabetes incidence have been documented (Padaiga et al. 1999). Prospective studies on children with increased genetic risk to type 1 diabetes have suggested that enteroviral infections may initiate the autoimmune process against beta cells, and that enteroviral infections often precede the appearance of islet autoantibodies (Salminen et al. 2003). Involvement of other viruses is not as evident (Hyöty, Taylor 2002, Aarnisalo et al. 2008), and there does not seem to be a correlation between childhood vaccinations and type 1 diabetes (Graves et al. 1999, Karvonen, Cepaitis & Tuomilehto 1999, Hviid et al. 2004, Morgan et al. 2016).

The impact of nutritional factors on the risk of autoimmune diabetes has been the focus of ongoing research since the 1940s. Breastfeeding, nicotinamide, zinc, and vitamins C, D, and E have been implicated as possible factors protecting against the disease, whereas N-nitroso compounds, cow's milk, increased linear growth, and obesity may increase the risk (Virtanen, Knip 2003). However, data on the significance of specific nutritional factors in the etiology of type 1 diabetes are scattered. For instance, children who later develop type 1 diabetes have an abnormal immune response to oral antigens, such as cow milk proteins, already early in infancy (Luopajärvi et al. 2008). Promising results of an attempt to reduce beta-cell autoimmunity with a dietary intervention were published a few years ago (Knip et al. 2010b). The investigators were able to detect a reduction in the signs of beta-cell autoimmunity in a population of children targeted with an early dietary intervention: weaning to an extensively

hydrolyzed casein formula instead of a cow's-milk-based formula. In contrast, in the first report of a larger trial with a similar design, partly by the same investigators, there was no difference in the cumulative incidence of autoantibodies against islet antigens by the age of 6 years between the two groups weaned to either hydrolyzed or normal cow's-milk-based formula (Knip et al. 2014).

The role of vitamin D in the pathogenesis of type 1 diabetes remains controversial. Vitamin D supplementation has been reported to be associated with protection from type 1 diabetes (Hyppönen et al. 2001). However, serum concentrations of vitamin D were similar in children progressing to type 1 diabetes during prospective follow-up and in matched controls in a recent Finnish study (Mäkinen et al. 2016). Low vitamin D concentration in mothers during pregnancy was associated with an increased risk of type 1 diabetes in offspring according to a Norwegian study (Sørensen et al. 2012), but no association could be detected in a Finnish study with a similar study design (Miettinen et al. 2012). Neither was there any association between type 1 diabetes in offspring and maternal intake of vitamin D during pregnancy (Marjamäki et al. 2010). The Finnish investigators speculated that the different results in the Norwegian and Finnish populations might reflect variation in the source of vitamin D (Niinistö et al. 2012), and maternal intake of n-3 polyunsaturated fatty acids could act as a confounding factor. Intake of n-3 fatty acids during childhood has been linked to the development of islet autoimmunity (Norris et al. 2007).

2.1.3 AUTOANTIBODIES AGAINST ISLET CELL AUTOANTIGENS

Clinical type 1 diabetes is preceded by a preclinical phase during which autoantibodies against a series of intracellular antigens present in the beta cells can be detected in the peripheral circulation (Knip, Siljander 2008). The first type 1 diabetes-associated autoantibodies recognized were the islet cell antibodies (ICA), representing a heterogeneous group of antibodies to several cytoplasmic islet cell antigens, demonstrated by immunofluorescence staining of pancreatic sections (Bottazzo, Florin-Christensen & Doniach 1974). Insulin autoantibodies (IAA) are often present before treatment with exogenous insulin is initiated and can be detected in 20–60% of patients with newly diagnosed type 1 diabetes (Palmer et al. 1983, Kimpimaki et al. 2001, Knip et al. 2002). Glutei acid decarboxylase antibodies (GADA) are frequent, especially in adult patients diagnosed with type 1 diabetes (Sabbah et al. 2000). The islet antigen 2 antibodies (IA-2A) are most prevalent in children and adolescents with recently diagnosed type 1 diabetes and in subjects carrying a strong genetic susceptibility for the disease (Knip et al. 2002). In combination with HLA testing, islet cell autoantibody assays can be used to identify subjects at an increased risk for type 1 diabetes (Kupila et al.

2001). Sustained positivity for multiple diabetes-associated autoantibodies is considered a pivotal sign of increased risk for disease progression, and approximately 70% of genetically susceptible children who tested positive for two or more autoantibodies progressed to clinical type 1 diabetes over a 10-year follow-up (Ziegler et al. 2013).

2.1.4 ZINC TRANSPORTER 8 ANTIBODIES (ZNT8A)

A fourth major autoantigen, the zinc transporter 8 (ZnT8), was discovered few years ago (Wenzlau et al. 2007). ZnT8 is intensely expressed in insulin granules of pancreatic beta cells (Chimienti et al. 2004) and is involved in both zinc accumulation and regulation of insulin secretion (Chimienti et al. 2006). A radiobinding assay targeting antibodies to ZnT8 detected the majority of subjects with newly diagnosed type 1 diabetes, but fewer than 2% of non-diabetic controls, 10% of patients with autoimmune disorders other than type 1 diabetes, and 3% of patients with type 2 diabetes tested positive for these autoantibodies (Wenzlau et al. 2007). Polymorphisms in the gene encoding the ZnT8 protein, SLC30A8, are associated with both increased and decreased risk for type 2 diabetes (Sladek et al. 2007, Flannick et al. 2014). In another study on ZnT8A-positive children, certain SLC30A8 polymorphisms were also associated with an increased risk of disease progression among subjects with preclinical diabetes (Achenbach et al. 2009). There are three known clinically important polymorphic amino acid variants in the carboxyl-terminal domain of ZnT8. The arginine and the tryptophane variants, which determine the autoantibody specificity to their corresponding isoepitopes, are the most prevalent (Wenzlau et al. 2008). Combined assays targeting multiple isotypes of ZnT8A have, however, been shown to have comparable sensitivity and specificity to assays analyzing each autoantibody variant separately (Vaziri-Sani et al. 2011).

2.1.5 CELL-MEDIATED BETA-CELL DESTRUCTION

The inflammation of pancreatic islet cells in type 1 diabetes was first described more than a century ago, but very few studies have characterized the lesions in patients with the disease or during the preclinical phase (Campbell-Thompson et al. 2013). Besides the autoantibodies produced by B-cells, T-cells are involved in the pathogenesis of type 1 diabetes. Autoreactive T-cells are detected in the circulation of patients with newly diagnosed type 1 diabetes, and therapies directed against T-cells have been shown to slow down the disease process and prevent or at least postpone the recurrence of beta-cell destruction after islet transplantation (Røep 2003). The initiation of the cell-mediated attack against beta cells is characterized by pathological mechanisms involving both proinflammatory and regulatory

T-cells (Campbell-Thompson et al. 2016). According to recent studies, the majority of patients with type 1 diabetes secretes minimal amounts of insulin and has functioning beta cells as well as signs of insulitis even many years after the diagnosis, which suggest that the destructive process is dynamic and continues after the appearance of clinical symptoms (Campbell-Thompson et al. 2016).

2.2 ADVANCED GLYCATION END PRODUCTS AND THEIR RECEPTOR

2.2.1 ADVANCED GLYCATION END PRODUCTS

French biochemist Louis-Camille Maillard reported in 1912 the first systematic studies on amino acids and reducing sugars undergoing complex reactions during heating, finally leading to the formation of brown substances (Hellwig, Henle 2014). The non-enzymatic browning of food is called the Maillard reaction in his honor. Further studies have led to extensive utilization of the information of the Maillard reaction in the food industry as well as in the field of medicine (Hellwig, Henle 2014). The advanced glycation end products (AGEs) are formed by non-enzymatic glycation reaction, and they are present in excess amounts in Western diets (Henle 2005). AGEs are also produced endogenously within the human body (Henle 2005). Specifically, proteins exposed to monosaccaharides containing an aldehyde group undergo non-enzymatic glycation and oxidation. Reversible Schiff bases and Amadori products are formed primarily. These molecules are exposed to further molecular rearrangements, which finally leads to the formation of irreversible AGEs (Schmidt et al. 1996). Structurally, AGEs are heterogeneous, have characteristic yellow-brown pigmentation, and have a tendency to form cross-links (Schmidt et al. 1992). Specifically, AGEs are created through a non-enzymatic reaction between reducing sugars and proteins, free amino groups, lipids, or nucleic acids (Uribarri et al. 2010). AGE formation becomes concrete and visible when foods turn progressively brown during heating (Henle 2005). AGEs are a novel candidate for a dietary element contributing to the autoimmune process leading to type 1 diabetes.

A novel variant of human hemoglobin was described in electrophoretic investigations of the blood in 1955. It was later designated as HbA1C, and in 1968, the association with diabetes mellitus was discovered (Hellwig, Henle 2014). The elucidation of the structure of HbA1C was the base of the investigations of the Maillard reaction in physiological systems. Further research led to the conclusion that the formation of AGEs is a physiological component of normal metabolism, but if the levels of AGEs in tissues and

circulation are excessive they can cause pathogenic phenomena (Uribarri et al. 2010). Some amount of AGEs is present in uncooked, especially animal-derived foods, and cooking, grilling, broiling, roasting, searing, and frying induce and accelerate new AGE formation within these foods (Goldberg et al. 2004). It is still somewhat unclear how AGE content of consumed food affects the AGE concentration in serum or the AGE burden in the whole body. A single AGE-rich meal can result in measurable elevation of serum AGE concentration (Koschinsky et al. 1997, Uribarri et al. 2007b) In animal models, a high AGE diet clearly correlates with serum AGE concentration (Cai et al. 2008), a finding also made in humans (Uribarri et al. 2007a). However, in another study where healthy adult subjects were randomized to a high-AGE or low-AGE diet for six weeks, no significant changes were present in AGE concentration in serum or urine in the high-AGE group, and only a modest decline was seen in the low-AGE group (Semba et al. 2014). In most studies, the best-characterized AGE compounds, carboxymethyllysine (CML), pentosidine, or derivatives of methylglyoxal (MG), serve as AGE markers (Uribarri et al. 2007a, Vlassara, Uribarri 2014).

Naturally occurring reducing sugars have different glycation rates. For instance, glucose is slow relative to fructose, glucose-6-phosphate, or glyceraldehyde-3-phosphate, which are present at low levels in cells, but can form AGEs at a faster rate (Suarez et al. 1989).

2.2.2 RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS, STRUCTURE AND SUBTYPES

AGEs can exert their biological effects by binding to tissues and engaging the receptor for advanced glycation end products (RAGE) (Vlassara, Brownlee & Cerami 1985, Schmidt et al. 1992, Schmidt et al. 1996). RAGE is a member of the immunoglobulin superfamily of cell surface molecules and a multi-ligand receptor involved in immune and inflammatory responses (Chavakis et al. 2003). The advanced glycation end product-specific receptor gene (AGER) encoding RAGE is located within the HLA class III region near the junction with the class II loci (Sugaya et al. 1994). RAGE shares structural homology with other immunoglobulin-like receptors (Chavakis et al. 2003). The AGER gene product is translated into a 404-amino acid (aa) -long protein composed of an extracellular domain (aa 1-342), a transmembrane helix (aa 343-363), and a cytosolic region (aa 364-404) (Hudson et al. 2008). RAGE forms oligomers at the cell surface, which is suggested to be essential for the pattern recognition function (Xie et al. 2008). The RAGE molecule comprises five distinct protein domains (**Figure 1**): an extracellular region composed of a signal peptide, three immunoglobulin-like domains, including a variable-like V-type domain containing the ligand binding site, and two constant-like C-type domains, including a single transmembrane domain and a short

cytoplasmic tail (Hudson et al. 2008). RAGE is highly expressed in many tissues during development, but in adult humans and animals high expression is limited to the lungs (Sparvero et al. 2009).

