Aspects of autoantibody epitopes in type 1 diabetes

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Aspects of autoantibody epitopes in type 1 diabetes

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Title: Aspects of autoantibody epitopes in type 1 diabetes

Abstract: Type 1 diabetes (T1D) is strongly associated with autoantibodies against insulin (IAA), glutamic acid decarboxylase 65 (GADA), insulinoma-associated protein 2 (IA-2A) and most recently identified zinc transporter 8 (ZnT8A). Together or alone, they are important both to predict T1D and to classify diabetes at the time of clinical onset. The single nucleotide polymorphism (SNP) rs13266634 in the SLC30A8 gene encodes either an arginine (R) or a tryptophan (W) at the amino acid position 325 in the ZnT8 protein. Autoantibodies that recognize ZnT8R (ZnT8RA), ZnT8W (ZnT8WA), or both, but restricted by the polymorphic site of the ZnT8 protein are common in T1D patients. However, the epitope specificity and affinity of ZnT8A are poorly understood. The autoantigenicity of the ZnT8 polymorphism is unique and comparable protein sequence variations are not found for insulin, GAD65 or IA-2. Neuropeptide Y (NPY) was reported as a minor autoantigen in T1D. This neurotransmitter has a SNP, rs16139, at amino acid position 7 in the NPY gene. The major allele is coding for leucine (L), and the minor for proline (P). The latter has been associated with impaired glucose tolerance and increased risk for type 2 diabetes (T2D). However, the possible autoantigenicity of the NPY polymorphism in T1D has not been investigated. The overall aim of this thesis was to investigate the autoantibody epitopes of ZnT8 and NPY in newly diagnosed T1D patients as well as in patients with long-term T1D and T2D. Our data in paper I and IV suggest that the polymorphic 325 variant is a major determinant of a conformation-dependent ZnT8A epitope. However, human sera with ZnT8-specific autoantibodies against either the R or the W variant did not recognize ZnT8 (318-331) peptides. It was therefore suggested that the conformational epitope recognized by the ZnT8A requires yet other amino acids beyond the 318-331 peptide of ZnT8. In addition, newly diagnosed T1D patients with specific ZnT8WA displayed higher affinity compared to patients with specific ZnT8RA as demonstrated in reciprocal competitive displacement experiments. In order to investigate the presence of NPY autoantibody (NPYA) variants (L or P at amino acid position 7), we developed radiobinding assays for both variants. We identified NPY-LA (23%) and NPY-PA (19%) in long-term T1D and 12% and 23% in T2D patients, respectively. The frequency of NPYA in newly diagnosed T1D patients at 1-19 years of age was 17% for NPY-LA and 24% for NPY-PA. In statistical regression analyses adjusted for gender, HLA and autoantibody status, NPYA were more common in children with older age at onset compared to children at younger age at onset. We suggest that NPYA may prove useful in the screening of young adults and in patients with long-term diabetes. This thesis has revealed novel insights of the ZnT8A affinity to the 325-epitope and the possible importance of autoantibody affinity in T1D. Novel insights also include NPY as a minor autoantigen of significance to diabetes etiology and pathogenesis. Both epitope-specific ZnT8A and NPYA are suggested to be included in future attempts to identify islet autoimmunity and to predict clinical onset of autoimmune T1D.

Key words: Autoimmune (type 1) diabetes, ZnT8 autoantibodies, Affinity, Minor autoantigens Neuropeptide Y,

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”I livets lopp finns det inget målsnöre. De snören vi passerar är starten till ett nytt lopp.”

Okänd

"The minute you think of giving up, think of the reason why you held on so long."

Unknown

ABSTRACT

Type 1 diabetes (T1D) is strongly associated with autoantibodies against insulin (IAA), glutamic acid decarboxylase 65 (GADA), insulinoma-associated protein 2 (IA-2A) and the most recently identified zinc T8 transporter (ZnT8A). Together or alone, they are important both to predict T1D and to classify diabetes at the time of clinical onset. The single nucleotide polymorphism (SNP) rs13266634 in the SLC30A8 gene encodes either an arginine (R) or a tryptophan (W) (R325W) at the amino acid (aa) position 325 in the ZnT8 protein. Autoantibodies that recognize ZnT8-arginine (ZnT8RA), ZnT8-tryptophan (ZnT8WA), or both, but restricted by the polymorphic site of the ZnT8 protein are common in newly diagnosed T1D patients. However, the epitope specificity and affinity of ZnT8A are poorly understood. The autoantigenicity of the ZnT8 polymorphism is unique and comparable protein sequence variations are not found for insulin, GAD65 or IA-2.

Neuropeptide Y (NPY) was reported as a minor autoantigen in T1D. This neurotransmitter has a SNP, rs16139, at aa position 7 in the NPY gene. The major allele is coding for leucine (L), and the minor for proline (P). The latter has been associated with impaired glucose tolerance and increased risk for type 2 diabetes (T2D). However, the possible autoantigenicity of the NPY polymorphism in T1D has not been investigated.

The overall aim of this thesis was to investigate the autoantibody epitopes of ZnT8 and NPY in newly diagnosed T1D patients as well as in patients with long-term T1D and T2D.

Our data in paper I and IV suggests that the polymorphic 325 variant is a major determinant of a conformation-dependent ZnT8A epitope. However, human sera with ZnT8-specific autoantibodies against either the R or the W variant did not recognize ZnT8 (318-331) peptides. It was therefore suggested that the conformational epitope recognized by the autoantibodies requires yet other amino acids beyond the 318-331 peptide of ZnT8. In addition, T1D patients with specific ZnT8WA displayed higher affinity compared to patients with specific ZnT8RA as demonstrated in reciprocal competitive displacement experiments. We suggest that future studies should include the ZnT8A variant specificity in both humoral and cellular tests to understand the possible role of autoantibody affinity to predict T1D.

In order to investigate the presence of NPY autoantibody (NPYA) variants (L or P at aa position 7), we developed radiobinding assays for both variants. We

identified NPY-LA (23%) and NPY-PA (19%) in long-term T1D and 12% and 23% in T2D patients, respectively. The frequency of NPYA in newly diagnosed T1D patients at 1-18 years of age was 17% for NPY-LA and 24% for NPY-PA. In statistical regression analyses adjusted for gender, HLA and autoantibody status, NPYA were more common in children with older age at onset compared to children at younger age at onset. We suggest that NPYA may prove useful in the screening of young adults and in patients with long-term diabetes. The autoantibody response against the L7P polymorphic site was rarely specific to any of the two amino acid variants.

This thesis has revealed novel insights of the ZnT8A affinity to the 325-epitope and the possible importance of autoantibody affinity in T1D. Novel insights also include NPY as a minor autoantigen of significance to diabetes etiology and pathogenesis. Therefore, both epitope-specific ZnT8 and NPY autoantibodies are suggested to be included in future attempts to identify islet autoimmunity and to predict the clinical onset of autoimmune T1D.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

I Sverige insjuknar två barn i autoimmun diabetes (typ 1 diabetes) varje dag. Typ 1 diabetes (T1D) är en kronisk autoimmun sjukdom som kan få livshotande konsekvenser vid utebliven behandling. Sverige har den högsta incidensen av (T1D) i hela världen efter Finland. Förutom att den delvis är ärftlig vet man inte vilken eller vilka faktorer som triggar sjukdomen. Sedan mitten av 1920-talet finns behandling i form av insulin, vilken måste injiceras i kroppen i samband med varje måltid under hela livet. Är inte sjukdomen under ständig kontroll kan ett barn få allvarliga komplikationer som hjärnödem och i värsta fall hamna i koma. Insulin är en livsnödvändighet, som inte finns att tillgå i alla delar av världen.

Attackerande immunförsvar bryter ned bukspottkörteln

T1D uppkommer för att immunsystemet, som vanligen skyddar oss genom att bekämpa bakterier och virus, istället attackerar frisk vävnad. En av anledningarna till att det händer är att toleransen mot kroppsegen vävnad bryts. Ett exempel är när immunsystemet dödar de insulinproducerande (β celler) cellerna i bukspottskörteln. Bukspottkörteln är ett viktigt organ eftersom det är källan till insulinproduktion och dess reglering. Insulin är ett hormon som utsöndras i respons av en måltid varpå insulinet kan liknas vid en nyckel som låser upp porten till vår vävnad. Utan nyckeln hålls porten stängd och näring från måltiden kan inte passera in i vävnaden. Kroppen kan således inte tillgodogöra sig näringen.

Autoantikroppar är viktiga markörer

Det är sedan länge känt att symptom av T1D först uppkommer när ungefär 80-90% av bukspottkörteln är nedbruten och inte längre kan producera tillräckligt med insulin. Nedbrytningen av bukspottkörteln kan starta redan vid 9 månaders ålder eller så sent som efter tonåren. Tiden från att nedbrytningen har startat till insjuknande kan dock variera från veckor till flera år hos personer med risk för att utveckla T1D. Denna obestämda tid representeras av autoantikroppar som är

riktade mot särskilda proteiner i bukspottkörteln. Dessa specifika autoantikroppar finns i blodet hos ett barn som kommer att insjukna och kan med hjälp av laboratoriska metoder detekteras. Autoantikroppar fungerar därför som en markör både innan insjuknande (under tiden vid nedbrytning) och vid insjuknande då diagnos fastställs. Det medför att vi idag bättre kan förutspå ett barns utveckling av sjukdomen. Autoantikropparnas roll och varför de finns i blodet är dock ännu okänd.

Det främsta målet med dagens forskning är att förhindra sjukdomsutvecklingen av T1D och därmed kunna rädda bukspottkörteln innan insulinkällan har sinat. I denna avhandling har en del (studie I och IV) fokuserats på att studera autoantikropparna mot Zink transportör 8 (ZnT8A) och den andra delen (studie II och III) på att identifiera en ny autoantikropp riktad mot Neuropeptid Y (NPYA).

Zink transportör 8 (ZnT8) – proteinet med högst specificitet

Under 2007 visade en forskargrupp från USA att ett särskilt protein, ZnT8, i bukspottkörteln agerar måltavla för immunförsvarets attack. ZnT8-proteinets uppgift är att transportera zink in i insulin-vesiklarna i cellerna, vilket i möss har visats vara ytterst nödvändigt för insulinproduktion. Upp till 80 % av de barn som drabbas av T1D har ZnT8A i blodet, men när och hur denna markör uppstår är inte känt. Tre autoantikropps-varianter, mot ZnT8 har hittills upptäckts arginin (R), tryptofan (W) och glutamin (Q). Man kan ha en, två eller alla tre varianter. Vad som orsakar att man får en eller flera är inte klarlagt. Samtliga autoantikropps-varianter har tidigare visats binda till en särskild del (epitop) av ZnT8-proteinet. Denna epitop styrs av en genförändring (polymorfi), som bestämmer vilken variant av R, W eller Q i genomet som man har. ZnT8 autoantikropparna har förmågan att urskilja de olika varianterna i genomet och studier har visat att har man R i genomet så har man oftare R autoantikroppar. Det betyder att de tre varianterna av autoantikropparna kan se skillnad på endast en aminosyra (R, W eller Q) och har därmed extremt hög specificitet. Trots att detta är känt har den exakta epitopen ännu inte identifierats. Syftet i studie I och IV var därför att närmre studera hur ZnT8 autoantikroppar binder till epitopen av ZnT8-proteinet samt mäta autoantikroppsbindingen till epitopen.