RAGE has several isoforms formed via alternative splicing of the RNA, including a soluble form called endogenous secretory RAGE (esRAGE) (Yonekura et al. 2003). The messenger RNA (mRNA) coding for esRAGE molecule is C-terminally truncated, whereas the N-terminally truncated, membrane-bound form of RAGE lacks the ability to bind AGEs. Other RAGE ligands exist as well (Yonekura et al. 2003). Hudson et al. (2008) discovered that there are actually a number of splice variants of the RAGE gene in cells and tissue, but the exact function and clinical relevance of all of these variants remains to be defined. However, most soluble RAGE (sRAGE) either produced by cell lines or in human blood, is derived from proteolytic cleavage of the membrane-bound molecules and is not recognized by an anti-esRAGE antibody (Raucci et al. 2008). The soluble forms of RAGE in vivo act as decoy receptors and reduce the interaction between membrane-bound RAGE and its ligands by competitively binding RAGE ligands from the circulation (Yap et al. 2012). These soluble forms of RAGE are considered cytoprotective since most of the known RAGE-mediated signaling pathways are proinflammatory (Stern et al. 2002, Maillard-Lefebvre et al. 2009, Jiang et al. 2015).

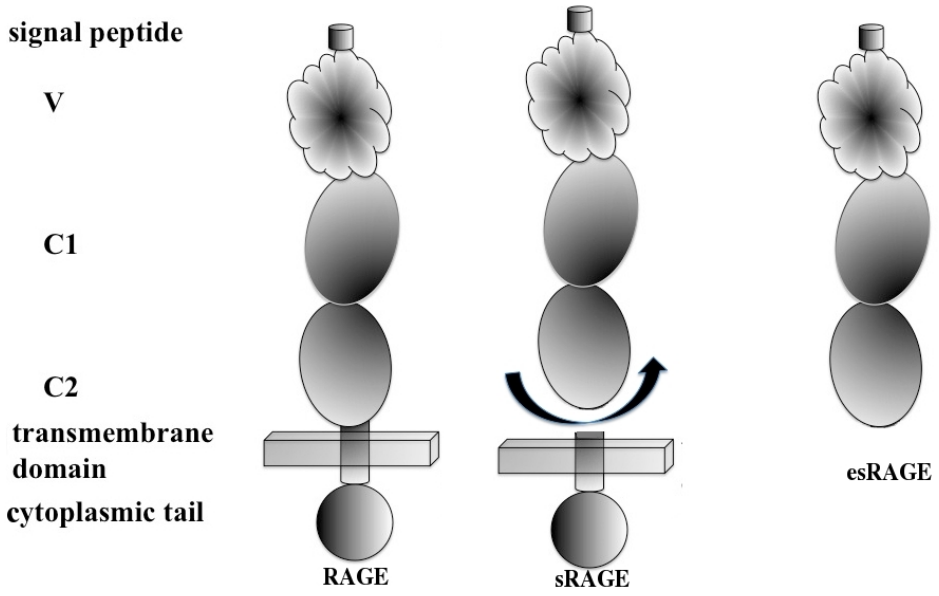

Figure 1. Schematic representation of the RAGE molecule. V = V-type domain, C1 = C1 domain, C2 = C2 domain. RAGE = full-length RAGE molecule, sRAGE = proteolytically cleaved sRAGE, esRAGE = esRAGE splice variant.

2.2.3 RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS IN HEALTH AND DISEASE

AGEs are associated with oxidative stress and inflammation, processes that play key roles in most chronic diseases, including cardiovascular disease, diabetes, chronic kidney disease, and neurodegenerative diseases (Uribarri et al. 2015). The relationship between AGEs and oxidative stress is a two-way street; AGEs induce oxidative stress, but oxidative stress leads to AGE formation as well (Vlassara, Uribarri 2014). There are two main mechanisms for AGEs to induce pathological phenomena. AGEs may cross-link proteins, directly altering their structure and function. AGEs also activate intracellular signals through various receptor-mediated and non-receptor-mediated mechanisms, which lead to oxidative stress and increased production of inflammatory cytokines (Vlassara, Uribarri 2014).

RAGE is highly expressed during development, especially in the brain, but its expression level decreases in adult tissues. In the pre- and postnatal nervous system of rodents, the expression of RAGE is active. RAGE co-localizes with amphoterin, alternatively named high mobility group box 1 (HMBG1) (Hori et al. 1995, Chou et al. 2004). Amphoterin-induced neurite growth in cell culture can be prevented via blocking RAGE/amphoterin interaction. This suggests that the effect of amphoterin in neuronal development is RAGE-mediated (Hori et al. 1995). RAGE is also expressed in the nervous system of human adults (Chen et al. 2007). To date, there are, however, few studies on the involvement of RAGE in physiological development of nervous systems and a wealth of research on RAGE and RAGE ligands in pathologies of the nervous system. Full-length RAGE is upregulated in Alzheimer´s disease, and the RAGE ligand amyloid β peptide is one of the main components of neuritic plaques integral to the pathology of the disease. RAGE has been shown to be essential for the transport of degenerative substances across the blood-brain barrier (Srikanth et al. 2011).

In healthy adult tissues, RAGE is usually expressed at a low basal level. Furthermore, upregulation of RAGE expression is associated with a variety of pathological conditions. However, RAGE is expressed at high basal levels in normal adult pulmonary tissue (Buckley, Ehrhardt 2010). Interestingly, the isoforms of RAGE molecule expressed in the lung appear to be different structurally and functionally than in all other tissues, implying that RAGE plays a unique role in the physiology of the lung (Gefter et al. 2009). Research on animal models of lung fibrosis as well as human studies have indicated a protective function for RAGE against pulmonary fibrosis (Englert et al. 2008, Queisser et al. 2008), although some studies report just the opposite. Therefore, the involvement of RAGE in pulmonary fibrosis is somewhat controversial (Bargagli et al. 2009). In a number of other tissues,

including the peritoneum, kidney, and liver, RAGE has been suggested to promote fibrosis (Li et al. 2004, De Vriese et al. 2006, Xia, Liu & Zhu 2008).

There is a series of clinical studies linking RAGE expression with different types of cancer such as gastric cancer (Kuniyasu et al. 2002), colon cancer (Kuniyasu, Chihara & Takahashi 2003), common bile duct cancer (Hirata et al. 2003), pancreatic cancer (Takada et al. 2001), prostate cancer (Ishiguro et al. 2005), and oral squamous cell carcinoma (Bhawal et al. 2005). Cancer cells express and secrete RAGE ligands, such as S100/calgranulins and amphoterin, which cause cellular activation, increased expression of cytokines and growth factors, cell migration, and activation of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) (Sparvero et al. 2009). Blockade of RAGE/ligand interaction reduces tumor cell growth and metastases, and invasive tumors have high levels of RAGE in cancers originating from many different organs (Logsdon et al. 2007). In contrast, other studies have suggested that RAGE may also act as a tumor suppressor, particularly in lung cancer (Bartling et al. 2005). Compared with the normal lung, reduced levels of RAGE have been reported in non-small cell carcinoma (Bartling et al. 2005, Buckley, Ehrhardt 2010), and RAGE downregulation correlated with higher tumor stages in lung cancer (Bartling et al. 2005). The paradox is that while RAGE ligands are overexpressed in lung cancer the receptor itself is downregulated or cleaved proteolytically (Buckley, Ehrhardt 2010).

The hypothesis that sRAGE and esRAGE act as decoy molecules and counteract the effects of membrane-bound RAGE is supported by an increasing amount of research data. However, the serum concentrations of sRAGE and esRAGE have been reported to be both elevated and decreased in various disease states relative to healthy controls (Prasad 2014). Decreased concentrations of sRAGE have been reported in acute systemic inflammation, including Kawasaki disease and systemic onset juvenile idiopathic arthritis (Wittkowski et al. 2007), patients with active rheumatoid arthritis (Pullerits et al. 2005) and in the cerebrospinal fluid of patients with multiple sclerosis, at clinical onset of the disease (Glasnovic et al. 2014). Low sRAGE concentration is associated with Alzheimer´s disease (Emanuele et al. 2005), systemic lupus erythematosus (Ma et al. 2012), Sjögrens syndrome (Stewart et al. 2008), hypercholesterolemia (Santilli et al. 2007), and essential hypertension (Geroldi et al. 2005). Initially low sRAGE is suggested to be a risk factor for atherosclerosis and coronary disease, complications of coronary and cerebrovascular diseases, and early death (McNair et al. 2010, Hudson et al. 2011, Selvin et al. 2013). However, again, some studies present contradictory results (Nin et al. 2009, Thomas et al. 2011), especially when the study subjects are affected by diabetes. Chronic kidney disease and significant renal impairment increase the concentrations of both sRAGE and esRAGE according to many studies, which might be a confounding factor in

some cohorts (Schmidt 2015). Concentration of sRAGE is positively associated with longevity according to a study on healthy centenarians and controls (Geroldi et al. 2006). The investigators measured sRAGE from samples of healthy centenarians, non-diabetic patients who experienced an acute myocardial infarction (AMI) before the age of 40 years, and healthy subjects aged less than 40 years. Interestingly, of the three groups, healthy centenarians had the highest and young AMI patients the lowest concentrations of sRAGE (Geroldi et al. 2006). It is tempting to speculate that healthy centenarians have high innate sRAGE concentration, whereas low sRAGE might be a risk factor for AMI and other diseases affecting life expectancy. Another study demonstrated an association between a polymorphism of the promoter region of the RAGE gene and longevity in males (Falcone et al. 2013), although the polymorphism does not seem to have any direct effect on the circulating sRAGE concentration (Gaens et al. 2009). Several studies support a role for RAGE gene polymorphisms in vascular disease (Kalea, Schmidt & Hudson 2009).

2.2.4 ADVANCED GLYCATION END PRODUCTS AND TYPE 1 DIABETES

AGEs are suggested to play a role in etiopathogenesis and both microvascular and macrovascular complications of diabetes (Goldin et al. 2006). In vitro studies have demonstrated that AGEs and the glycation reaction promote beta-cell dysfunction and apoptosis (Matsuoka et al. 1997, Kaneto et al. 1996). Furthermore, dietary restriction of AGEs reduced the incidence of diabetes in an animal model, non-obese diabetic (NOD) mice (Peppa et al. 2003). Increased levels of CML were an independent predictor of diabetes progression in a cohort of ICA-positive schoolchildren from the general population, together with diabetes-associated autoantibodies and increased HLA risk (Beyan et al. 2012). The investigators observed as well that monozygotic twins discordant for diabetes had similar CML levels, which were higher than in the control population, suggesting that high CML might be, at least in part, genetically determined (Beyan et al. 2012). One way for hyperglycemia to cause cell damage in type 1 and type 2 diabetes is to enhance the formation of AGEs, which contribute to the complications of diabetes by inducing intracellular oxidative stress (Vlassara, Palace 2002).