Neuropeptid Y – ny måltavla för immunförsvaret?

Neuropeptid Y (NPY) är en neurotransmittor som mestadels uttrycks i det centrala nervsystemet, men även i det perifera nervsystemet som innerverar bukspottkörteln. NPY uttrycks tillsammans med insulin i insulin-vesiklar och har till uppgift att bland annat stimulera aptit. Ett flertal studier har identifierat att en särskild polymorfi i NPY-genen var vanligare hos överviktiga och typ 2 diabetes (T2D) patienter. Denna polymorfi medför att man istället för aminosyran Leucin (L) har Prolin (P) på en position i NPY-proteinet. Utöver NPYs roll inom T2D har NPY associerats med T1D. Under 2008 rapporterade en studie att ungefär 9% av nyligen insjuknade typ 1 diabetespatienter hade autoantikroppar mot NPY. Därför var syftet i studie II att utveckla en ny metod för att identifiera NPY autoantikroppar. I studien undersöktes NPY autoantikropparna i patienter som hade haft typ 1 eller typ 2 diabetes under 10-40 år. I studie III studerades NPY autoantikroppar hos patienter som nyligen insjuknat i T1D. Vi ville även studera likheter och skillnader mellan NPY autoantikropparna och de tidigare identifierade autoantikropparna vilka bland annat innefattade de tre ZnT8 autoantikropps-varianterna.

ZnT8 autoantikropparna skiljer sig i affinitet

I samarbete med ett företag och en annan forskargrupp i Lund producerade vi olika långa delar (peptid och protein) av ZnT8-proteinet. Alla dessa olika proteindelar omfattade den antagna epitopen till vilken ZnT8 autoantikropparna binder. För att identifiera den exakta epitopen samt hur autoantikropparna binder till epitopen utvecklade vi en ny laborativ metod. Metoden tillät oss att studera ZnT8 autoantikropparna i blodet hos nyligen insjuknade typ 1 diabetespatienter.

I studie I och IV, rapporterade vi att epitopen av ZnT8 omfattade en större del än vad som tidigare uppskattats. Epitopen omfattade mer än 15 aminosyror och sträcker sig troligen utöver aminosyra position 318 till 331 av ZnT8-proteinet. Vi kunde även bekräfta att ZnT8 autoantikropparna är konformationsberoende. Det betyder att ZnT8-proteinet har en särskild struktur som känns igen av autoantikropparna. Vidare fann vi att ZnT8*W* autoantikropps-varianten hade starkare bindning (högre affinitet) till ZnT8-proteinet i jämförelse med ZnT8*R* autoantikropps-varianten hos patienter med T1D.

NPY autoantikroppar finns i blodet hos patienter med typ 1 diabetes

I studie II utvecklades två laborativa antikropps-metoder som skulle kunna identifiera två varianter av NPY autoantikroppar, *Leucin (L)* och *Prolin (P)*. Metoderna verifierades i labbet och ansågs ha en hög precision. Vi fann att patienter som haft typ 1 eller typ 2 diabetes i upp till 40 år hade NPY autoantikroppar i blodet. NPY autoantikropparna kunde identifieras hos 29% av T1D patienterna i jämförelse med 13% av T2D patienterna. 15% av patienterna med T1D hade båda varianterna av autoantikroppar, NPY-*L* och NPY-*P*.

Utöver våra fynd i studie II identifierade vi att 26% av nyligen insjuknade T1D patienter hade NPY autoantikroppar (studie III). NPY autoantikropparna var vanligare bland barn som var *äldre* än 10 år vid diagnostillfället i jämförelse med barn som var *yngre* än 10 år. NPY autoantikropparna visade sig även förekomma oftare bland flickor än hos pojkar. Genom att analysera T1D patienter för NPY autoantikroppar kunde ca 1% av de tidigare autoantikropps-negativa patienterna betraktas som autoantikropps-positiva. Således bidrog NPY autoantikroppen till en förbättrad diagnostisk känslighet, vilket betyder att NPY autoantikroppen som ensam markör skulle kunna identifiera barn som riskerar att utveckla T1D.

Slutsats och framtidsperspektiv

Sammanfattningsvis så har vi utvecklat två nya metoder. Dessa har tillåtit oss att studera ZnT8 autoantikroppars affinitet samt att identifiera en ny autoantikropp riktad mot neurotransmittorn, NPY, hos patienter med T1D. Vi fann att affiniteten skiljer sig beroende på vilken variant av ZnT8 autoantikropp T1D patienter hade. Det har spekulerats om att affiniteten av autoantikroppar kan vara viktig eftersom den kan ha betydelse vid utvecklingen av T1D. Vi anser därför att fler affinitets-studier av autoantikroppar bör genomföras för att se huruvida affinitet kan bidra till att förutspå att ett barn kommer att insjukna i T1D. Vi har även visat att NPY autoantikroppar både finns hos patienter med lång duration och bland dem som nyligen har insjuknat i T1D. Vi påvisade även NPY autoantikroppar hos patienter som inte hade någon av de andra autoantikropparna. Vi föreslår att analys av NPY autoantikroppar bör göras som ett komplement till redan etablerad autoantikropps-analys. Detta skulle kunna gynna personer som vid diagnostillfället inte har identifierats med andra autoantikroppar, eller som har en ökad risk att insjukna. Kostnaden av NPY autoantikropps-analysen bör dock vägas mot nyttan av att identifiera autoantikropps-positiva individer vid diagnos.

Slutligen är det av stor betydelse att öka vår kunskap om diabetesrelaterade markörer, såsom autoantikroppar, eftersom de är viktiga diagnostiska verktyg. Genom att studera autoantikropparnas roll för att förutspå T1D kan vi förhindra att barn som håller på att insjukna hamnar i ett livshotande tillstånd.

LIST OF PAPERS

Papers included in this thesis

I. **Skärstrand H**, Lernmark Å and Vaziri-Sani F. **Antigenicity and epitope specificity of ZnT8 autoantibodies in type 1 diabetes**. *Scand J Immunol*. 2013 Jan;77(1):21-9.

II. **Skärstrand H**, Dahlin LB, Lernmark A, Vaziri-Sani F. **Neuropeptide Y autoantibodies in patients with long-term type 1 and type 2 diabetes and neuropathy**. *J Diabetes Complications.* 2013 Nov-Dec;27(6):609-17.

III. **Hanna Skärstrand**, Fariba Vaziri-Sani, Ahmed J Delli, Carina Törn, Helena Elding Larsson, Sten Ivarsson, Åke Lernmark and the Skåne study group. **Neuropeptide Y is a minor autoantigen in newly diagnosed type 1 diabetes mellitus patients**. *Submitted for publication.*

IV. **Hanna Skärstrand**, Ewa Krupinska, Tatu Haataja, Fariba Vaziri-Sani, Jens O Lagerstedt, and Åke Lernmark. **ZnT8 autoantibody epitope specificity and affinity examined with recombinant ZnT8 variant proteins in specific ZnT8R and ZnT8W autoantibody positive type 1 diabetes patients**. *In manuscript*.

Papers not included in this thesis

I. Kanatsuna N, Taneera J, Vaziri-Sani F, Wierup N, Larsson HE, Delli A, **Skärstrand H**, Balhuizen A, Bennet H, Steiner DF, Törn C, Fex M, Lernmark Å. **Autoimmunity against INS-IGF2 protein expressed in human pancreatic islets.** *J Biol Chem*. 2013 Oct 4;288(40):29013-23.

II. Pourhamidi K, **Skärstrand H**, Dahlin LB, Rolandsson O. **HSP27 concentration are lower in patients with type 1 diabetes and and correlate with large nerve fiber dysfunction**. *Diabetes Care*. 2014 Mar;37(3).

III. Flannick J, Thorleifsson G, Beer NL, Jacobs SB, Grarup N, Burtt NP, Mahajan A, Fuchsberger C, Atzmon G, Benediktsson R, Blangero J, Bowden DW, Brandslund I, Brosnan J, Burslem F, Chambers J, Cho YS, Christensen C, Douglas DA, Duggirala R, Dymek Z, Farjoun Y, Fennell T, Fontanillas P, Forsén T, Gabriel S, Glaser B, Gudbjartsson DF, Hanis C, Hansen T, Hreidarsson AB, Hveem K, Ingelsson E, Isomaa B, Johansson S, Jørgensen T, Jørgensen ME, Kathiresan S, Kong A, Kooner J, Kravic J, Laakso M, Lee JY, Lind L, Lindgren CM, Linneberg A, Masson G, Meitinger T, Mohlke KL, Molven A, Morris AP, Potluri S, Rauramaa R, Ribel-Madsen R, Richard AM, Rolph T, Salomaa V, Segrè AV, **Skärstrand H**, Steinthorsdottir V, Stringham HM, Sulem P, Tai ES, Teo YY, Teslovich T, Thorsteinsdottir U, Trimmer JK, Tuomi T, Tuomilehto J, Vaziri-Sani F, Voight BF, Wilson JG, Boehnke M, McCarthy MI, Njølstad PR, Pedersen O, Groop L, Cox DR, Stefansson K, Altshuler D. **Loss-of-function mutations in SLC30A8 protects against type 2 Diabetes**. *Nat Genet* 2014 Mar (1–8).

ABBREVIATIONS

APC, Antigen presenting cell

BCR, B cell receptor

CiPiS, Celiac Prediction in Skåne

Cl, Confidence interval

cDC, Conventional dendritic cell

DC, Dendritic cell

DiPiS, Diabetes Prediction in Skåne

FDR, First degree relative

Fc, Fragment crystallizable

FOXP3, Forkhead box protein P3

GABA, Gamma aminobutyric acid

GADA, Glutamic acid decarboxylase autoantibodies

GFP, Green fluorescent protein

GLP-1, Glucagon-like peptide-1

HLA, Human leukocyte antigen

HMBG1, High mobility group B1

HPLC, High performance liquid chromatography

IAA, Inulin autoantibodies

IA-2A, Insulinoma-associated protein 2 autoantibodies

IvGTT, Intravenous glucose tolerance test

ICA, Islet cell protein 12

IFN-γ, Interferon-gamma

Ig, Immunoglobulin

IL, Interleukin

INS, Insulin gene

LD, Linkage disequilibrium

MBP, Maltose binding protein

MS, Mass spectrometry

MHC, Major histocompatibility complex

NPY, Neuropeptide Y

NPY-L, Neuropeptide Y-Leucine

NPY-P, Neuropeptide Y-Proline

OGTT, Oral glucose tolerance test

OR, Odds ratio

PAGE, Polyacrylamide gel electrophoresis

PAMP, Pathogen-associated molecular pattern

pDC, Plasmacytoid dendritic cell

PLN, Pancreatic lymph node

PRP, Tyrosine phosphatase family

ROC, Receiver operating characteristic

RBF, Residual β-cell function

SDS, Sodium dodecyl sulfate

SOX, Sry-related HMG (high mobility group) box

T1D, Type 1 diabetes

TCR, T cell receptor

TEDDY, The Environmental Determinants of Diabetes in the Young

TLR, Toll-like receptor

Treg, Regulatory T cells

VAMP-2, Vesicle-associated membrane protein 2

VDJ, Variable diverse joining

ZnT8, Zinc transporter 8

ZnT8A, Zinc transporter 8 autoantibodies

ZnT8Q, Zinc transporter 8 Glutamine autoantibodies

ZnT8R, Zinc transporter 8 Arginine autoantibodies

ZnT8W, Zinc transporter 8 Tryptophan autoantibodies

GENERAL INTRODUCTION

Autoimmune (type 1) diabetes

Autoimmune diabetes, referred to as Type 1 diabetes (T1D) is a chronic autoimmune disorder caused due to the pancreatic beta-cells incapacity of producing insulin [1, 2]. The lack of insulin leads to a state of hyperglycemia [3], in which the consequences include the life-threatening state of ketoacidosis. The frequency of ketoacidosis at disease onset is 15-74% worldwide [4], more often affecting children less than five years of age [5]. T1D is a complex heterogenetic disease with a manifestation most commonly at the puberty [6], but can may be diagnosed as early as at one year of age or late in the adulthood [7, 8]. T1D results from the interplay between genetic susceptibility including both Human leukocyte antigen (HLA) and non-HLA related genes in a combination with environmental factors [9-11]. The disease complexity was already in 1950 postulated by Harris writing "we were dealing not with a single disorder but several genetically distinct diseases". However, previous view of the events occurring during the time of progression to clinical onset of T1D may need to be revised.

A milestone contributing to the knowledge of today was the discovery of pancreatic islet autoantibodies in 1974 [12]. Recent research suggests that the clinical onset of diabetes in T1D is occurring after a prodromal period of islet autoimmunity [13, 14]. The first step is that individuals with propensity for T1D defined by HLA risk genes, DR3-DQ2, DR4-DQ8 or both on chromosome 6, are exposed to a hypothetical trigger (Figure 1). The trigger is thought to be an environmental factor such as a virus [15-21]. The triggering event is inducing an autoimmune reaction against specific autoantigens in the pancreatic islet beta cells [22, 23]. The islet autoimmunity is reflected by the presence of autoantibodies to insulin (IAA), glutamic acid decarboxylase 65 (GADA), insulinoma-associated protein 2 (IA-2A) and Zinc transporter 8 (ZnT8A) [24-26].

Progression to clinical onset of diabetes may take months to years [27] but may be predictive by autoantibodies [28, 29]. Progression may be accelerated by an increased number of islet autoantibodies. The more autoantibodies, the higher is the risk for clinical onset of diabetes [14, 29]. The number of islet autoantibodies in combination with a virus infection dramatically increases the progression to clinical onset of T1D [23]. In addition, the intestinal microbiota composition, which affects the gut permeability to keep out infectious agents may contribute to the progression of clinical T1D onset [30, 31]. Insulitis characterized by immune

cell infiltration in the pancreatic islets appear to be a late event rather associated with clinical onset of diabetes [32] than islet autoimmunity as previously hypothesized [33]. At onset about one third of the T1D patients have insulitis [34] and show markedly reduced numbers of β cell in the islets [10, 35] perhaps due to a higher rate of β cell death [36] .

Hyperglycemia will ensue once β cells are lost or when the function of the remaining β cells is reduced. Severe clinical symptoms of diabetes such as vomiting, dehydration and brain oedema still occur in most children and young adults who develop clinical diabetes. Fortunately, the severe onset may be prevented in longitudinal studies of children with genetic risk for islet autoimmunity and T1D [14, 37-39].

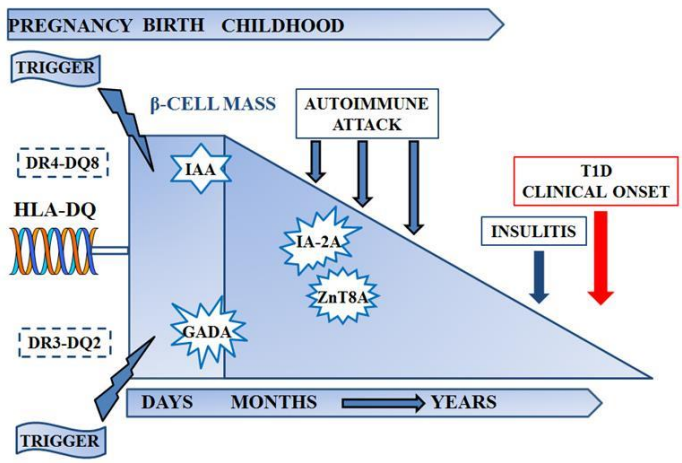

Figure 1. Schematic figure of possible factors contributing to the progression to clinical onset of T1D. It may be suggestive that a child is born with genetic susceptibility assessed by HLA-DQ and exposed by an environmental trigger such as a virus early in life, either during the intra-uterine environment or after birth. The trigger results in a prodromal stage of islet autoimmunity including appearance of autoantibody resulting in insulitis and eventually clinical onset of T1D.

The incidence of clinical onset T1D varies across the world with the highest incidence noted in Finland affecting >60/100,000/year followed by Sardinia and Sweden. The lowest incidence of 0.1/100,000/year was reported in China [6, 40]. It is striking that the average incidence rate is increasing by 3% per year independently of the geographical region. In Europe the highest incidence rate is among the youngest children between 0-4 years of age [40] and the reason is unidentified [6].

Up until 1921 when the scientists Frederick Banting, Charles Best and John Macleod were able to extract and isolate insulin from a healthy dog and injected it into a diabetic dog, subsequently lowering the blood glucose, diabetes was a fatal disease [41].

BACKGROUND

Genetic susceptibility– "the head quarter"

The susceptible genes may be considered to be *the head quarter* (the military base), which has the duty of controlling and navigating the immune system (the fighters) in T1D. It is well established that HLA class II genes on chromosome 6p21 are the most important genetic factors for T1D. It is believed that the genetic susceptibility of T1D is necessary, yet not sufficient for T1D to develop. The risk of being diagnosed with T1D among monozygotic twin patients was reported to approximately 40% [42, 43], although the cumulative incidence showed a frequency of 65% at the age of 60 years [44]. There is a 5% risk for a child to develop T1D by the age of 20 years if there is a family member with T1D compared to a risk of 0.3% in a family without T1D [45]. Therefore, the majority of the T1D patients do not have first degree relative (FDR) affected by T1D. Interestingly, the risk for an offspring to develop T1D is higher if the T1D affected parent is the father rather than the mother [46]. However, it should be pointed out that the association between T1D and HLA is likely to be secondary to the association between autoantigen reactivity and HLA.

Human leukocyte antigen (HLA)

In Caucasians, 40-50% of the general population carry HLA susceptibility genes, nevertheless, only about 10% of the individuals develop T1D [43]. More than 40% of the genetic risk for T1D is explained by the HLA genes [47, 48], which has been classified into three families, class I, class II and class III. The class II family, also analyzed in this thesis, has three regions, the DR, DQ and the DP. The alleles within these regions are particularly polymorphic and often found in linkage disequilibrium (LD) meaning they are commonly inherited together as haplotypes. The alleles in the DR- and DQ- regions of class II have been associated with an increased risk of T1D [49, 50], although to a various extent. In particular, the haplotypes, DR3 (DRB1*03) together with DQ2 (DQA1*05:01-DQB1*02:01) and DR4 (DRB1*04) together with DQ8 (DQA1*03:01-DQB1*03:02) confer the highest genetic risk present in nearly 90% of Caucasian T1D patients under the age of 35 years [51, 52]. The genotype, DR3-DQ2/DR4-DQ8, are present in 30% of T1D patients with on onset age below 15 years compared to 2% overall i [37, 53] but 3.2% in the general Swedish population [54].

The structure of the HLA class II molecule is represented by an alpha (α) and a beta (β) chain encoded by the DQA1/A2 allele and DQB1/B2 allele, respectively. For several years it has been known that allelic variations of DQ, as well as of DR and DP affect the antigen recognition and presentation to the immune cells [55], as well as linked to autoimmune responses [56]. More specifically, the autoimmune response may be modulated through the interactions between the HLA DR/DQ/DP molecule(s) expressed on the surface of antigen presenting cells (APC) and the T-cell receptor (TCR) on the T cells [57].

The immune system –"the fighters"

The immune system consists of two types of *fighters*, the fast non-specific (innate) and the slower specific (adaptive) immune fighters. Their main mission is to recognize and defend the body´s tissue against foreign pathogens by eliminating them in various ways. The innate immune defense is the first-line defense already activated and ready to prevent pathogens to replicate instantly or within a few hours. These innate immune cell fighters consist of monocytes, neutrophils and tissue macrophages acting through their surface membrane-bound toll-like receptors (TLRs) via recognition of the pathogen-associated molecular patterns (PAMPs) on the surface of the pathogens. The PAMPs helps the innate immune cells to distinguish between self- and non-self-antigens [58]. However, the recognition of what is a self-antigen (autoantigen) or a foreign antigen (antigen) is not obvious to the immune system in subjects at risk for T1D or in patients with the disease [9].

The adaptive immune response is represented by the lymphocytes, T- and B cells, and takes often between 24-48 h (T cells) and 14 days (B cells producing IgG antibodies) to develop once the primary response has been initiated (Figure 2). The adaptive immunity refers to the specific modifying (adapting) of these cells to become specialized in their response to eliminate particular pathogens. This specialization is an important feature of the adaptive immune response. The efficiency of the adaptive immunity is dependent on the communication with the innate immune system, in particular a type of a monocyte called dendritic cell (DC) [59].

Antigen presentation followed by T cell activation

DCs are specialized in their function to present pathogens to the adaptive immune cells and are therefore also termed antigen presenting cells (APCs). APCs are the guards surveying their surroundings in the body for foreign antigens. By phagocytosis they capture the antigen which leads to an activation, maturation and

migration to secondary lymphoid organs of the DC. There are two major classes of DCs identified, the plasmacytoid DCs (pDCs) and the conventional DCs (cDCs) [60]. The class of pDCs produces Interferon-1 (IFN-1) following activation of TLR-7 and TLR-9 while cDCs express high levels of the major histocompatibility complex (MHC), herein referred to as HLA, class II heterodimer molecules on their surface essential for antigen presentation [60].

In the lymphoid organs, such as lymph nodes, the APCs present the processed fragmented antigen, the epitope, lodged between α and the β chains as a trimolecular complex. This trimolecular HLA class II complex is presented on the APC surface [61]. A large pool of naive $CD4^+$ (helper) T cells with TCR specific for a unique antigen resides in the lymph nodes waiting for the APC. When the T cell recognizes the unique and matched antigen presented by the APC they interact via the CD4 and HLA class II molecules as well as with the TCR. This leads to T-cell priming and further clonal expansion and differentiation of T cells. Also, APCs are important for the T-cell proliferation and differentiation by producing cytokines and up-regulating co-stimulatory molecules [60]. The activation of T cells results in a differentiation into different T-cell subsets. The development of different T cell subset is dependent on the stimulatory signals and factors in the milieu surrounding the T cell.