2.2.5 RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS AND TYPE 1 DIABETES

Multiple studies link RAGE to both type 1 and type 2 diabetes (Litwinoff et al. 2015). Interestingly, various effects of a high-fat diet, including excess weight gain and insulin resistance, were prevented in RAGE -/- knock-out mice

(Song et al. 2014), suggesting a role for RAGE in the pathogenesis of type 2 diabetes and obesity, and also in the clinical characteristics of type 1 diabetes. The results of numerous studies on sRAGE in diabetes are not unequivocal, but at least in most studies on adult populations circulating sRAGE concentrations seem to be elevated in the diabetic state, while esRAGE might be decreased (Prasad 2014). There are a limited number of studies reporting sRAGE concentrations in children and adolescents with diabetes, but there are studies reporting elevated (Dettoraki, Gil & Spiliotis 2009, Heier et al. 2015), decreased (Giannini et al. 2012) and similar (Zorena et al. 2013) levels in children with type 1 diabetes compared with healthy controls. The associations between sRAGE, esRAGE, and type 1 diabetes are listed in **Table 1.** Evidence suggests that sRAGE concentrations may be associated with events before or close to birth (Chiavaroli et al. 2012, Rogers et al. 2013). However, likely the higher sRAGE seen in most studies comparing diabetic patients and healthy controls is due to upregulation of the RAGE gene expression since circulating AGEs are elevated in type 1 diabetes (Berg et al. 1997, Berg et al. 1998). Recent studies have suggested that specific RAGE gene polymorphisms are associated with increased risk of type 1 diabetes and its complications (Pettersson-Fernholm et al. 2003, Damiani et al. 2007, Forbes et al. 2011), although studies in different populations have produced somewhat conflicting results (Kang, Tian & Jia 2012, Yuan, Yuan & Liu 2012). Studies reporting associations between polymorphisms of the gene encoding the RAGE molecule (*AGER*) and diabetes are listed in **Table 2.**

Table 1. Studies reporting sRAGE and esRAGE concentrations of patients with type 1 diabetes compared with non-diabetic controls.

Study	Population with type 1 diabetes	sRAGE concentration in type 1 diabetes	esRAGE concentration in type 1 diabetes
(Challier et al. 2005)	Adult	↑	
(Katakami et al. 2005)	Adult		↓
(Katakami et al. 2008)	Adult	↔	↓
(Dettoraki, Gil & Spiliotis 2009)	Pediatric	↑	
(Skrha et al. 2012)	Adult	↑	↑
(Giannini et al. 2012)	Pediatric	↓	↓
(Zorena et al. 2013)	Pediatric	↔	
(Koutroumani et al. 2013)	Pediatric	↔	↓, ↑*
(Lam et al. 2013)	Adult	↑	↑
(Heier et al. 2015)	Pediatric	↑	↓, ↔ *

Table 2. Studies reporting associations between AGER polymorphisms and risk for type 1 or type 2 diabetes or complications of diabetes.

Polymorphism	Study	Type of diabetes	Association of the minor allele
rs2070600	(Kankova et al. 2001, Prevost et al. 2005, Forbes et al. 2011, Yuan, Yuan & Liu 2012)	Type 1, type 2	predisposing, type 1 risk, type 1 and type 2 complications
rs9469089	(Forbes et al. 2011)	Type 1	protective
rs17493811	(Forbes et al. 2011)	Type 1	predisposing
rs1800624	(Pettersson-Fernholm et al. 2003, Falcone et al. 2004, Damiani et al. 2007, Yuan, Yuan & Liu 2012)	Type 1, type 2	protective, diabetes risk and complications
rs1800625	(Hudson et al. 2001, Sullivan et al. 2005)	Type 2	predisposing, insulin resistence, complications
63 base pair deletion from −407 to −345	(Poon et al. 2010)	Type 2	protective, complications
rs1800625, rs3134940 haplotype	(Kankova et al. 2005)	Type 2	predisposing, complications

Some associations were not confirmed in subsequent studies in other populations (Kalea, Schmidt & Hudson 2009).

In an animal model, the blocking of RAGE activation and RAGE/ligand interaction via administration of external soluble RAGE prevents advanced stages of autoimmune diabetes (Chen et al. 2004). Animal studies have implicated a role for RAGE in the activation of adaptive immune responses to auto- and alloantigens (Chen et al. 2008). Absence of RAGE in RAGE-/- knock-out mice and cell cultures is manifested by reduced activation of T-cells to alloantigens, and RAGE is also involved in T-cell differentiation towards a Th1 phenotype (Chen et al. 2008). A preliminary pilot study by our group showed a decline in sRAGE concentration at seroconversion to autoantibody positivity both in an animal model of autoimmune diabetes and in a small group of prediabetic children (Forbes et al. 2011).

3 AIMS OF THE STUDY

This thesis set out to define the significance of a novel autoantibody in a clinical phenotype of newly diagnosed children with type 1 diabetes and the implication of a suggested new link between genetic and environmental risk factors in type 1 diabetes. The aim was to investigate possible roles for advanced glycation products (AGEs) and their receptor (RAGE) in the pathogenesis of type 1 diabetes and in addition the aspects of ZnT8 autoimmunity in children with type 1 diabetes. In detail, we

1. defined characteristics of humoral autoimmunity against ZnT8 in a large Finnish population of children and adolescents with newly diagnosed type 1 diabetes.
2. assessed the relationships between humoral beta-cell autoimmunity, RAGE gene polymorphisms, and soluble RAGE in children with newly diagnosed type 1 diabetes.
3. defined the associations between circulating concentrations of RAGE and the humoral autoimmune response in children who have progressed to clinical diabetes during prospective observation in the DIPP study.
4. characterized the natural course of soluble RAGE in preclinical type 1 diabetes.

4 SUBJECTS AND METHODS

4.1 SUBJECTS

4.1.1 CHILDREN AND ADOLESCENTS WITH NEWLY DIAGNOSED TYPE 1 DIABETES (STUDIES I AND II)

The 2115 study subjects in the first two publications were derived from the Finnish Pediatric Diabetes Register and Sample Repository, which in the original articles is referred to as the Finnish Pediatric Diabetes Register and Biobank. Approximately 90% of children and adolescents diagnosed with diabetes under the age of 15 years in Finland are registered, and the family and the care-giving unit provide information to the register. Serum samples from children and adolescents with newly diagnosed type 1 diabetes as well as from their first-degree relatives are collected for the Sample Repository. Data on the degree of metabolic decompensation at the time of diagnosis and family history of type 1 diabetes and other forms of diabetes are collected with a structured questionnaire (Parkkola et al. 2013). The participants in Studies I and II were diagnosed with type 1 diabetes between 2 February 2002 and 7 January 2009. Only children and adolescents with a sample drawn within 30 days of the diagnosis were included in the studies. The mean duration from the diagnosis of type 1 diabetes to sampling was 5 days. Islet cell antibodies, insulin autoantibodies, autoantibodies to GAD, islet antigen 2, and ZnT8 were analyzed from the serum samples of the index children and their family members as part of the register study protocol.

The study questionnaire does not collect data on ethnicity, but the vast majority of the families reported the birthplaces of both parents and all grandparents to be in Finland. Only 3% of the families reported having roots outside Finland, and only 25% of those families had one or more family members who were born outside Europe. The majority of the study subjects were males, 1194 of 2115 subjects (56%). Mean age at diagnosis of type 1 diabetes was 7.96 years (median 8.16 years, range 0.28–14.98 years).

4.1.2 CONTROL SUBJECTS IN STUDY II

The control population in Study II comprised 316 healthy children and adolescents who had tested negative for diabetes-associated autoantibodies.

They were siblings of non-diabetic participants in the DIPP study who carry HLA genotypes conferring an increased risk for type 1 diabetes (Kupila et al. 2001). Five siblings came from families with an affected parent at the time of the recruitment of the index child to the DIPP study. Demographic data including age, sex distribution, and distribution of HLA genotypes were similar in the control subjects and in the children afflicted with type 1 diabetes.

4.1.3 THE DIPP CHILDREN (STUDIES III AND IV)

The subjects included in Studies III and IV were derived from the population-based DIPP study. The objectives of the DIPP study are to (1) identify newborn infants who carry HLA genotypes conferring an increased susceptibility to type 1 diabetes, (2) recognize the early stages of disease progression at the appearance of the first markers of humoral autoimmunity, and (3) attempt to delay the onset of clinical symptoms in subjects at genetic risk and with signs of islet cell autoimmunity (Kupila et al. 2001). All families of eligible newborn infants in three university hospitals were offered an opportunity for HLA screening for type 1 diabetes susceptibility, and the families received oral and written information on type 1 diabetes and the DIPP study. If neither of the parents was of Caucasian origin, or the parents had difficulties in understanding Finnish, Swedish, or English, or the infant had severe congenital disease, the family was not eligible to take part in the study. The exclusion criteria were set because of different risk genotypes in non-Caucasian populations and practical issues involved in the implementation of the study protocol. The unaffected older siblings of the index cases were also invited to genetic screening, and if they had genotypes associated with increased type 1 diabetes risk, they were invited to participate in the immunological screening. More than 90% of the eligible families gave their informed consent to genetic screening. Around 10-14% of the HLA-tested infants carried increased risk genotypes and roughly 80% of the parents gave their consent to participate in the prospective follow-up (Kupila et al. 2001).

All samples from the children participating in the follow-up study were screened for ICA, and if the child became positive for ICA, all of his/her subsequent and preceding samples were analyzed for GADA, IAA, and IA-2A. ZnT8A was analyzed from the samples of all autoantibody-positive children included in the current study. The immunological surveillance samples were drawn at the ages of 3, 6, 12, 18, and 24 months, and thereafter, at an interval of 6-12 months. If the children seroconverted to autoantibody positivity, the interval between study center visits was reduced to 3 months. If there were autoantibodies present in the cord blood, which decreased in subsequent

samples and disappeared by the age of 15 months, they were considered maternal (Kupila et al. 2001).

From the DIPP cohort, 114 children who progressed to overt type 1 diabetes during the prospective follow-up and 114 controls matched for time and city of birth, sex, and HLA genotype-based risk group were included in Study III. There were equal numbers of boys and girls. One sample taken before seroconversion to positivity for diabetes-associated autoantibodies, the first autoantibody-positive sample, the first sample positive for multiple autoantibodies (which, in 76 of the 114 cases, was also the first autoantibody-positive sample), and a sample taken close to the diagnosis were analyzed from the children who progressed to type 1 diabetes. The diagnosis of diabetes was based on the World Health Organization (WHO) criteria. Sample 4 was drawn at the earliest 12 days before and at the latest 13 days after the diagnosis (mean -2.7 days). Samples of the matched controls were analyzed at corresponding time-points. The samples included in the third study were collected between 26 July 1995 and 24 September 2008.

The study population in Study IV comprised 211 children followed in the DIPP study from birth. Among these children, 168 (96 boys, 72 girls) were diagnosed with type 1 diabetes by the end of follow-up, and 43 (29 boys, 14 girls) remained non-diabetic, but had seroconverted to persistent autoantibody positivity for multiple autoantibodies. The 114 prediabetic children included in the third paper were also included in this study. Similarly to Study III, a sample taken before seroconversion to autoantibody positivity and the first autoantibody-positive sample were selected for analysis, and thereafter, annual samples until the diagnosis of type 1 diabetes or the end of follow-up. The diagnosis of diabetes was based on the WHO criteria. The samples included in the fourth substudy were collected between 1 August 1995 and 8 December 2008. Information on the diabetes status of the children and adolescents was available until the end of the year 2014.

Table 3. Summary of study subjects.

Study (subjects/controls)	n	Girls (%)	Boys (%)
Study I (subjects)	2115	921 (44)	1194 (56)
Study II (subjects)	2115	921 (44)	1194 (56)
Study II (controls)	316	134 (42)	182 (58)
Study III (subjects)	114	57 (50)	57 (50)
Study III (controls)	114	57 (50)	57 (50)
Study IV (subjects)	211	86 (41)	125 (59)

4.1.4 ETHICAL ASPECTS

No additional blood samples were needed from the children and adolescents to conduct these studies (I-IV). The analyses were performed from back-up samples collected from the participants in the Finnish Pediatric Diabetes Register and Sample Repository and in the DIPP study. In both of these earlier studies, the contents covered investigations related to type 1 diabetes and its complications. The parents and adult siblings in the families taking part in the Finnish Pediatric Diabetes Register gave their signed informed consent prior to participation. Index cases and siblings between 10 and 18 years of age provided their consent prior to participating in the register and sample repository. The project has been approved by the Ethics Committee for pediatric diseases and psychiatry at the Helsinki and Uusimaa Hospital District. Each participant has the right to withdraw from the Register and Sample Repository at any time and to request that his/her information and/or samples be deleted and destroyed. All parents of the children included in the DIPP study signed an informed consent. The DIPP study has been approved by the Ethics Committee in each participating hospital.

4.2 METHODS

4.2.1 THE ZNT8A ASSAY (STUDIES I-IV)

ZnT8A were analyzed with a radiobinding assay (Wenzlau et al. 2008). A 35S-methionine-labeled chimeric recombinant plasmid (4.1) encoding the C-terminal region (aa 268–369) of the ZnT8 aa325-Arg allele and the ZnT8 aa325-Trp allele (provided by J. Hutton, University of Colorado Denver, CO, USA) was used in the radioligand assay. The disease specificity of this assay was 100% and the disease sensitivity 60% according to the 2010 Diabetes Autoantibody Standardization Program. Autoantibody levels were expressed as RU based on a standard curve derived from a pool of samples strongly positive for ZnT8 and diluted in normal human serum. The cut-off limit for positivity was set at 0.61 RU, corresponding to the 99th percentile in 250 non-diabetic Finnish children and adolescents. The inter-assay coefficient of variation was 11.2% and the intra-assay coefficient of variation was 7.6%. In the comparisons of ZnT8A levels between groups, the antibody-negative cases were excluded from the analyses.

4.2.2 IAA, GADA, IA-2A, AND ICA ASSAYS (STUDIES I-IV)

Autoantibodies to insulin, GAD, and IA-2 were analyzed with specific radiobinding assays, as described earlier (Savola et al. 1998). The 99th percentiles in 354 Finnish non-diabetic children and adolescents were used as the cut-off limits for antibody positivity and were 2.80 RU for IAA, 5.36 RU for GADA, and 0.77 RU for IA-2A. Disease sensitivities and specificities of the assays for type 1 diabetes were 44–50% and 96–98% for IAA, 82–92% and 94–97% for GADA, and 64–72% and 97–100% for IA-2A, respectively, according to the 2002–2010 Diabetes Antibody Standardization Program workshops. The intra-assay coefficient of variation was 8.4% for IAA, 7.3% for GADA, and 9.7% for IA-2A. The inter-assay coefficient of variation was 9.0% for IAA, 11.7% for GADA, and 12.0% for IA-2A. ICA was analyzed with indirect immunofluorescence of a human group 0 donor pancreas with 2.5 Juvenile Diabetes Foundation units as the detection limit (Bonifacio, Lernmark & Dawkins 1988) .

4.2.3 SERUM CONCENTRATIONS OF SRAGE (STUDIES II-IV)

Serum samples were analyzed undiluted according to the manufacturer's instructions (Human RAGE ELISA, R&D Systems, Minneapolis, MN, USA). The interassay coefficient of variation was 7.6%, while the intra-assay coefficient of variation was 3.5%. The analysis covers both esRAGE and sRAGE components of the circulating RAGE pool.

4.2.4 POLYMORPHISMS OF THE AGER GENE (STUDY II)

AGER genotyping was performed with the Sequenom multiplex platform (Sequenom, Hamburg, Germany) on 1444 patients according to the manufacturer's instructions. To ascertain the quality of Sequenom genotyping, all three single-nucleotide polymorphism (SNP) markers studied were tested for Mendelian inconsistencies (if the genotypes of parents were available), Hardy-Weinberg equilibrium, and missingness of genotyping results. Mendelian and missingness tests were based on a more extensive set of 72 SNPs (from AGER and other genomic regions). All markers were in Hardy-Weinberg equilibrium, and per-marker missingness of genotyping results was at a level of less than 2%, which is acceptable. Families with more than 2% Mendelian errors or more than 50% missingness in 72 SNPs were removed from the analysis (less than 4%), leaving 1390 case patients who passed the Sequenom quality control.

4.2.5 SERUM CONCENTRATIONS OF CML (STUDY III)

Serum concentrations of CML were measured with an indirect enzyme immunoassay specific to CML-modified human serum albumin (650.8 μmol/mol lysine) as described previously (Norman et al. 2009), with the modification that 739 mg/ml of rabbit polyclonal anti-CML antibody was used and reactions were terminated after 5 min with 1.8 mol/l H_2SO_4. Samples were diluted to either 1:48,000 or 1:96,000. The intra- and interassay coefficients of variation were 13% and 28%, respectively. The linearity of dilution of the assay was $r2 = 0.96$.

4.2.6 HLA TYPING (STUDIES I-IV)

HLA typing of the major protective and predisposing DR-DQ haplotypes was performed with a PCR-based lanthanidelabeled hybridization method using time-resolved fluorometry for detection. This method identifies the following

alleles: DQB1*02, *0301, *0302, *0303, *0304, *04, *0501, *0502, *0503, *0601, *0602, *0603, *0604, DQA1*0201, *03, *05, and DRB*0401, *0402, *0403/06, *0404, *0405, *0407, *0408 (Hermann et al. 2003). The presence of the (DR3)-DQA1*05- DQB1*02 haplotype is shortened to DR3 in the text and that of HLA-DRB1*04-DQB1*0302 to DR4, according to convention.

4.3 STATISTICAL ANALYSES

SPSS Statistical Software Package (version 19.0; SPSS, Chicago, IL, USA) was used to perform statistical analyses in Study I. The analyses in Studies II-IV were performed with IBM SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL, USA). Comparisons between groups were conducted using Student's t-test for independent or paired samples, when appropriate, if there were only two groups, or the non-parametric Mann–Whitney U-test or the Wilcoxon signed-rank test. Student's t-test for paired samples for parametric variables and Wilcoxon signed-rank test for non-parametrical variables were used to compare the subsequent samples from the patients or samples from the patients and matched controls. In Study III, Bonferroni correction for multiple comparisons (p^c) was applied, when appropriate. ANOVA analysis followed by post hoc analysis with Tukey's or Tamhane's test was used when there was more than two groups. Kruskall–Wallis test was used for comparing differences between more than two groups in non-parametric variables. Differences in proportions between groups were tested using the Chi-squared test. Correlations were analyzed using the Pearson correlation coefficient (r) for normally distributed variables or the Spearman rank correlation test (r_s) for skewed or non-parametric variables. Logistic or linear regression was applied when appropriate to assess the unique contribution of a specific variable to the model. For all analyses, *p*-values of less than 0.05 were considered significant.

5 RESULTS

5.1 HUMORAL AUTOIMMUNITY AGAINST ZNT8 (STUDIES I AND II)

5.1.1 CHARACTERISTICS OF HUMORAL AUTOIMMUNITY AGAINST ZNT8 AND OTHER ISLET ANTIGENS

Of the 2115 newly diagnosed children and adolescents with type 1 diabetes, 63% (n=1325) tested positive for ZnT8A, with a mean level of 17.9 RU (range 0.63–459.4 RU). The frequencies and levels of ZnT8A were similar in boys and girls. Children who had ZnT8A were older than the negative children (mean 8.2 years versus 7.5 years, $p < 0.001$). When the subjects were divided into tertiles by age, (0–4.99, 5–9.99, and 10–14.99), the youngest age group had significantly lower titers of ZnT8A than the two older groups (mean 11.1 RU versus 19.0 RU and 21.0 RU, respectively, $p < 0.001$). Of children younger than 5 years, only 53% tested positive for ZnT8A. This was significantly less ($p < 0.001$) than in the older age groups, where the frequencies were 69% and 63%, respectively. Least likely to have ZnT8A at diagnosis were the children diagnosed with type 1 diabetes at very young age. Only one-third of children younger than 2 years had ZnT8A at diagnosis ($p < 0.001$), and if they did, their titers were significantly lower than in the rest of the population (10.1 RU versus 18.2 RU, $p = 0.003$).

The combined analysis of GADA, IA-2A, and IAA yielded a disease sensitivity of 94% in our population. The measurement of ZnT8A increased the sensitivity of humoral beta-cell autoimmunity to 96%. There were 133 subjects (6.3%) who tested negative for GADA, IAA, and IA-2A. One-third of the triple-negative subjects had ZnT8A. The frequencies of various antibody combinations are given in Table 4.

Subjects with at least two diabetes-associated autoantibodies constituted approximately two-thirds (67%) of the population, when only GADA, IAA, and IA-2A were considered. Eighty-one percent of the children and adolescents were positive for multiple autoantibodies when the ZnT8A assay results were added. If all five autoantibody specificities were included in the analysis, 93% of the population was positive for more than two autoantibodies, but only 90% with the four previously available assays without the ZnT8A assay. A total of 44 children and adolescents (2.3%) tested negative for all four traditional autoantibodies including ICA. The ZnT8A assay added only 0.3% to the detection rate of humoral beta-cell autoimmunity since only seven subjects had ZnT8A as their single

autoantibody specificity. Among the ICA-negative patients (n=163), only 20% had ZnT8A. Frequencies of all the autoantibody specificities in the study population are presented in **Figure 2**. Subjects who tested negative for autoantibodies had a similar frequency of family members affected by type 2 diabetes and a similar body mass index relative to the rest of the population (data not shown). All five autoantibodies were detectable in 406 cases (19%). There was a correlation between ZnT8A and ICA levels (r_s = 0.42, p < 0.001), between ZnT8A and IA-2A levels (r_s= 0.36, p < 0.001), and between ZnT8A and GADA levels (r_s= 0.15, p < 0.001).

The serum concentration of sRAGE was not associated with the number of autoantibodies detectable at the diagnosis of type 1 diabetes (p = 0.31). However, children testing positive for ZnT8A had higher and subjects with IAA lower sRAGE concentrations than the rest of the population (1196 versus 1128 pg/ml, p < 0.001; 1137 vs. 1198 pg/ml, p = 0.001, respectively), but the associations were no longer significant when the effect of age at diagnosis was considered. The other autoantibodies were not associated with the sRAGE concentrations. The data on mean sRAGE concentration in autoantibody-positive and -negative subjects are summarized in **Table 5.**

Table 4. ZnT8A and their combination with other antigen-specific autoantibodies in three age categories, and frequency in the population.