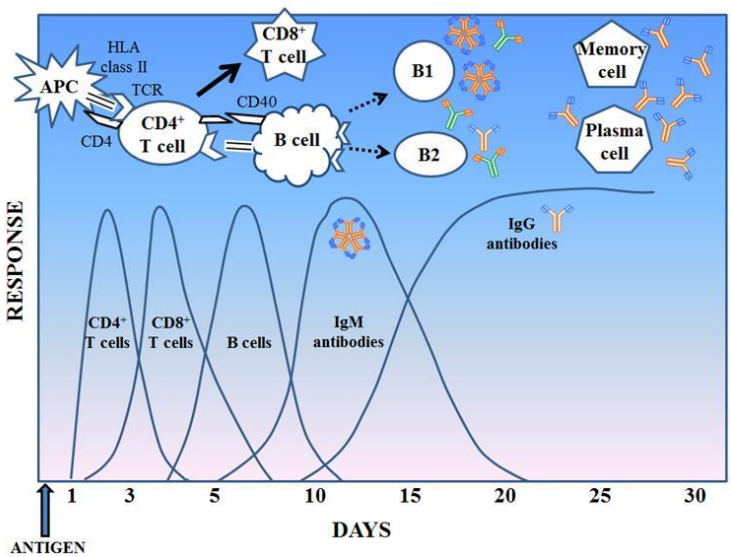

Figure 2. Schematic figure of the immune response to a primary antigen over the first 30 days. During the first 12-20 hours prior antigen stimulation the Antigen presenting cells (APCs) become activated for antigen presentation to the $CD4^+$ T cells in the lymph nodes. The $CD4^+$ T cells are primed through the interaction between the HLA class II molecule and the TCR followed by clonal selection expansion of the $CD4^+$ T cells. The activated $CD4^+$ T cells can interact with B cells for proliferation. Eventually, antibody production and by terminally differentiated plasma cells and specific memory B cells are generated.

T-cell subsets

T cells can polarize to become distinct subtypes of $CD4^+$ T cells. The differentiation has been simplified to indicate that T helper-1 response (Th-1 cells) is induced by e.g. cytokines such as interferon-gamma (IFN-γ) and interleukin-2 (IL-2). In addition to IL-2, $CD4^+$ T helper-2 (Th-2 cells) are thought to be promoted by IL-4, and IL-12. The differentiation reaction is also affected by the strength and duration of the TCR stimulation [62]. During the polarization specific receptors for chemokines that are necessary for migration to sites of inflammation are expressed. $CD4^+$ T cells are frequently involved in the activation of other cell types, such as the B cells.

Another subset of T cells are the $CD8^+$ T cells, which can differentiate into cytotoxic $CD8^+$ T cells with the assessment of killing virus-infected cells to prevent expansion of the virus [62]. Depending on factors such as duration of TCR stimulation and cytokine signaling, activated T cells are progressively pushed through differentiation [62]. During an immune response it was hypothesized that non-polarized T cells may become partly differentially into memory cells during the primary antigen stimulation. The T cell differentiation is completed if the T cell encounters the specific antigen a second time. The survival of T cells has been speculated to be dependent on a threshold of stimulation, which the T cells must reach above [62]. Both the $CD4^+$ and $CD8^+$ T cells are capable to establish memory, thereby upon re-activation respond quicker than before. T cells that have developed specificity against an antigen can be detected several years after the primary response

In recent years, the knowledge of naturally occurring regulatory T cells (Treg) has expanded. Tregs uniquely express $CD4^+CD25^+$ together with Forkhead box protein P3 (FOXP3) and are therefore useful markers distinguished from the $CD4^+$ and $CD8^+$ T cells [63]. Following the Treg production in thymus, the survival of the Treg in the periphery depends on the antigen recognition and IL-2, similar to the conventional $CD4^+$ T cells. Upon antigen stimulation via their TCR, Tregs enter a suppressive mode although non-specifically. Although, the Tregs have the capability to suppress the immune response in an antigen-specific manner, the exact know function remains elusive [63, 64].

Antigen presentation, T-cell help followed by B-cell activation

B cells express Immunoglobulin (Ig) as a complex of the unique B-cell receptor (BCR) on their surface, which is important for the B-cell recognition and activation. In contrast to T cells, the B cells recognize soluble antigens in its native form via their membrane-bound BCR [65]. Upon antigen recognition the B cells become activated via either the T-cell dependent or the T-cell independent way

[66]. The T-cell dependent activation requires that an antigen has already been processed to smaller peptides by an APC prior to a T-cell activation and also B-cell activation. The T-cell independent activation occurs when the B cell binds to an antigen directly through recognition via the Ig molecule on the surface. Following antigen recognition the B cell can present the processed peptide-antigen via the HLA class II on the surface, thereby acting as an APC. B cells interact with antigen-specific $CD4^+$ T cells to become stimulated and undergo clonal expansion and differentiation [67]. Furthermore, the B cells require co-stimulatory signals by proliferative cytokines, IL-4 and IL-5 [62].

Although, the B cell capacity of antigen presentation in comparison to the DCs may not be as efficient it was shown that their antigen presentation was enhanced following specific recognition of antigen [68] and that it is required for a proper T cell response following the antigen stimulation [69]. In addition, the surface Ig molecule is involved in the uptake and concentration of antigen, allowing specific B cells to present antigen to T cells with very high efficiency. It is evident that the B cells can respond to antigens via many mechanisms dependent on the nature of the antigen as well as the cellular milieu where upon the antigen presentation and activation occurs [65].

The factors that determine the fate of the activated B cells are still unidentified. However, it is known that the B cells may differentiate along two separate pathways [70]. They could differentiate to extrafollicular plasmablasts to initiate a rapid antibody production essential for an early immune response. The antibodies generated at this stage are often of low affinity [71]. B cells may also migrate to the germinal center to differentiate into plasma cells and undergo affinity maturation or become memory B cells with a long-lasting memory of protection through their continuous IgG secretion [70].

Plasma cells

Plasma cells produce antibodies which are of the same type as the Ig molecule expressed on their surface [72]. The antibodies are secreted into the blood and can bind to circulating soluble antigen resulting in neutralization of the antigen. The binding of the antibody and antigen are recognized via the Fragment crystallizable (Fc) region of the antibody which interacts with the Fc receptor of the monocytes. This interaction allows the monocytes to engulf the antigen-antibody complex for destruction. Most long-lived plasma cells in the bone marrow are dependent upon T cell activation [65]. It was proposed that two particular subtypes of B cells, B1 and B2 cells exist with the differences in their need of T cells. B1 cells express higher amount of polyspecific IgM resulting in responses of low affinity but with broader specificity. B1 cells receptors produce antibodies in a T-cell independent

manner while the B2 cells are the conventional B cells activated through the T-cell dependent way [66].

During the differentiation after the B cells have been activated by a primary immune response, they undergo genetic somatic hypermutations [71]. This is a process happening every time they are stimulated with the antigen to proliferate. Somatic hypermutations are nucleotide substitutions that occur in the re-arranged variable (v) -region of the Ig molecule to improve the affinity to the specific antigen. These mutations do not completely happen randomly but favor hotspots associated with the structure. Through the process of somatic hypermutations the B cells are able to develop a secondary antibody repertoire with an increased diversification, which occurs first after day 6 of the primary response (Figure 2) [71]. Typically, the expressed antibodies in a given response carry anywhere up to 20 amino acid (aa) substitutions within the genes of the v-region, with the amount of mutation broadly increasing as the response matures. Eventually, the B cells with the highest affinity generated by the somatic hypermutations will be the B cells surviving. The selection is thought to be a competitive process in which B cells compete with free antibody to capture decreasing amounts of antigen [65]. Generally, the B cells are driven to somatic hypermutations in the germinal centres although not in all germinal centres [73].

Memory B cells

An important feature of the adaptive immune response is the establishment of "memory" by the memory B cells. The memory B cell encompasses the specificity for an antigen that it has already encountered. Therefore, they have the ability to quickly become re-activated in a response to the secondary meeting of the antigen. Memory cells express high-affinity antibodies and can quickly differentiates into a plasma cell during the secondary response [65]. The plasma cells are relatively short-lived compared to the memory B cells that can survive more than 10 years.

Taken together, an inclusive explanation of the mechanisms by which B cells recognize and respond to antigen are important for both treatments and prevention of autoimmune diseases, such as T1D as well as for the improvement of vaccination strategies [65].

Tolerance induction

Normally self-tolerance is induced during fetal and neonatal life being a crucial part of the maturation of the T and B cells in the thymus and bone marrow, respectively. Constantly during the process of T cell maturation tissue-derived self-antigens are presented by the HLA class II molecule at the surface of the

APCs to the TCR on the T cells [74]. This is to make sure that the immune system constantly generate new T and B cells in order to maintain central and peripheral self-tolerance [75, 76]. The T cells binding with a high affinity to HLA class II molecule presenting self-antigens are eliminated by thymic medullary epithelial cells and by the cDCs and pDCs [77]. This is a mechanism to prevent fully mature and differentiated self-reactive T cells in the end of the maturation. Furthermore, the T cells escaping the central tolerance in the thymus are secondly tested to additional self-antigens in the periphery [76]. In the periphery the T cells are prevented from further maturation through anergy (preventing further response) or Treg-mediated suppression by the DCs and macrophages [78]. A proper induction of the central and peripheral tolerance is highly critical for the generation of tolerance, immunity and autoimmunity [74]. The self-antigens presented on the HLA class II are selected based on the HLA class II affinity, the presence or absence of HLA-activity, and the arrays of available peptides.

The B cells are also going through a process of self-tolerance induction. In the bone marrow the B cells are controlled by deletion and receptor editing with high or moderate avidity to self-antigens. The B cells that are capable of somatic recombination by the variable diverse joining (VDJ) gene re-arrangements of the heavy-chain are further matured into pre-B-cells expressing the surface Ig molecule [79]. Receptor editing is the predominant mechanism of central tolerance by the B cells [80]. The B cells that are maintaining self-reactivity undergo clonal deletion and thereby programmed cell death by apoptosis [79].

Autoimmunity in T1D

DCs have been suggested to be implicated in the pathogenesis of autoimmune diseases, including T1D [60]. It is known that the DCs constantly present cell-derived antigens from the β cells to the T cells located in the pancreatic lymph nodes (PLN). However, it is unclear why these cells induce an active immune response instead of tolerance in response to self-antigens. It was suggested that the DCs are activated via TLR-2 and TLR-4 through DNA-binding protein high mobility group B1 (HMBG1), which is released from dying pancreatic β cell death [60]. However, the DCs are not capable to drive the autoimmune reaction alone. In several studies, the significance loss of T cell-tolerance in autoimmune diabetes has been established [81, 82]. It is commonly suggested that in order for the T cells to become autoreactive they have escaped the central tolerance in the thymus and have encountered β-cell antigens in the pancreatic lymph nodes. These β-cell antigens may have become exposed to the T cells due to an unknown trigger harming the β cells, which may have resulted in β-cell death [83]. If autoreactive T cells in the PLN recognize the β-cell antigens as foreign they undergo activation and clonal expansion following migration to the pancreatic islets to mediate β-cell destruction.