Autoantibodies and combinations	all (n=2115)	0-4.99 y (n=569)	5-9.99 y (n=808)	10-14.99 y (n=738)	*p*
No autoantibodies	89 (4)	18 (3)	25 (3)	46 (6)	<0.01
Only one autoantibody	308 (15)	73 (13)	112 (14)	123 (17)	NS
Only ZnT8A	44 (2)	7 (1)	20 (2.5)	17 (2)	NS
Two autoantibodies	576 (27)	144 (25)	240 (30)	192 (26)	NS
ZnT8A+, GADA +	89 (4)	10 (2)	43(5)	36 (5)	<0.01
ZnT8A+, IAA +	23 (1)	10 (2)	7 (1)	6 (1)	NS
ZnT8A +, IA-2A +	191 (9)	25 (4)	94 (12)	72 (10)	<0.001
Three autoantibodies	733 (35)	204 (36)	259 (35)	270 (37)	NS
ZnT8A+, IAA+GADA+	51 (2)	20 (4)	16 (2)	15 (2)	NS
ZnT8A+, IAA+, IA2A+	114 (5)	68 (12)	35 (4)	11 (2)	<0.001
ZnT8A+, GADA+, IA2A+	405 (19)	32 (6)	172 (21)	201 (27)	<0.001
Four autoantibodies	409 (19)	130 (23)	172 (21)	107 (14)	<0.001
ZnT8A+, n (%)	1325 (63)	302 (53)	559 (69)	464 (63)	<0.001
ZnT8A+, GADA-	371 (18)	111 (20)	156 (19)	104 (14)	<0.01
ZnT8A+,IAA-	728 (34)	74 (13)	329 (41)	325 (44)	<0.001
Znt8A+, IA-2A-	207 (10)	48 (8)	86 (11)	73 (10)	NS

The p-values refer to differences between age groups (ANOVA) and groups defined by autoantibody positivity (t-test). NS=non-significant. Modified from Study I.

Table 5. Mean sRAGE concentrations in autoantibody-positive (+) and -negative (-) subjects.

Autoantibody	n	Mean sRAGE concentration pg/ml (SD)		*p*
	+/-	+	-	
ICA	1952/163	1169 (428)	1198 (471)	NS
IAA	945/1170	1137 (423)	1198 (436)	0.001
GADA	1417/698	1184 (428)	1145 (437)	NS
IA-2A	1608/507	1171 (434)	1172 (423)	NS
ZnT8A	1325/790	1196 (433)	1128 (426)	<0.001

The p-values refer to differences between groups defined by autoantibody positivity (t-test). NS=non-significant. Modified from Study II.

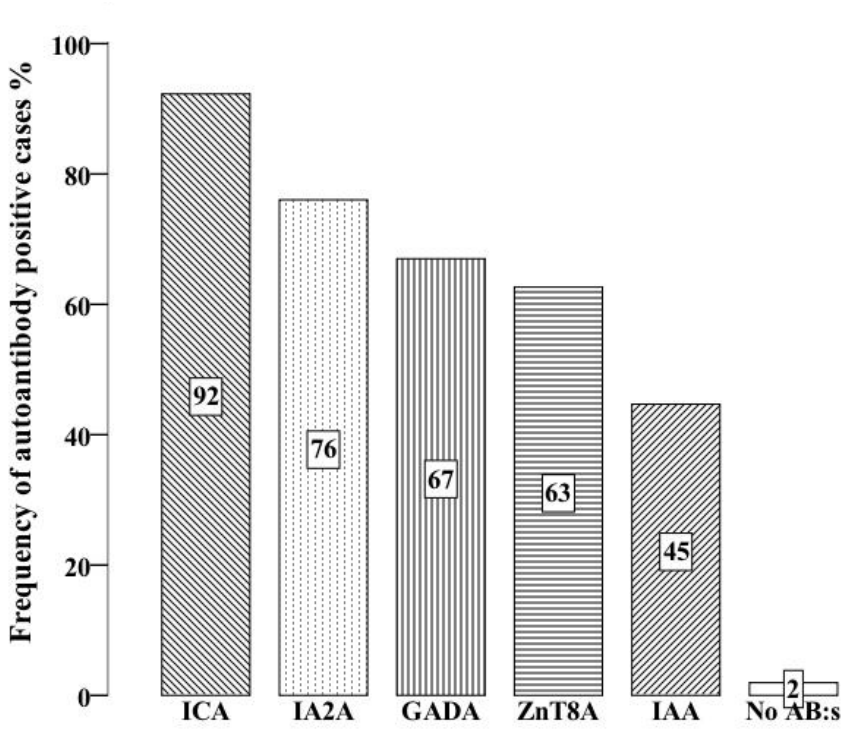

Figure 2. Bars represent the proportion of study subjects testing positive for each islet cell autoantibody, and the last bar the frequency of autoantibody-negative subjects.

5.1.2 ZNT8A IN RELATION TO METABOLIC STATE, HLA GENOTYPE, AND FAMILY HISTORY OF DIABETES

The subjects testing positive for ZnT8A had less frequently diabetic ketoacidosis defined as a blood pH <7.30 than the ZnT8A-negative children and adolescents (16% versus 20%, p = 0.012). Even after adjustment for age, sex, and HLA genotype, the difference remained significant (p = 0.006). There were differences in plasma glucose and pH levels of the ZnT8A-positive and -negative subjects as well, which were significant or almost significant (p = 0.024 and 0.054, respectively), but the mean absolute values were quite similar (25.0 mmol/l versus 26.0 mmol/l and 7.35 versus 7.34). The ZnT8A levels in the subjects with ketoacidosis and those without were similar (18.1 RU versus 17.9 RU, p = 0.98).

Subjects carrying the HLA DR3 allele (n=784) tested less often positive for ZnT8A than the DR3-negative subjects (58% vs. 65%, p = 0.001). The difference between DR4-positive (n=1466) and -negative cases was insignificant (62% versus 64%, p = 0.40). Among subjects heterozygous for HLA DR3/DR4 (n=455), 55% tested positive for ZnT8A at diagnosis. The difference in ZnT8A frequency between the children with the highest HLA-conferred genetic risk for type 1 diabetes and the rest of the population (55% versus 65%, p < 0.001) remained significant in a regression model with sex and age as other covariants (p <0.001). The mean ZnT8A levels in the four genotype groups were similar. The DR13-DQB1*0604 haplotype (n=170), which is neutral in relation to risk for type 1 diabetes, was associated with increased frequency of ZnT8A positivity (77% vs. 61%, p < 0.001). Subjects carrying the haplotype DR1-DQB1*0501 (n=563) tested more often positive for ZnT8A than the rest of the population (66% versus 61%, p = 0.05) as well. DR8-DQB1*04 haplotype (n=362) had a negative association (57% versus 64%, p=0.02) and the two other common neutral haplotypes, i.e. DR9-DQA1*03-DQB1*0303 and DQA1*0201-DQB1*02, no association with the ZnT8A frequency. In a logistic regression model of the effect of each neutral haplotype on the frequency of ZnT8A, only DR13-DQB1*0604 contributed significantly to the model (p < 0.001).

No associations were observed between the prevalence or levels of ZnT8A on one hand and a positive family history for type 1 or type 2 diabetes on the other.

5.2 SERUM SRAGE CONCENTRATIONS IN CHILDREN WITH NEWLY DIAGNOSED TYPE 1 DIABETES (STUDY II)

5.2.1 CIRCULATING SRAGE CONCENTRATION IN RELATION TO AGE, METABOLIC STATE AT DIAGNOSIS, DEMOGRAPHIC AND HLA GENOTYPE DATA, AND HUMORAL AUTOIMMUNITY AGAINST ISLET CELL ANTIGENS

Boys and girls with newly diagnosed type 1 diabetes had similar circulating sRAGE concentrations (1173 vs. 1168 pg/ml, p = 0.80). Neither was there any difference in the circulating sRAGE concentrations between the patients with newly diagnosed type 1 diabetes and the control subjects (1171 vs. 1153 pg/ml, p = 0.48). However, the affected children showed a modest correlation between age at diagnosis and sRAGE concentration (r = 0.10, p < 0.001). The youngest tertile in the subjects with newly diagnosed type 1 diabetes, i.e. the children <5 years of age, had the lowest sRAGE concentrations (1113 pg/ml), and the difference was significant when their concentrations were compared

with those of the children 5–9.99 years of age (1178 pg/ml, $p = 0.01$) or with the children and adolescents in the oldest age group (10–14.99 years of age, 1208 pg/ml, $p < 0.001$). Children who were diagnosed with type 1 diabetes before 2 years of age had even lower concentrations (1027 vs. 1181 pg/ml, $p < 0.001$) when compared with the older ones. The sRAGE concentrations divided into tertiles according to age are presented in **Figure 3.** There was no significant linear correlation between age and sRAGE concentrations in the control population (r = -0.006, $p = 0.95$). The non-diabetic children <2 years of age had somewhat higher concentrations than the rest of the unaffected subjects (1329 vs. 1140 pg/ml, p = 0.04), whereas the situation was the opposite among the children with type 1 diabetes. If only children <2 years of age were included in the analysis, there was a significant difference in the sRAGE concentrations between diabetic children (n = 133) and control subjects (n = 21) (1027 vs. 1329 pg/ml, $p = 0.005$).

Diabetic ketoacidosis was associated with lower sRAGE concentrations (1081 vs. 1190 pg/ml, $p < 0.001$) and severe diabetic ketoacidosis with even lower concentrations (1009 vs. 1178 pg/ml, $p < 0.001$). The associations between metabolic state at diagnosis, demographic data, and sRAGE concentrations are presented in **Table 6.**

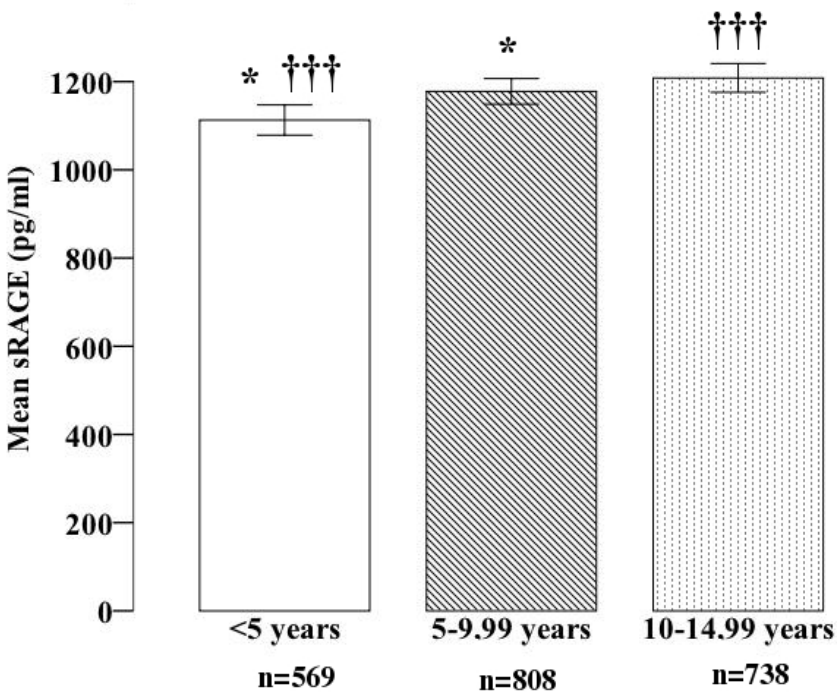

Figure 3. Subjects aged <5 years (n=569) had the lowest sRAGE concentrations. Mean serum sRAGE concentrations are displayed as bars with 95% CI. *p=0.01 in children aged <5 years vs. 5-9.99 years (n = 808). $^{\dagger\dagger\dagger}p < 0.001$ in children <5 years vs. children and adolescents aged 10-14.99 years (n=738). The p-values are derived from one-way ANOVA, post hoc analysis with Tamhane´s test.