In addition to the DCs and T cells, B cells may play an essential role in T1D. It is possible that impaired B-cell tolerance is another factor contributing to the development of T1D [72]. In order for the B cells to become autoreactive several mechanisms such as the clonal deletion, B-cell receptor editing as well as clonal anergy and ignorance may not properly be functioning. The mode of tolerance may also likely be determined by the affinity of the receptor for the antigen and cross-linking of the receptor. Moreover, the property (soluble or membrane-bound) of the antigen may have significance [72]. Taken together, the interplay between genetic predisposition factors and the lack of immunological tolerance in response to β-cell antigens by the immune system may likely contribute to the autoimmunity in T1D.

Autoantigens – "the victims"

The *victims* of the autoimmune attack are the autoantigens in the pancreatic islet cells. The victims with specific focus in this thesis are the major autoantigen Zinc transporter 8 (ZnT8) in paper I and IV and the minor autoantigen Neuropeptide Y (NPY) in paper II and III. These autoantigens are described more thoroughly.

Major autoantigens are major victims

Until today there are four *major autoantigens*, defined as positive for antigen-specific autoantibodies in >35% in newly diagnosed T1D patients [84]. The autoantigens targeted by the autoreactive immune cells are either soluble, such as insulin or membrane-bound proteins, such as the ZnT8 expressed within the islet of Langerhans (Figure 3) [72].

Insulin is the main specific islet autoantigen in T1D exclusively expressed within the pancreatic islets by the β cells (Figure 3). The insulin protein is encoded by the insulin (INS) gene and produced as a prepro-hormone consisting of the signal peptide, B-chain, C-peptide and A-chain. The signal peptide is cleaved off already in the ER. In the end of the synthesis and maturation of the insulin molecule the C-peptide is cleaved off. C-peptide and insulin are released from the β cells in equimolar amounts. The A- and B-chains are connected via disulfide (SH)-bridges representing the active insulin molecule, which has been suggested to be critical to insulin autoimmunity [82, 85].

The neural enzyme, *glutamic acid decarboxylase* (GAD), is involved in the synthesis of the neurotransmitter gamma aminobutyric acid (GABA). In mammals two isoforms, GAD65 and GAD67, have been identified with molecular weights of 65 kDa and 67 kDa, respectively. Although the sequences of them share 65%

identity, they are different in their cellular distribution and regulation [86-88]. GAD65 is expressed both in the pancreatic neurons and in the pancreatic islet cells (Figure 3) [86]. In the β cells, GAD65 has been identified in three distinct forms; a hydrophilic soluble form, a hydrophobic form that is soluble or has low membrane avidity and a fixed membrane-bound form [89].

Insulinoma-associated protein 2 (IA-2) is a plasma membrane protein belonging to the tyrosine phosphatase family (PTP) [90]. It consist of an intracellular transmembrane and an extracellular domain encoded by 979 aa found in neuroendocrine tissues and in secretory vesicles of β cells (Figure 3) [91]. IA-2 exists in two isoforms, the 40 kDa IA-2 protein (previously designated Islet cell antigen 512) and the 37 kDa IA-2β protein (phogrin) [92]. Although, it is known that the IA-2 lacks enzymatic activity the exact function has not been elucidated [11].

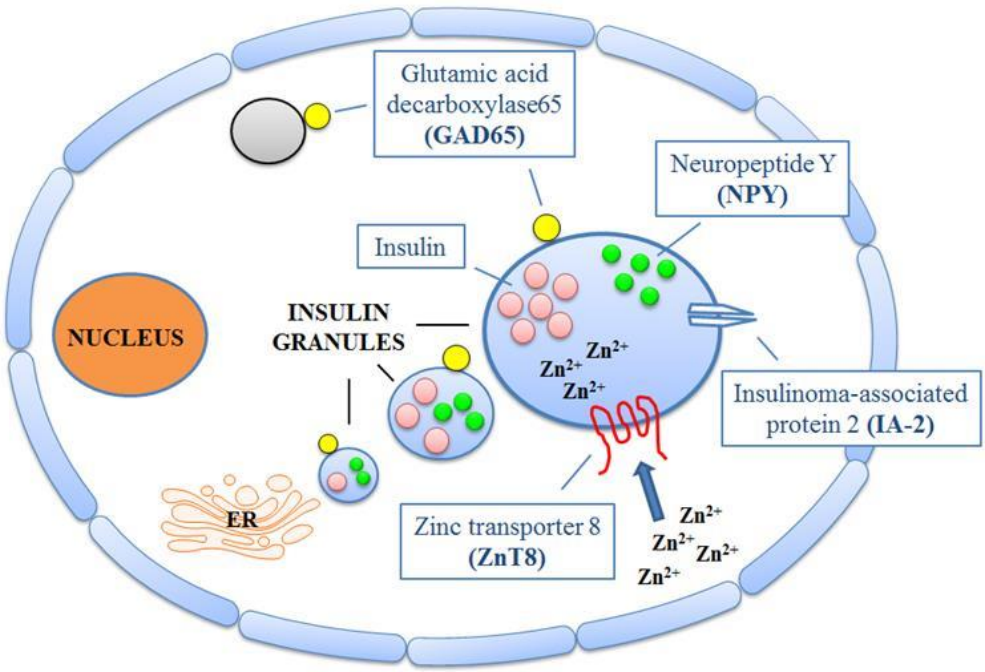

Figure 3. Localization of the major autoantigens, insulin, GAD65, IA-2 and the ZnT8 as well as the NPY in the β cell. The proinsulin molecule is synthesized in the ER and matured to insulin in the insulin granules. Insulin is thought to co-crystallize with Zn^{2+} to form a hexamer structure. The Zn^{2+} ions are transported into the insulin granules by the ZnT8 transporter that is localized in the membrane of the granules. IA-2 is also a transmembrane protein while GAD65 adheres to the membrane surface by hydrophobic determinants. The neurotransmitter NPY is expressed and co-secreted with the insulin molecules.

Zinc transporter 8 (ZnT8)

Regulation of zinc

Zinc is a trace element crucial for the maintenance of normal physiological function and cellular homeostasis [93, 94]. Zinc has a biological role by being the constituent in many proteins, such as in growth factors and hormones as well as having catalytic and regulatory functions by acting as a cofactor in signaling pathways [95]. The concentration of zinc is 2-4 g in the body, although the zinc in plasma is only 12-16 μM [96]. The small amount of zinc needs to be tightly regulated by two zinc transporter families, the SLC30A (ZnT) family and the SLC39 (ZIP) family. The ZnT family transport zinc ions *from* the cytosol either into intracellular compartment such as organelles or to extracellular compartments outside the cell [97]. The ZIP family exerts their function in the opposite direction by facilitating the zinc influx *into* the cytosol from either extracellular or intracellular compartments of the cell. The expression and activity of the two family proteins are specifically regulated at various levels such as at transcription, translation and post- transcription and translation levels in response to the demand of zinc [98].

Structure and expression of ZnT8

The 369 aa long ZnT8 protein is predicted to comprise of a six-transmembrane domain with both the C- and the N-terminal ends located in the cytosol of the cell. There is a histidine-rich loop located between the fourth and fifth transmembrane region with potential binding-site for zinc in the cytosol [99]. The ZnT8 protein belongs to the ZnT family of transporters and is expressed in the islets of Langerhans mainly in the β cells but also in the α cells [100, 101] as well as in the kidney and testis [102]. In addition, ZnT3, ZnT5 and ZnT7 have been reported to be expressed in the β cells of rodents [98]. ZnT8 is encoded by the SLC30A8 gene on chromosome 8. The aa at position 325 in the C-terminal part of ZnT8 is controlled by two different single nucleotide polymorphisms (SNP) rs13266634 and the adjacent rs16889462 in SLC30A8 [103, 104]. The SNP rs13266634 causes an aa substitution in position 325 from arginine (R), encoded by CGG (C-allele), to tryptophan (W), encoded by TGG (T-allele). The C/C-genotype (R/R) is present in 46-48% and the T/T-genotype (W/W) in 10-13%, while the heterozygous C/T-genotype is found in 40-42% of the general Caucasian population [105, 106].

The nearby SNP rs16889462 is causing an aa change to glutamine (Q) from CGG (R) to CAG (Q) in the middle base triplet (G<A) [104]. The frequency of this SNP has not been thoroughly studied, although the A/A-genotype (Q/Q) was reported in less than 1% of the general population in China compared to the G/G- (R/R) and A/G-genotype in 91% and 9%, respectively [107]

Function of ZnT8

For many years it has been noted that an unbalanced zinc homeostasis has been associated with diabetes, both type 1 and 2 [108, 109]. Zn deficiency has been shown to affect glucose metabolism and insulin secretion [110]. Also, it is known that zinc is essential for the crystallization of the insulin hexamer with two zinc ions located in the middle of the hexamer [111]. The main function of ZnT8 is to transport zinc ions from the cytosol into the insulin granules [98]. However, the implication of the ZnT8 transporter on glucose homeostasis and insulin secretion is still under investigation. Studies of ZnT8 knock-out mice with the intentions to explore the role of the ZnT8 are ambiguous. In one knock-out mouse, mature dense core insulin granules were replaced by immature and pale insulin-like granules. These data suggested that the ZnT8 transporter was essential for the formation of insulin crystals [112]. In two other knock-out mice, the zinc content of the insulin granules was reduced [113, 114] and showed reduced insulin secretion [115] as well as abnormal glucose tolerance [114]. Another study showed that the granules in isolated islets displayed normal glucose-stimulated insulin secretion although showed the structure of rod-like crystals [113]. Even though, extensive studies of the ZnT8 in mice have been performed, the exact role of the ZnT8 transporter on insulin secretion is unclear [113, 115]. The α cells in pancreas secret glucagon and thereby counter-regulate the effects of insulin on plasma glucose. The α cells were not seen to be affected in the ZnT8 knock-out mice. In both α cell-specific and global knock-out mice the secretion and circulated levels of glucagon as well as the glucose metabolism were normal [114, 116, 117].

Recently, an extensive study of 150,000 participants, loss-of-function mutations in the human SLC30A8 were found to confer protection against T2D [118]. Carriers of these mutations (Arg138* or Lys34Ser*50) resulting in protein-truncation and, reduced risk for T2D by 65% [118]. These observations in humans appear inconsistent with the findings in the knock-out mice. The human findings suggest that the ZnT8 transporter might be a candidate for therapeutic intervention in T2D [118].

Taken together, it is evident that zinc is required for insulin synthesis, processing, and maturation as well as maintaining the glucose homeostasis. Yet, whether the function of the ZnT8 transporter impacts the cause of hyperglycemia and diabetes remains elusive.

ZnT8 SNP rs13266634 in diabetes

The SNP at rs13266634 in the SLC30A8 was found to be associated with T2D and lowered insulin secretion in healthy subjects [119]. Recently, it was shown that T2D patients had lower plasma zinc levels compared to healthy controls and that the zinc levels were modified by the polymorphism in SLC30A8 [120]. Studies have demonstrated lower peripheral blood insulin levels in both β-cell specific

ZnT8 knock-out mice [121] and in humans carrying the SNP rs13266634 (R325W) risk C-allele [106]. Consistent with these findings, humans carrying the C-allele (R) were proposed to have an impaired proinsulin conversion in the β cell [122] potentially causing a lower level of peripheral insulin. In addition, it was shown that both humans with the C-allele (R) and β-cell specific Slc30a8 deficient mice had an increased hepatic insulin clearance as a result of a lower amount of zinc. A possible absence of zinc was suggested to lead to a lower suppression of the insulin uptake and degradation by the liver [121]. The physiological impacts of SNPs in other β-cell expressed ZnT transporters (ZnT3, ZnT5, ZnT7, ZIP6-8 and ZIP14) remain unknown [98]. In 2007, ZnT8 was identified as a major autoantigen in T1D when autoantibodies directed against the aa 325 controlled by the SNP rs13266634 were identified in >60% of newly diagnosed T1D patients [123]. However, the SNP rs13266634 was not found to be associated with T1D in the genetic consortium (GM) genome-wide association scanning [124].

Minor autoantigens are minor victims

There are several *minor autoantigens*, defined as positive for antigen-specific autoantibodies in <35% in newly diagnosed T1D patients [84]. A few are mention below while additional minor autoantigens are presented in Table 1.

The islet cell protein 12 (ICA12) consist of 622 aa and has a homology to the SOX (Sry-related HMG (high mobility group) box) family of transcription factors [125] and was therefore also named SOX13. Both ICA12 and SOX13 are membrane-bound proteins, although to the chromatin structure. They are located intracellularly and have been identified in the pancreatic islets [125]. Vesicle-associated membrane protein 2 (VAMP-2) is another protein considered to be connected to diabetes. VAMP-2 is involved in the intracellular regulation of the exocytosis by aiding in the docking process of the insulin granule with the cell membrane [126] as well as in exocytosis of the glucagon-like peptide-1 (GLP-1) [127]. In addition to VAMP-2, the neurotransmitter Neuropeptide Y (NPY) was suggested as a minor autoantigen in newly diagnosed T1D patients [84] and is described more thoroughly below.

Table 1. Candidate minor autoantigens in type 1 diabetes. The autoantibody prevalence and short autoantigen description of previously reported autoantigen are presented.

T1D CANDIDATE MINOR AUTOANTIGENS	AUTOANTIBODY PREVALENCE	COMMENTS	REFERENCES
Vesicle-associated membrane protein 2 (VAMP-2)	**21%**	Associated with secretory vesicles in pancreatic β cells.	[84]
Neuropeptide Y (NPY)	**9%**	Neurotransmitter that inhibits glucose-stimulated insulin secretion in pancreas.	[84]
SOX13/ICA12	**9-30%**	A membrane-bound protein intracellularly expressed in pancreas.	[128] [129] [130]
Islet cell antigen 69 (ICA69)	**5-30%**	Polypeptide expressed in β cells as well as in brain, heart, thyroid and kidney.	[131]
Glima 38	**19%**	Amphiphilic glycated β-cell membrane protein expressed in islet and neuronal cell lines.	[132]
Heat shock protein 60 (HSP60)	**16%**	Expressed by both prokaryotes and eukaryotes as a response to stressful stimuli (high temperatures and infections).	[133]
CD38 (ADP ribosyl cyclase/ cyclic ADP-ribose hydrolase)	**13-19%**	A type II transmembrane glycoprotein which is a signal transducing cell surface receptor and a marker of differentiation and activation in hematopoietic cells. Involved in Ca^{2+} release.	[134] [135] [136]

Neuropeptide Y (NPY)

Structure and expression of NPY

NPY is a neurotransmitter consisting of 36 aa long mature protein encoded by the NPY gene on chromosome 7q15.1. NPY is synthesized as a prepro-protein containing a hydrophobic signal peptide region (aa 1-28) and ended by a C-flanking peptide (aa 68-97) (Figure 4) [137]. According to a three-dimensional model of the mammalian NPY, based on the known structure of the avian homologue, it was proposed that the tertiary structure is characterized by hydrophobic interactions between an N-terminal helix and a C-terminal alpha-helix [138]. The NPY conformation obtained is composed of two short attached α-helices involving aa residues 15-26 and 28-35 [139]. It was suggested that both the

C- and N-terminal regions, closely located together in space, are needed for bioactivity as well as receptor binding and activation [138]. NPY is widely expressed within the central and peripheral nervous systems and was demonstrated to exist in islet cells and pancreatic nerve fibers [140, 141].

Function of NPY

NPY exerts pivotal roles involved in vasoconstriction, insulin secretion and food intake [142-144]. NPY has been shown to inhibit glucose-stimulated insulin secretion in rat islets and was suggested with neuromodulator functions [142]. Moreover, NPY administration into the cerebroventricular system was shown to stimulates feeding in rats [145] via the Y5 receptor [144].

NPY SNP rs16139

The SNP rs16139 (T1128C) identified an aa substitution from leucine (L) to proline (P) (L7P) in the signal peptide region [146]. The minor allele (P), encoded by the C-allele, correlated with elevated NPY levels in plasma [147] perhaps due to an increased biosynthesis [148, 149]. It was suggested that the L7P affects NPY secretion as a consequence of a modified secondary and tertiary structure [148]. It is therefore of interest that the minor allele was associated with atherosclerosis [150] and impaired glucose tolerance [151]. In obese humans (BMI >28 kgm^{-2}) it was shown that the risk of the genotype- (heterozygous for L7P genotype) obesity interaction caused a 12-fold increased risk for abnormal glucose regulation [152]. This polymorphism has also been associated with a three- to four-fold increased risk for T2D [151, 153], but was not find to increase the risk for T1D [154]. However, the L7P conferred an increased risk for retinopathy [150] and nephropathy [155] in patients with T2D. NPY has previously not been investigated in relation to neuropathy complications.

The minor allele of SNP rs16139 may be considered relatively common in the general population [152]. In Europeans the heterozygous T/C-genotype (L/P) was found in 5-12% and in 11-12% of T2D patients [146, 151, 153, 156]. In Swedish T1D patients the homozygous major (L/L) genotype, encoded by T/T, were reported in 90%, while 9% had the heterozygous and 1% the minor C/C-genotype (P/P) [154].

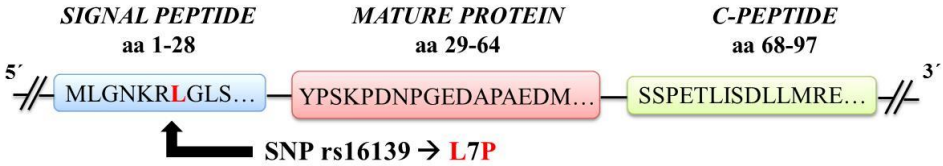

Figure 4. The amino acid (aa) sequence of the neurotransmitter Neuropeptide Y (NPY). NPY is synthesized as a prepro-protein containing a signal peptide region (1-28), mature protein (29-64), three aa (65-67) necessary for post-translational processing and a C-flanking peptide (68-97). The signal peptide contains a SNP at rs16139, which causes an amino acid substitution from a leucine (L) to a proline (P) (L7P) associated with increased risk for T2D and diabetic retinopathy and nephropathy.

Islet autoantibodies – "weapons or signal corps"

The islet autoantibodies in T1D can be perceived either as *weapons* helping the fighter (immune system) to harness their victims (autoantigens) or as *signal corps* sending out warning signals. The mission is to survey and warn about the ongoing autoimmune war. The possible role of autoantibodies in the autoimmune destructive process remains elusive.

Islet autoantibodies as markers of T1D

Islet autoantibodies are commonly referred to as biomarkers of an ongoing autoimmune response [157-159], although the rate of progression to clinical onset of T1D after seroconversion to islet autoantibodies has been undefined. An islet autoantibody could appear at any time point in life, although most commonly during 9 months and 3 years of age [160] in pre-clinical stage of autoimmunity. The appearance of multiple autoantibodies following the first autoantibody often appears within a year but may occur later [161-163].

Major autoantibodies

Up until today there are four *major* autoantibodies identified in the pathogenesis of T1D, defined as prevalent in >35%, identified in newly diagnosed T1D patients.

IAA

In competitive radiobinding assay endogenous IAA were detected in serum from newly diagnosed T1D patients and postulated as a marker of β cell damage [157]. Insulin autoantibodies may be detected in children as early as at 9 months of age.

Although, IAA are possible to identify before the age of 6 months they may origin from the mother and not be de novo [164, 165]. The risk of clinical onset of T1D was shown to be 27% in off-springs positive for IAA at 9 months of age and 100% in off-springs with multiple autoantibodies at 9 months of age [165]. Several studies have reported that IAA primarily are detectable in children with an onset age less than five years [26, 165, 166]. The levels of IAA are commonly declining with increasing age [164].

Both proinsulin and insulin autoantibodies are detected in newly diagnosed T1D patients [157], although the immune recognition remain unclear as human studies are complicated by the fact that B and T cells may not reflect an ongoing insulitis [85]. There are several autoantibody epitopes of insulin including the sites of aa 8-13 of the A chain and aa 28-30 on the B chain [167, 168].

T cells were shown to recognize the aa residues of 10-18 [169], 9-23 [170] and 15-24 of the signal peptide region [171, 172]. In the case where (pro)insulin peptides from the B chain are presented it seems that the $CD4^+$ T cells prefer presentation on the HLA DR4 and DQ8 molecules [173]. Also, the epitopes that are presented and processed from the antigen may depend on the type of DCs (thymic or intra-islet) [82]. In addition, several studies have indicated a strong association between the IAA and HLA DQ8, which is in LD with HLA DR4 as previously mentioned [174].

The major problem with the IAA RBA is the inability of the research community to standardize this assay [175]. The Islet autoantibody standardization program (IASP) (previously DASP) is a workshop allowing participating laboratories to assess the sensitivity/specificity of their autoantibody assays as well as comparisons to peer group findings [176].

GADA

Autoantibodies directed against GAD65 in human islets of newly diagnosed T1D patients were first identified in 1982 [177] followed by the detection in the diabetic prone Bio Breeding (BB) rat [178] and in sera from newly diagnosed T1D patients [158, 179]. Since then, studies have demonstrated that GADA are present in about 70-80% of newly diagnosed T1D patients [180-182] compared to 2-4% in the general population [183, 184] and with a lower frequency of 55% in acute-onset T1D patients in the Chinese population [185]. The frequency of GADA was 7% in healthy subjects at risk, primarily representing FDR. About 70% of the GADA positive FDRs progressed to clinical onset of T1D within 8 years compared to 4% who did not progress [186]. GADA was suggested to be associated with a slower progression to clinical onset of T1D [187]. GADA was shown to be more frequently displayed in older children compared to younger children [182, 188] and is the most common autoantibody among adults [11].