Table 6. Associations between sRAGE concentrations and factors reflecting the metabolic state at diagnosis of type 1 diabetes and demographic data of patients.

		Mean sRAGE level pg/ml (SD)				
	n	**+**	**-**	**r_s**	***p****	***p**†*
Severe DKA	86	1009 (397)	1178 (433)		<0.001	<0.001
DKA	365	1081 (439)	1190 (429)		<0.001	<0.001
Blood pH	*2092*			0.11	<0.001	<0.001
Blood glucose	2077			-0.05	0.02	0.01
zBMI	2075			0.06	<0.01	<0.01
Family history of type 1 diabetes	263	1198 (432)	1167 (432)		NS	
Family history of type 2 diabetes	34	1147 (399)	1171 (432)		NS	

The *p** -values represent the results of Student's t-test analysis comparing two groups or Spearman correlation test and *p*† -values are derived from logistic regression (dichotomous variables) or linear regression (continuous variables) analyses with age, sex, DR3 positivity, and DR4 positivity as other covariates, and reflect the independent effect of the sRAGE concentrations. zBMI = age-adjusted body mass index. NS=non-significant. Table modified from Study II.

Subjects with the highest genetic risk for type 1 diabetes, the HLA-DR3/ DR4 heterozygotes, had somewhat lower sRAGE concentrations than the rest of the population (1127 vs. 1180 pg/ml, p = 0.008), when both the study population and the control subjects were analyzed together. The association with sRAGE concentration remained significant (p = 0.02) in a logistic regression model with the presence of the HLA-DR3/DR4 genotype as the dependent variable, and age at diagnosis and sex as other covariates. Lower circulating sRAGE concentrations were associated with the presence of the DR3 haplotype as well, when compared with DR3-negative subjects (1131 vs. 1190 pg/ml, p = 0.001), and the difference also remained significant (p =0.002) when the effects of age and sex were included in a logistic regression analysis. The inverse association between the circulating sRAGE concentrations and the HLA-DR3/DR4 genotype was present both in the population with type 1 diabetes (1133 vs. 1181 pg/ml, p = 0.02) and the controls, although in the latter the association was not statistically significant

(1084 vs. 1171 pg/ml, $p = 0.13$). The association between the DR3 haplotype and lower sRAGE concentrations was significant in both populations (1144 vs. 1186 pg/ml, $p = 0.03$ in the diabetic population and 1033 vs. 1214 pg/ml, $p < 0.001$ in controls). Carrying the HLA-DR4 haplotype was associated with higher serum concentrations in the control population (1186 vs. 1055 pg/ml, $p = 0.01$), but the difference remained non-significant (1175 vs. 1152 pg/ml, $p = 0.64$) in the study population. However, the HLA-DR4/non-DR3 genotype was associated with higher sRAGE concentrations in both patients with type 1 diabetes (1192 vs. 1152 pg/ml, $p = 0.03$) and controls (1224 vs. 1070 pg/ml, $p = 0.001$).

5.2.2 *AGER* POLYMORPHISMS AND SRAGE

We observed that two SNPs of the *AGER* gene, previously associated with an increased risk of type 1 diabetes, i.e. rs2070600 and rs9469089, were strongly associated with the sRAGE concentrations ($p < 0.001$ and $p = 0.001$, respectively, ANOVA), whereas the third one analyzed, rs17493811, was not. The genotype frequencies of the three AGER gene polymorphisms and their associations with sRAGE concentration are shown in **Table 7.** The three SNPs were analyzed in 1390 subjects with type 1 diabetes. Carrying the minor allele of rs2070600 or rs9469089 had a unique, significant effect on the sRAGE concentration with sex, age, DR3 positivity, and DR4 positivity as other covariates (ß -0.32, $p < 0.001$; ß 0.09, $p = 0.002$, respectively). Subjects carrying the minor allele of rs2070600, which is associated with increased risk for type 1 diabetes, were younger at diagnosis than the rest of the population (7.4 vs. 8.0 years, $p = 0.03$). Protective minor allele of rs9469089 was not associated with age at diagnosis (7.8 vs. 7.9 years, $p = 0.61$). The major G alleles in both rs9469089 and rs2070600, which have opposite effects on sRAGE concentrations and type 1 diabetes risk, are present in most DR3 and DR4 haplotypes in the control population, while the minor C and A alleles are rare in both DR3 and DR4 haplotypes, as summarized in Table 8. The location of the rs2070600 and the rs9469089 SNPs in the AGER gene is shown in **Figure 4.**

Table 7. Genotype frequencies of *AGER* polymorphisms and mean sRAGE in each genotype group of *AGER* polymorphisms of rs2070600 and 9469098.

SNP	Genotype	n (%)	sRAGE (pg/ml)
rs2070600	GG	1078 (78)	1245 ± 437***
	AG	299 (22)	931 ± 332***
	AA	13 (1)	439 ± 190†††
rs9469089	GG	941 (68)	1144 ± 433*†
	CG	423 (30)	1215 ± 440‡
	CC	26 (2)	1395 ± 518

Concentrations of sRAGE are presented as mean ± SD. There were significant differences in the sRAGE between subjects carrying different genotypes of rs2070600 and rs9469098 SNPs. ***p <0.001 vs. AA homozygotes, $^{\dagger\dagger\dagger}p$ <0.001 vs. AG heterozygotes; *p =0.02 vs. CG heterozygotes, $^{\dagger}p$ =0.01 vs. CC homozygotes. $^{\ddagger}p$ =0.02 vs. GG homozygotes. Table modified from Study II.

Table 8. Population frequencies of AGER SNPs in HLA haplotypes.

	rs9469089	
	C	G
HLA-DR3	3 (1%)	315 (99%)
HLA-DR4	57 (19%)	249 (81%)
	rs2070600	
	A	G
HLA-DR3	0 (0%)	328 (100%)
HLA-DR4	32 (10%)	289 (90%)

Table represents the frequencies of AGER SNPs in HLA haplotypes in 1761 pseudo-controls derived from Finnish trio families; mother, father, and child. Table modified from the supplemental materials of Study II.

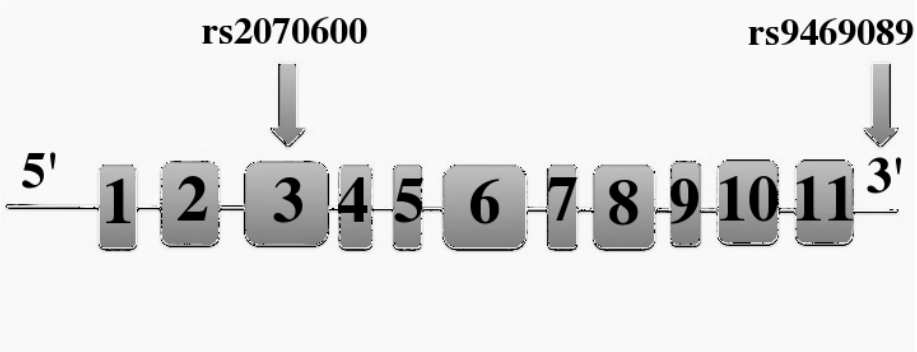

Figure 4. Location of the rs2070600 and the rs9469089 SNPs in the AGER gene. The boxes 1-11 represent the exons in the AGER gene. The arrows point to the locations of the rs2070600 and the rs9469089 SNPs. The rs2070600 is located in exon 3 and the rs9469089 at the 3´end of the gene in the untranslated region.

Carriers of the minor allele of the rs2070600 had higher frequency of IAA than carriers of the major G allele (55% vs. 43%, p <0.001). ZnT8A were more common among carriers of the minor allele of the rs9469089 than the rest of the population (66% vs. 58%, p = 0.01), while the IAA frequency was lower when comparing the two allelic groups (38% vs. 48%, p = 0.001). The associations between the SNPs and autoantibody frequencies remained significant in logistic regression analyses with the age, sex, and HLA genotype as other covariates (data not shown).

5.3 CHANGES IN SRAGE CONCENTRATIONS IN CHILDREN PROGRESSING TO CLINICAL TYPE 1 DIABETES (STUDY III)

5.3.1 DECLINE IN SRAGE AT TIME OF SEROCONVERSION TO POSITIVITY FOR DIABETES-ASSOCIATED AUTOANTIBODIES

At all time-points: 1) before seroconversion (sample 1), 2) in the first autoantibody-positive sample (sample 2), 3) in the first sample positive for multiple autoantibodies (sample 3), and 4) in the sample taken close to diagnosis (sample 4), the children who progressed to type 1 diabetes had higher sRAGE concentrations than the controls. The difference was not significant at the time of the second sample. Data on sRAGE concentrations at each time-point for the cases and controls are summarized in **Table 9.** When all four samples from each participant were included in the analysis,

an inverse correlation between age and sRAGE concentration existed in the control group (r_s = -0.23, p < 0.001), but not in the children who progressed to type 1 diabetes (r_s = 0.03, p = 0.55).

In the prediabetic children, the sRAGE concentrations were lower in the second sample than in the sample taken before seroconversion (1232 vs. 1384 pg/ml, p = 0.001, p^c =0.01). The sRAGE concentrations were even lower in the third sample of the prediabetic children, but the reduction between the second and the third sample was not significant (p = 0.72). In the controls, the difference in the sRAGE concentrations between the first two samples was not significant (p = 0.22). However, in the controls, a decline in sRAGE concentration between the second and third sample (1167 vs. 1111 pg/ml, p = 0.008, p^c = 0.10) was observed. The changes observed in sRAGE concentration in between different samples of the controls were not significant after the Bonferroni correction. The changes in the sRAGE concentrations in the cases and controls are shown in **Figure 5.**

Table 9. Mean age in years and mean sRAGE concentration in pg/ml in cases and controls at each sampling point.

	Mean age, years (SD) progressor/control	**Mean sRAGE, pg/ml (SD) progressor/control**	***p***	***p*^{c}**
Sample 1	1.4 (1.4)/1.4 (1.4)	1403 (562)/1228 (465)	<0.01	0.03
Sample 2	2.1 (1.6)/2.1 (1.6)	1235 (456)/1136 (412)	NS	NS
Sample 3	2.5 (1.8)/2.5 (1.9)	1216 (435)/1042 (380)	<0.01	<0.01
Sample 4	5.6 (2.9)/5.3 (2.9)	1270 (454)/1106 (471)	0.01	0.04

The first sample is taken before seroconversion, the second sample is the first autoantibody-positive sample. The third sample was drawn at the time of seroconversion to multiple (≥2) autoantibodies and the fourth sample close to the diagnosis of diabetes in the children progressing to clinical type 1 diabetes and at corresponding ages in the control children. Only pairs with both case and control samples available are included. The *p*-values are derived from Student's t-test for paired samples, and the p^c represents the Bonferroni-corrected value. NS=non-significant. Table modified from Study III.