The levels have been shown to be relatively stable after clinical onset and persistent over several years [11, 189, 190]. GADA confer the highest genetic risk to HLA DR3-DQ2, but also to DR4-DQ8 although to a lesser extent [174, 183]. Additionally, GADA was associated with the combination of the two haplotypes, but found to be associated to DQ2 alone and not DQ8 alone [182, 183, 191, 192].

The GADA RBA and ELISA (RSR Limited, Cardiff, UK) have shown to be standardized. A GADA standard is available to demonstrate harmonization between GADA analyses in many different laboratories worldwide [193, 194].

IA-2A

Autoantibodies against IA-2 are present in up to 85% of newly diagnosed T1D patients [182, 195]. The autoantibodies against both isoforms, IA-2 and IA-2β (IA-2βA) are directed against the intracellular domains of the proteins sharing 74% of the intracellular and 26% of the extracellular domain structure [196, 197]. Due to the structure similarities and shared epitopes, the immunoassay used for detection of IA-2A does not distinguish between the two variants, yet, the sera reacting to the IA-2, representing most of the sera, also react to the IA-2β [11]. The highest risk assessment of progression to clinical onset of T1D was found in subjects with high titers of IA-2A, IAA and antibodies against IA-2β [198].

The frequency of IA-2A may vary dependent on the genetic background in different populations. However, IA-2A has been associated with the HLA haplotype, DR4-DQ8 [195, 199], although stronger with DR4 than DQ8 [200]. IA-2A was found to be negatively associated with DQ2 [174] indicating differences in the antigen presentation between the HLA molecules. Also, it was reported that the prevalence of IA-2A was associated with a SNP rs6598 located in the GIMAP5 gene. T1D patients with the minor allele A of the polymorphism had an increased prevalence of IA-2 autoantibody levels compared to patients without the minor allele [201]. The prevalence of IA-2A has been reported to decline with increasing age [91, 202].

In similar for GADA, the IA-2A RBA and ELISA (RSR Limited) were standardized [193]. An IA-2A standard is available to demonstrate harmonization between IA-2A analyses in many different laboratories worldwide [194].

ZnT8A

The ZnT8A are the most recently identified major autoantibody [123]. There are three variants of ZnT8A, the ZnT8R autoantibodies (ZnT8RA), ZnT8W autoantibodies (ZnT8WA) and ZnT8Q autoantibodies (ZnT8QA) directed against the polymorphic residue of aa position 325 of the C-terminal part of the ZnT8 protein [189, 203, 204]. In newly diagnosed T1D patients, the frequency of the ZnT8A has been reported to 58-83% [189, 205, 206] with a general lower prevalence in the Chinese population (24%) [207]. Consistently, ZnT8RA (50–

54%) appear to be more frequent than ZnT8WA (41–50%) [208-210] and ZnT8QA (32–36%) [209, 210] in Caucasian population. Although ZnT8QA are found in combination with ZnT8RA or ZnT8WA, it is rare to find patients who have only ZnT8QA and no other islet autoantibody [206, 211] .

Despite the absence of the association between the SNP rs13266634 in ZnT8 and T1D, several authors have independently reported a correlation between the polymorphic genotype and the autoantibody specificity of ZnT8RA and ZnT8WA [208, 212-214]. T1D patients with the C-allele more often than expected had ZnT8RA, and patients with the T-allele had ZnT8WA. As 30-44% of ZnT8A positive subjects react with all three variants [208], that is, despite that subject is homozygous for the R-variant, there may still be autoantibodies that react with the W-variant [210]. Previously, it was shown that the C/C-genotype were more often present (65%) T1D patients with an earlier age at diagnosis (<5 years) compared to patients with an older age at diagnosis [105].

The prevalence of ZnT8A has been reported to decline with increasing age [123] following the pattern of IA-2A [190] [189]. In newly diagnosed T1D patients, the ZnT8A was associated with older age at onset (>8 years) [215] and was rarely found in patients below 2 years of age [206]. The prevalence of ZnT8A were correlated with the HLA DQ6.4 (DQA1-B1*X-06:04) [206, 215] and DQ8 (DQA1-B1*03-0302) [206], but negatively associated with DQ2 (DQA1-B1*05:02) and DR3/DR4 [206, 215], similar to IA-2A.

It was demonstrated that children with newly diagnosed T1D who were followed for 6 years post-diagnosis showed decreased levels of ZnT8A (ZnT8WA and ZnT8QA), which was associated with a decrease in the C-peptide level. This may indicate that the presence of ZnT8A caused a reduction in residual β-cell function (RBF) [25]. In addition, the ZnT8QA-variant were associated with impaired glucose metabolism measured by Oral glucose tolerance (OGTT) or intravenous glucose tolerance (IvGTT) tests [216]. Moreover, it was shown that ketoacidosis was less common in patients with ZnT8A compared to ZnT8A negative patients [215].

In 2009 the ZnT8A were standardized for the first time followed by a pilot workshop in 2007. The ZnT8A RBA workshop (DASP) concluded that the ZnT8A showed both high sensitivity and specificity [217].

Minor autoantibodies

More than 90% of patients with T1D have multiple major autoantibodies [206], and less than 5% of T1D patients were found negative for any of the four major islet autoantibodies at clinical onset [209]. However, it has been suggested that islet autoantibody negative individuals may be positive for yet other

autoantibodies against β cell-specific autoantigens [209]. Several minor autoantibodies, defined as prevalent in <35%, has been identified in newly diagnosed T1D patients (Table 1). Of the minor autoantigens referred to in Table 1, the autoantibodies against ICA12, also named as SOX13, varied between 9-30% in T1D patients compared to 2-4% in controls [128, 130, 218]. Though, these autoantibodies were found not to be specific for T1D, but rather several autoimmune diseases [130]. Autoantibodies against VAMP-2 and NPY were found in 21% and 9% in newly diagnosed T1D patients, respectively [84].

Appearance of islet autoantibodies

Multiple autoantibodies

It has been suggested that the prodromal stage of autoimmunity to clinical onset is shortened in young children with four islet autoantibodies compared to young adults [27]. Recently, it was reported that 70-90% of healthy children with HLA risk genes who had multiple (two or more) autoantibodies progressed to clinical onset of T1D within 10 years compared to 15% in children with a single autoantibody [14, 219]. The risk of developing T1D was as little as 0.4% if the children had no islet autoantibodies. The same study reported that the progression to clinical onset was faster in children who showed multiple seroconversion before the age of 3 years (75%) compared to children above 3 years of age (61%) [14]. In large studies such as The Environmental Determinants of Diabetes in the Young (TEDDY) study children born with genetically susceptible risk for T1D that are carefully monitored by regular autoantibody screening are prevented from life-threatening symptoms such as ketoacidosis [38].

Which autoantibody confers the highest risk?

The risk of T1D can vary dependent on which autoantibody is present. In children with multiple autoantibodies, the combination of IAA and IA-2A conferred an increased risk (84%) of progression to clinical onset within 10 years compared to the combination of IAA and GADA (55%) [14]. Other studies showed that IA-2A was associated with an increased risk of progression to T1D compared to the other islet autoantibodies in healthy subjects at risk [220, 221]. Additionally, it was reported that IA-2A appeared earlier in children who had a shorter prodromal stage (<3 years) of islet autoimmunity compared to children with a longer prodromal stage (>10 years) [222]. This may be explained by the genetic background of either HLA [223] or non-HLA related genes [222].

Autoantibody patterns

In healthy children at increased risk of T1D (defined by HLA) [224] as well as in newly diagnosed T1D patients the major islet autoantibodies appear in a sequential pattern which may differ between individuals [161]. The time of first autoantibody

appearance has been associated with the rate of progression to clinical onset. The younger the child is at the first autoantibody appearance, the younger the child is at clinical diagnosis [163, 225]. In healthy children at risk the first autoantibody seem to appear is the IAA, which peaks around two years of age followed by the GADA at 3-5 years of age [224]. IA-2A is the autoantibody least commonly displayed as the first autoantibody of the autoantibodies against insulin, GAD65 and IA-2 [191]. The autoantibodies can appear already around the age of 9 months in a child, more often directed against insulin than any of the other autoantigens [165], as previously mentioned. Children born to mothers or fathers with T1D often developed IAA as their first autoantibody to be followed later by the appearance of other autoantibodies one after the other. These eventually multiple autoantibody positive children have an extraordinary risk for diabetes [14, 165, 224].

Autoantibody titers

The levels or end-point titers of islet autoantibodies are rarely reported. This is a weakness as high level sera may show false negative results due to the prozone phenomenon [226]. The autoantibodies may vary in levels depending on the stage of islet autoimmunity. Transient autoantibodies may also be displayed and were found to be associated with very high levels of autoantibodies at birth [165]. In children diagnosed before the age of 2 years the IAA titers, but not GADA and IA-2A titers, were higher compared to children diagnosed at an older age [227].

Risk assessment by autoantibody characterization

While autoantibodies are valuable tools in prediction, classification, and prognosis, they provide limited knowledge about the disease process at the cellular level. Broader knowledge about autoantibody isotypes, epitopes and affinity may reflect the maturation of the autoimmune response resulting in clinical onset of diabetes.

Autoantibody isotypes

It is known that different autoantibody isotypes play different roles. IgM variant is the first isotype produced followed by the isotype switch to the IgG (Figure 2), which is dependent on the cytokines provided by the T cells. It was hypothesized that the initial autoantibody response in pre-diabetes and in healthy children was dominated by the IgM isotype followed by an isotype switch to specific IgG, but only in the pre-diabetes children at risk. In healthy non-risk children the levels of IgM would decline and eventually disappear. There also studies suggesting that the IgG response in T1D are of both IgG1 and IgG3 type, predominated by the IgG1 [228, 229].

Autoantibody epitopes

The risk of clinical manifestation of T1D may be reflected in a developing pattern of epitope-specific autoantibodies [26]. It has been speculated whether the autoantibodies may have an increasing effect on the antigen presentation to T cells via receptor-mediated antigen uptake, and thereby lower the threshold for the T cell response [230]. This may lead to that antibody-mediated antigen internalization modifies the antigen presentation resulting in a greater diversity of epitopes presented to the T cells. It was suggested that this process can be a mechanism for the disrupted immunological tolerance against the islet proteins in the β cells [26].

The presence of multiple epitopes was associated with the progression of T1D [231]. IA-2A detected in the early stage recognized epitopes in only the juxtamembrane domain while they later in the disease stage also recognized epitopes in the IA-2β domain [220, 232, 233] indicating epitope spreading. In an additional study, epitope spreading of IA-2A was observed in younger children at clinical onset compared to older children, who were reported to have steady epitopes [234]. The GADA have been shown to be predominately directed against conformational epitopes located in the middle of the GAD65 sequence although, linear epitopes are also recognized by GADA positive individuals [235, 236]. It was shown that the conformational epitope were dominantly recognized by T1D patients in the early stage while epitope spreading to the linear epitopes of GAD65 were observed later [237]. Also, the GADA binding to the conformational epitope were more profound in the children with higher risk of clinical onset compared to children with lower risk [26].