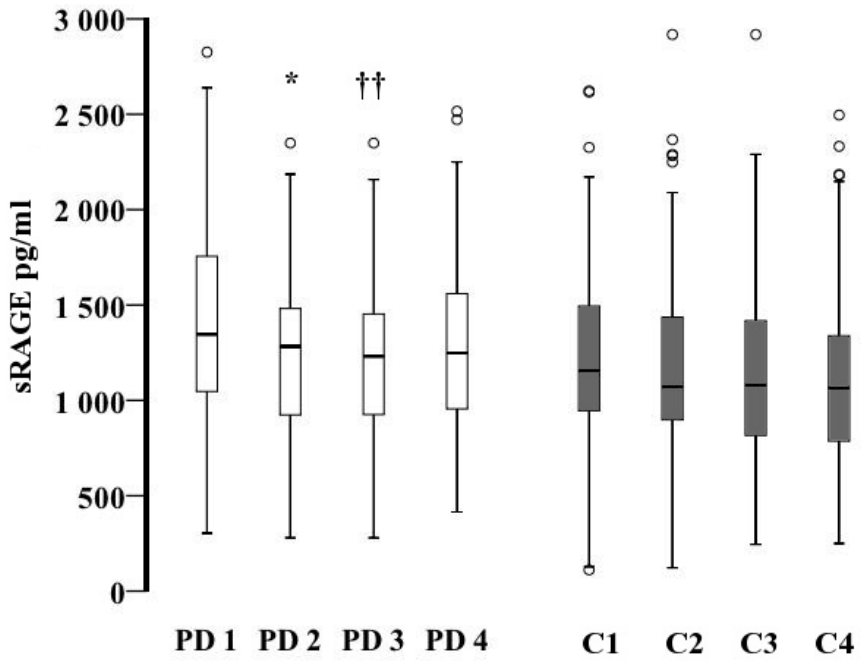

Figure 5. Changes in sRAGE concentrations in prediabetic children (PD) and in control children (C) at time-points 1-4. Box plots illustrate the changes in sRAGE concentrations in prediabetic children (PD) and in control children (C) at time-points 1-4. The line within the boxes is the median, the bottom of each box the 25th percentile, and the top of the box the 75th percentile. The whiskers represent the 5th and 95th percentiles, and the small circles are outliers. The first time-point is before seroconversion, the second at the first autoantibody-positive sample. The third sample is the first one with multiple (≥2) autoantibodies, and the fourth sample is taken close to diagnosis of diabetes in prediabetic children and at corresponding ages in control children. There is a decrease in sRAGE concentrations in the children progressing to diabetes after the first sample. There is a trend for declining concentrations also in the control children, but the changes become non-significant after correction or multiple comparisons. *p=0.01 vs. the first sample, $^{††}p$=0.005 vs. the first sample. The p-values are Bonferroni-corrected. Figure modified from Study III

There was no correlation between sRAGE concentration at different time-points and duration of the prediabetic period from seroconversion to diagnosis of type 1 diabetes (r_s = -0.07 to 0.07, p = 0.48–0.68).

5.3.2 CONCENTRATIONS OF CML AND SRAGE/CML RATIO IN PREDIABETES

Concentrations of CML were lower in the progressors than in the controls in all samples, although a significant difference was seen only in the last sample (2699 vs. 3424 μmol/mol lysine, p = 0.001, p^c = 0.004). In both cases and controls, the concentrations of CML remained stable, with the exception of the last sample of the progressors, taken close to the diagnosis of type 1 diabetes. The CML concentrations were significantly lower at diagnosis of type 1 diabetes than in the sample taken before seroconversion (2606 vs. 3095 μmol/mol lysine, p < 0.001, p^c = 0.003), in detection of the first autoantibody (2709 vs. 3225 μmol/mol lysine, p < 0.001, p^c < 0.001), and in the first sample positive for multiple autoantibodies (2664 vs. 3422 μmol/mol lysine, p < 0.001, p^c = 0.001). The ratio between sRAGE and CML concentration was higher among prediabetic children than in the autoantibody-negative controls in the first and last sample, i.e. before seroconversion and close to diagnosis of type 1 diabetes. Information on sRAGE/CML analyses is summarized in **Table 10.**

Table 10. Number of case-control pairs included in the analysis and mean sRAGE/CML ratio in progressors and controls at each sampling point.

	n	sRAGE / CML ratio (SD) in progressors	sRAGE / CML ratio (SD) in controls	*p*	p^c
Sample 1	74	0.83 (2.1)	0.40 (0.15)	<0.01	<0.01
Sample 2	76	0.47 (0.35)	0.35(0.16)	0.02	NS
Sample 3	53	0.50 (0.40)	0.35 (0.18)	NS	NS
Sample 4	71	0.61 (0.37)	0.35 (0.15)	<0.001	<0.001

The initial sample is taken before seroconversion and the second sample is the first autoantibody-positive sample. The third sample is taken at the time of seroconversion to multiple (≥2) autoantibodies and the fourth sample close to diagnosis of diabetes in progressors and at the corresponding ages in controls. The *p*-values are derived from Wilcoxon signed-rank tests between the groups, and the p^c represents the Bonferroni-corrected values. Table modified from Study III.

5.4 CIRCULATING SRAGE IN PREDIABETIC CHILDREN AND AUTOANTIBODY-POSITIVE CHILDREN REMAINING NON-DIABETIC (STUDY IV)

5.4.1 CHANGES IN SRAGE BEFORE AND AFTER SEROCONVERSION

There was no association between sRAGE concentration before seroconversion and number of diabetes-associated autoantibodies detectable at seroconversion [p=0.33 (ANOVA)]. Neither was there any difference in sRAGE concentrations between the subjects testing positive or negative for any specific autoantibody at seroconversion, or any correlation between sRAGE and autoantibody titers (data not shown). No association was present between time interval from seroconversion to diagnosis of type 1 diabetes and sRAGE concentration before seroconversion in the samples of the progressors [p =0.26 (linear regression)].

The characteristics of the progressors and non-progressors are listed in **Table 11**. The non-progressors were older than the children who progressed to clinical type 1 diabetes at seroconversion and at the end of follow-up (**Table 11**). Both groups had similar sRAGE concentrations before seroconversion (mean sRAGE 1384 pg/ml vs. 1282 pg/ml p =0.31, t-test), but after the first autoantibody-positive sample the sRAGE concentrations of the progressors were lower (mean sRAGE 1172 pg/ml vs. 1296 pg/ml, $p < 0.001$, t-test). When the sampling age and sex were included in a linear regression model as other covariates, the difference in sRAGE concentrations between the groups remained significant (p = 0.001).

Table 11. Demographic data of the children who did or did not progress to type 1 diabetes during prospective follow-up.

	Progressors	**Non-progressors**	***p***
n	**168**	**43**	
Boys (%)/girls (%)	96 (57) / 72 (43)	29 (67) / 14 (33)	0.30*
Median age at seroconversion (interquartile range), years	1.5 (1.0-2.8)	3.0 (1.5-4.1)	<0.001**
Median number of samples after seroconversion (interquartile range)	3 (2-5)	4 (2-6)	<0.001**
Median time from seroconversion to diagnosis of type 1 diabetes /end of follow-up (interquartile range), years	3.7 (1.9-6.6)	6.7 (5.4-8.4)	<0.001**
Median age at end of follow-up (interquartile range), years	3.7 (1.9-6.6)	6.7 (5.4-8.4)	<0.001**

The non-progressors were older at seroconversion and at the end of follow-up than the progressors. There were less samples/patient included in the study from the progressors as compared with the non-progressors. The p-values refer to the following statistical tests used to analyze the data: p*= Chi-square test, p**= Mann-Whitney U-test. Table modified from Study IV.

The concentrations of sRAGE were higher in the sample taken before seroconversion than in the first autoantibody-positive sample in the progressors (mean sRAGE 1382 pg/ml vs. 1226 pg/ml, $p < 0.001$, paired t-test). In the non-progressors, the serum concentrations of sRAGE were similar before seroconversion and in the first autoantibody-positive sample (mean sRAGE 1280 pg/ml vs. 1210 pg/ml, $p = 0.31$, paired t-test). There was a weak positive correlation between age and sRAGE concentration after seroconversion when all children were analyzed together [$rs=0.09$, $p=0.001$]. However, when each group was analyzed separately, no significant correlation was detected (data not shown).

6 DISCUSSION

6.1 STUDY POPULATION AND METHODS

Finland has the highest incidence of type 1 diabetes in the world (Soltesz et al. 2007) and comprehensive, uniform, and reliable registers for patient information available for scientific research (Harjutsalo, Sjöberg & Tuomilehto 2008), which makes the country ideal for diabetes research. The Finnish Pediatric Diabetes Register and Sample Repository contain data on the majority of the children diagnosed with type 1 diabetes in Finland since the establishment of the register in 2002 (Hekkala et al. 2010). Therefore, we had a unique opportunity to study the biologically complicated RAGE/AGE axis in a large, yet well-documented and coherent population of patients with newly diagnosed type 1 diabetes. The results of Studies I and II can be rather safely extrapolated to the Finnish pediatric diabetic population because of the high coverage (92%) of the Diabetes Register and Sample Repository. Since at least the HLA-conferred genetic risk is quite similar in other Caucasian populations, one may generalize the current results to many other pediatric populations with type 1 diabetes as well, although the distribution of risk alleles in the general population is somewhat different even in countries with close geographical proximity (Nejentsev et al. 1998).

Due to the unique situation in Finland with the highest incidence of type 1 diabetes, a well-organized healthcare system and a positive attitude among the Finnish population towards research, the nation provides an excellent basis for prospective, observational studies such as DIPP. The high rate of parental consent to genetic HLA testing of offspring and participation in the prospective follow-up ensures that the index cases in the DIPP study represent fairly reliably the general population in Finland (Kupila et al. 2001). The characteristics of the DIPP study enabled the selection of extensively matched controls for Substudy III and the long follow-up period in Study IV, which are rare even from a global perspective.

The laboratory methods used in this study are well documented and described in earlier studies, and our autoantibody laboratory participates in the international Diabetes Antibody Standardization Program workshops for quality control.

6.2 ZNT8A IN A COHORT OF FINNISH CHILDREN AND ADOLESCENTS WITH TYPE 1 DIABETES (STUDIES I AND II)

Study I was the first to report HLA DR3/DR4 heterozygosity to be associated with a decreased frequency of ZnT8A in patients with newly diagnosed type 1 diabetes. The ZnT8A assay did not significantly enhance the detection of beta-cell autoimmunity in this population, in contrast to an earlier study (Wenzlau et al. 2007, Andersson et al. 2011). The results of this study suggest that including ZnT8A in the panel of diabetes-associated antibodies is hardly crucial for the clinical recognition of beta-cell autoimmunity, provided that all four previously used autoantibody assays are available. However, if the ICA assay is not included, the ZnT8A assay provides a more significant addition to the other three biochemically determined autoantibodies in our population as well, with the autoantibody-negative cases decreasing by one-third. The question concerning ZnT8A has been whether or not it can replace ICA. The results here do not suggest that ICA be replaced by ZnT8A since among the ICA-negative patients only 20% had ZnT8A.

A steady increase in ZnT8A-positive patients at diagnosis after 5 years of age was observed. Around 10 years of age at diagnosis, the number of ZnT8A-positive patients reaches a plateau, and then starts to decrease. This is in concordance with previous studies showing decreased proportions of ZnT8A-positive patients diagnosed after the age of 15 years (Vaziri-Sani et al. 2010, Vermeulen et al. 2011).

According to our results, HLA DR3/DR4 heterozygosity and the HLA DR3 allele were associated with a decreased frequency of ZnT8A. ZnT8 is an important target of autoreactive T-cells in human type 1 diabetes and high-risk HLA molecules bind a series of ZnT8-derived peptide pools. Interestingly, binding to HLA DR3 is restricted to significantly fewer peptide pools than the ones binding to HLA DR4 (Dang et al. 2011). The negative association between ZnT8A and HLA DR3 allele and the lower frequency of ZnT8A in the younger patients were confirmed in a recent study by our group (Juusola et al. 2016). Surprisingly, though, in the more recently published study, testing positive for ZnT8A was positively associated with ketoacidosis at diagnosis, unlike in Study I. One explanation for this is that in the study by Juusola et al. the study population was collected about 15 years earlier, in 1986-1989, and both the age and other risk factors for ketoacidosis (Hekkala, Knip & Veijola 2007) and most likely also the distribution of age at diagnosis were different (Harjutsalo, Sjöberg & Tuomilehto 2008).

Considering Studies I and II together, associations existed between ZnT8A and both circulating concentrations of sRAGE and a polymorphism of

the AGER gene. The frequency of ZnT8A was higher among carriers of the minor allele of the rs9469089 SNP than among the other patients. Although both of these associations remained significant after controlling for the effect of age, sex, and HLA genotype with a logistic regression model, the clinical relevance of this finding remains open.

6.3 CIRCULATING CONCENTRATION OF SRAGE AND CML IN PRECLINICAL AND CLINICAL DIABETES (STUDIES II-IV)

Study III showed that children carrying an HLA genotype that is associated with an increased risk for type 1 diabetes and progressing to clinical disease have higher sRAGE concentrations than the matched controls, prior to the emergence of the first autoantibodies. Levels of CML were similar in cases and controls before the appearance of islet autoantibodies in the prediabetic children, but the sRAGE/CML ratio was different. Elevated concentration of sRAGE and higher sRAGE/CML ratio could reflect a defense mechanism against the commenced autoimmune process, or simply a dysregulated AGE-RAGE interaction in prediabetic children. There are studies suggesting that events early in life can affect later sRAGE concentration (Chiavaroli et al. 2012, Rogers et al. 2013). Dietary AGEs can induce inflammation (Uribarri et al. 2015), and even though we could not see a difference in the CML concentration between the cases and the controls before seroconversion, the difference present in the CML/AGE ratio is interesting since the AGE content of daily consumed food influences the AGE pool and the amount of oxidative stress in the human body (Uribarri et al. 2007a). The biological effects of AGE-RAGE interaction offer an interesting link between genetic and environmental factors and increasing incidence of type 1 diabetes in developed countries. The AGE content of commonly consumed foods has risen during the past decades due to increased processing (Goldberg et al. 2004). A recent study derived from the DIPP project identified an association between consumption of processed meat during pregnancy and increased risk for type 1 diabetes in offspring (Niinistö et al. 2015). AGE levels of the mother influences the AGE levels of the infant (Mericq et al. 2010). The increased amount of AGEs in industrially produced infant formula relative to breast milk has the same effect (Mericq et al. 2010, Sebekova et al. 2008). However, unequivocal evidence supporting the theory that breastfeeding is protective against type 1 diabetes is lacking (Knip, Simell 2012). These results suggest that the RAGE/AGE axis may be involved in the process leading to type 1 diabetes, but more information on possible excess AGE load or dysfunction in the AGE processing mechanisms in children progressing to type 1 diabetes is needed.

We observed a decline in the sRAGE concentrations coinciding with seroconversion in children who were later diagnosed with type 1 diabetes, confirming the results of an earlier animal study and a pilot study on a few individuals (Forbes et al. 2011). Such a decrease in sRAGE concentration was not detected in the autoantibody-negative matched controls or in the children seroconverting to autoantibody positivity later during childhood, not progressing to clinical type 1 diabetes during follow-up. A drop in the sRAGE concentration has also been reported in other acute inflammatory states such as Kawasaki disease (Wittkowski et al. 2007), rheumatoid arthritis (Pullerits et al. 2005), and multiple sclerosis (Glasnovic et al. 2014). In these conditions, however, the acute phase of the inflammatory process is clinically more apparent than in the case of the initiation of humoral beta-cell autoimmunity. However, this thesis work supports the hypothesis of RAGE involvement in the initiation of beta-cell autoimmunity, which was proposed in an earlier study (Forbes et al. 2011). It would be interesting to analyze the changes in sRAGE further if the exact time of seroconversion could be determined. The study visits in the DIPP study occur with an interval of a few months, which potentially affects our results. Presumably the change in sRAGE is underestimated here since the compensatory mechanisms observed in other diseases have already started in many cases.

The circulating sRAGE concentration at the time of diagnosis is associated with age at diagnosis of type 1 diabetes according to Study II. The inverse association between circulating sRAGE concentration and age at diagnosis as well as the associations between low sRAGE, ketoacidosis at diagnosis, and HLA DR3/DR4 heterozygosity indicate that reduced sRAGE concentrations reflect a more aggressive disease process. In Study III, an inverse correlation existed between age and circulating sRAGE concentration in the control group (mean age 2.8 years), in concordance with the results of Study II; children <2 years of age had the highest sRAGE concentrations in the series of non-diabetic children aged 0-15 years. The fact that the highest sRAGE concentrations in the control groups in Studies II and III were indeed seen in the youngest age group suggests that low sRAGE is truly associated with type 1 diagnosis at an early age. However, later on there was no correlation with age in the healthy controls. A 3-year observational study in an adult population indicated that the concentration of sRAGE is rather stable under normal conditions (Bower et al. 2014). According to Study IV, sRAGE concentrations remain stable also in prediabetes after the initial drop at seroconversion.

6.4 POLYMORPHISMS OF THE *AGER* GENE AND PHENOTYPE

The strong inverse association between sRAGE concentration and the predisposing minor allele of rs2070600 has been described previously (Gaens et al. 2009, Boor et al. 2010, Jang et al. 2007). The rs2070600 has a functional role in the RAGE/ligand interaction (Osawa et al. 2007). The minor allele of rs9469089, which was associated with protection against type 1 diabetes (Forbes et al. 2011), was associated with higher sRAGE concentration in Study II. This supports the theory of low sRAGE being a risk element for disease. The autoimmune process leading to type 1 diabetes might be influenced by the AGER genotype since it was associated with humoral autoimmunity against ZnT8 and insulin. Interestingly, two polymorphisms of the AGER gene were associated with an increased risk for type 1 diabetes according to an earlier study (Forbes et al. 2011): the minor allele of the rs2070600 and the major allele of the rs9469089, both of which were associated with decreased sRAGE concentration. This is in concordance with other findings in the current project and supports the hypothesis of RAGE playing a role in the pathogenesis of type 1 diabetes. This is a novel finding warranting further studies.

6.5 STRENGTHS AND LIMITATIONS OF THE STUDIES

Strengths of Study I include the large and well-documented population and the widely used laboratory analyses performed in a laboratory participating in regular international standardization efforts. The study generated novel findings regarding ZnT8A in relation to risk HLA genotypes and metabolic decompensation. Although no association between family history of type 2 diabetes and ZnT8A was observed, the negative result is of interest since 3% of patients with type 2 diabetes have Znt8A (Wenzlau et al. 2007). The study results confirm previous findings regarding the frequency of ZnT8A positivity observed in other diabetic populations and are generalizable to the Finnish pediatric population with type 1 diabetes. However, our study does not offer information on the clinical significance of ZnT8A positivity at diagnosis for the long-term outcome of the disease.

Study II was the first large study published to examine sRAGE and AGER polymorphisms in children and adolescents with type 1 diabetes, and the first one to report an association between sRAGE concentration and increasing age at diagnosis. One obvious limitation in of this study is the smaller size of the control population. We attempted, however, to minimize the effect of this limitation by carefully selecting the individuals in the control population.

They have similar distribution of age, sex, HLA risk genotypes, and residential area as the case population. The absolute differences in sRAGE concentrations between age groups or groups defined by the state of metabolic decompensation or HLA genotype are not large, although statistically significant. That is, however, often the case with biological variables, which have considerable variability between individuals. The same is true for the analyses of sRAGE changes in Study III. In addition, the 3- to 6-month interval between the study visits in the DIPP study is likely to affect the results, as mentioned earlier.

One of the strengths of Study III is the unique population of prediabetic children followed prospectively. Equally valuable is the control population, which we were able to match for many possible confounders. In contrast, in Study IV the differentiation of the two groups analyzed is not straightforward since it is likely that the vast majority of children with multiple diabetes-associated autoantibodies will eventually progress to clinical disease (Ziegler et al. 2013). Also in Study III, the direct statistical comparison of the changes between the groups was challenging. The studies are therefore more observational than analytical.

7 SUMMARY AND CONCLUSIONS

Study I was the first to report an inverse association between the HLA genotype with the highest type 1 diabetes risk in Caucasian populations and ZnT8A, and the first large study on ZnT8A in a pediatric population in Finland, the country with the highest incidence of type 1 diabetes incidence globally.

Although there are multiple studies suggesting that AGEs and RAGE might play a role in the pathogenesis of type 1 diabetes and the complications of diabetes, there are only a few studies on RAGE and AGEs in human pediatric populations. To our knowledge, the current thesis is the first to explore the involvement of circulating concentrations of sRAGE in the preclinical stage of type 1 diabetes in a human study population of considerable size, and the associations of sRAGE concentration and AGER polymorphisms in relation to the phenotype of children with newly diagnosed type 1 diabetes. The positive association between sRAGE concentration and age at diagnosis of type 1 diabetes together with the inverse association between sRAGE and high-risk HLA genotype and the risk for metabolic decompensation at diagnosis, reported in Study II, suggest that higher sRAGE is associated with protection against the most aggressive disease form characterized by young age at onset and diabetic ketoasidosis. Substudy II is the first to report a positive association between the protective allele of the AGER polymorphism rs9469089 and increased concentrations of the cytoprotective sRAGE, and the first to confirm the negative association between the minor allele of rs2070600 and sRAGE in a population of children with type 1 diabetes.

Substudies III and IV confirm the preliminary finding of a previous pilot study (Forbes et al. 2011) that a drop in sRAGE concentrations coincides with seroconversion to autoantibody positivity in children who progress to clinical type 1 diabetes. Furthermore, the results indicate that the sRAGE concentration is different in children progressing to type 1 diabetes already before the signs of humoral autoimmunity appear in the circulation compared with matched controls. The increased concentration of sRAGE in prediabetic children may be a protective mechanism against beta-cell destruction, while the decrease seen in serum RAGE close to the time of seroconversion to autoantibody positivity might reflect a failing protection, which is supported by the coinciding decrease in the sRAGE/CML ratio. The current findings suggest that sRAGE may be more involved in the initiation of autoimmunity against beta cells and less important in the later progression to clinical type 1 diabetes.

8 FUTURE PROSPECTS

The results of this thesis raise many questions for future studies. It would be of interest to assess the cumulative AGE load of prediabetic children prior to the seroconversion to autoantibody positivity and compare it with that of matched controls. Our collaborators at the National Institute for Health and Welfare (THL) already have plans for such a study. Subclass analysis of the islet cell autoantibodies could be one way to pinpoint the exact time of seroconversion, identifying affected individuals with the most relevant samples available, although the number of eligible subjects could be quite small. Many studies have shown the association between RAGE and kidney function (Zorena et al. 2013, Schmidt 2015). Our colleagues at the University of Queensland, Brisbane, Australia are analyzing the cystatin-C concentrations in the DIPP children included in this study. It will be interesting to explore whether there are any associations between the kidney function and sRAGE concentration in these children. Finally, we are planning to apply Mendelian randomization to the AGER polymorphism data of Study II to further analyze the effect of the SNPs.

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