ZnT8A have been found to react differently to the ZnT8 cytoplasmic fragment used for autoantibody detection dependent on the aa at position 325 [208]. This 325^{th} residue was suggested to be the major determinant in two epitopes of the C-terminal part [203]. In addition, the residues at R332, E333, K336, and K340 adjacent to the polymorphic aa 325 were found to be antigenic suggesting being a part of the 325-epitope (Figure 5) [238].

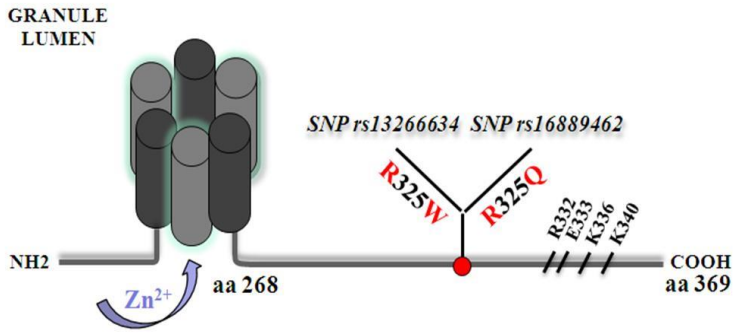

Figure 5. Schematic figure of the six-transmembrane ZnT8 protein and the SNPs rs13266634 and rs16889462. The T2D associated SNP at rs13266634 controls the amino acid (aa) substitution R>W at aa position 325 while the SNP rs16889462 causes the substitution of R>Q. Also, the aa residues 332, 333, 336 and 340 shown in the figure were suggested to be a part of the 325-epitope of the ZnT8 transporter (Paper I).

Autoantibody affinity

In children followed from birth displaying IAA as a first autoantibody and who stayed solely IAA positive had lower affinity compared to the children who developed multiple autoantibodies [168]. High affinity IAA were reactive to the epitopes on the insulin A chain and proinsulin while the low affinity IAA only bound to the C-terminal part of the B chain and not pro-insulin. The affinity of IAA was not found to be correlated with the titers of IAA. These data are consistent with the affinity studies on GADA demonstrating that children with multiple autoantibodies displayed higher affinity of GADA compared to children with single positivity for GADA who displayed lower affinity [239]. In affinity studied on the IA-2A, it was shown that the affinity in children at risk was at first not associated with epitope-specificity. However, to the time of follow-up the affinity of IA-2A was increased [240].

To our knowledge the affinity of the ZnT8 autoantibodies to the 325-epitope has not yet been studied in newly diagnosed T1D patients. Also, the crystal structure of the ZnT8 protein has not yet been identified. One of the reasons may be that the hydrophobic transmembrane part of the ZnT8 protein is complicating the crystallization process.

Taken together, the more islet autoantibodies present and the stronger their binding capacity is to the epitope defined by affinity, the higher is the risk for clinical onset of T1D. Therefore, it may be of high importance to study the affinity, autoantibody titer and epitope reactivity to assess the risk for T1D [198, 212, 241].

AIMS AND HYPOTHESES

Overall aim and hypothesis

The overall aim of this thesis was to better understand the involvement of autoantibody epitopes of ZnT8 and NPY in diabetes. By broadening our knowledge of islet-specific autoantibodies we may improve prediction of clinical onset in children at risk of developing T1D.

The overall hypothesis was that the major ZnT8 autoantibody epitope includes the polymorphic residue at aa position 325. NPY is a novel autoantigen in T1D, which may be useful to clarify the pathogenesis of islet autoimmunity as well as to predict T1D and possibly late diabetes complications.

Specific aims

The specific aims of the studies included in this thesis were to:

- Develop and optimize autoantibody radioligand binding assays (RBA) for detection of autoantibodies against both the ZnT8 and NPY autoantigen variants.
- Determine immunogenicity of the ZnT8 (318–331) variant peptides in mice.
- Analyze epitope-specificity, reactivity and affinity with ZnT8 variant autoantigens in competitive displacement studies by various recombinant ZnT8 peptides and proteins in human sera from newly diagnosed T1D patients.
- Determine frequencies and disease specificity of NPY autoantibodies in newly diagnosed T1D patients as well as in long-term T1D and T2D patients with neuropathy.
- Determine the association of NPY autoantibody variants with other islet autoantibodies and HLA genotypes in T1D patients.
- Study the influence of NPYA on the diagnostic sensitivity of T1D.

Specific hypotheses

The specific hypotheses of the studies included in this thesis were that:

- Younger T1D patients have ZnT8 autoantibodies with higher epitope-specificity and affinity compared to patients with older age at onset.
- The polymorphic NPY-P autoantibodies are associated with the SNP rs16139 (L7P).
- HLA-DQ haplotypes, such as DQ6.4, DQ5.1 or DQ4, paired with T1D high-risk HLA-DQ2 or HLA-DQ8 are associated with NPY autoantibodies.
- Minor NPY autoantibodies are related to age at diagnosis of T1D.
- Minor NPY autoantibodies are detected in diabetes patients with long disease duration and may be related to late complications.

METHODOLOGY

Study design and populations

Papers I and IV

In paper I, six newly diagnosed T1D patients below 18 years of age at diagnosis with autoantibodies against either ZnT8RA (negative for ZnT8WA and ZnT8QA) or ZnT8WA (negative for ZnT8RA and ZnT8QA) were included in the study. Previously, the patients (33% males) were genotyped for HLA [210]. Patient sera were analyzed for reactivity against the ZnT8 (318–331) peptides as well as against the *in vitro* transcribed translated ZnT8 (268–369) proteins in a competitive RBA.

In paper IV, newly diagnosed T1D patients in the BDD study [210] with autoantibodies against either ZnT8R (negative for ZnT8WA and ZnT8QA) or ZnT8W (negative for ZnT8RA and ZnT8QA) were subjected to ZnT8A end-point titration. Twelve patients with specific high titer ZnT8RA (3500-8240 U/mL) only and twelve patients with specific high titer ZnT8WA (5200-11400 U/mL) only were selected. The patients (5/12, 42% males) were diagnosed with T1D at the age 1-14 years (ZnT8RA), and 4-15 years (ZnT8WA) and were previously analyzed for IAA, GADA, IA-2A and all three autoantibody variants of ZnT8 (R, W and Q) as well as genotyped for HLA.

Papers II and III

In paper II, serum samples from 100 newly diagnosed T1D patients between 1–19 years of age were studied. In addition, serum samples from 48 long-term T1D (median duration 26 years) and 26 long-term T2D (median duration 23 years) patients were collected in 1998 at the Clinic of Diabetes at Skåne University Hospital in Malmö, Sweden. A subgroup of 32 patients with long-term T1D, first seen in 1998, was followed-up in 2005. Sera for control samples were obtained from 398 healthy blood donors in the ages between 19-81 years. Serum samples were analyzed for autoantibodies against the NPY-L and NPY-P variant, as well as for GADA, IA-2A and the ZnT8R, ZnT8W, ZnT8Q autoantibodies by RBA.

Previously, a subgroup of 31 long-term T1D patients underwent peripheral and cardiac autonomic nerve function tests during the follow-up in 2005. The peripheral nerve function (peripheral neuropathy) was evaluated by measurement of conduction velocity and amplitude of the sural and peroneal nerves in the lower extremities. The summation of nerve conduction attributes has been used elsewhere [242]. The cardiac autonomic nerve test (autonomic neuropathy) of the parasympathetic vagal nerve function (E/I ratio) was also tested [243]. E/I Z-score values less than −1.64 SD were considered as abnormal [243].

In paper III, a larger number of 673 newly diagnosed T1D patients recruited between January 1996 and April 2005 to the Skåne study. The T1D patients were classified with T1D according to the recommendation of ADA [244]. There were 54% (n=364) boys and 46% (n=309) girls in the study. The median age at T1D diagnosis was 10.2 years (range between 1-19 years). The same control group consisting of 398 healthy blood donors (median age 44 years, range between 19-81 years) were used in the present study. In addition, 608 healthy children (median age 9.4 years, range 9-12 years) from the Celiac Prediction in Skåne (CiPiS) study [245] were analyzed for NPY-L and NPY-P autoantibodies only.

Patient samples were analyzed for autoantibodies against the NPY-L and NPY-P variants by RBA. Previously, the serum samples from the Skåne study were analyzed for IAA, GADA, IA-2A, ICA and the three variants of ZnT8A as well as HLA-DQ genotyped. All serum samples for the islet autoantibody assays were stored at –20˚C until analyzed.

ZnT8 peptide and protein syntheses

The ZnT8 peptides and proteins consisted of synthetic ZnT8 (318-331) peptides and recombinant ZnT8 (275-369) proteins as well as *in vitro* transcribed and translated ZnT8 (268-369) proteins. These three various peptides/proteins described below were used in competitive displacement assays presented in papers I and IV.

Synthetic ZnT8 (318-331) peptides

In collaboration with Innovagen AB in Lund, Sweden, 15-mer ZnT8 peptides were synthesized to generate monoclonal ZnT8R-, ZnT8W- and ZnT8Q-specific antibodies in BALB/c mice. The ZnT8R-, ZnT8W- and ZnT8Q peptides covered the aa sequence (NH2-CHVATAAS**R/W/Q**DSQVVR-COOH) of aa 318-331 with either R, W or Q positioned in the middle of the peptide at aa position 325 using

standard Solid-phase peptide synthesis (SPPS) with 9-fluorenylmethyloxycarbonyl group (Fmoc) followed by mass-spectrometry (MS).

Recombinant ZnT8 (275-369) proteins

To our knowledge, the ZnT8 (275-369) proteins covering the aa sequence of 275-369 are the first recombinant C-terminal ZnT8 protein expressed, purified and successfully kept in solution without precipitation. The recombinant ZnT8 (275-369) proteins were generated in collaboration with Dr. Jens Lagerstedt at the Department of Experimental Medical Sciences, Lund University, Sweden.

Subcloning of ZnT8 (275-369) proteins

The ZnT8 C-terminal part of aa 275-369 was digested from full length ZnT8 (SLC30A8) and subcloned into the pETMBP_1 vector resulting in the pETMBP ZnT8R-aa275-369 vector containing the coding sequence for the maltose binding protein (MBP) green fluorescent protein (GFP) fusion protein (MBP-GFP). GFP was replaced by the ZnT8 cDNA in pETMBP ZnT8R-aa275-369. The tryptophan variant at position 325, MBP-ZnT8W was created using QuickChange Lightning Mutagenesis Kit.

Expression of ZnT8 (275-369) proteins

Vectors expressing (MBP-GFP, MBP-ZnT8R-aa275-369 or MBP-ZnT8W-aa275-369) transformed into Lemo21 (DE3) E.coli cells were incubated in EnPresso media overnight at 30° C with orbital shaking at 200 rpm. The growth media was supplemented with booster tablets, L-Rhamnose and IPTG. After 6 h of induction at 30° C, the cells were harvested by centrifugation at 4000 x g for 10 min at 4° C. Cells were washed twice with 0.9% NaCl and then stored at -20° C.

Purification and identification of ZnT8 (275-369) proteins

Cells were resuspended in 20 mL lysis buffer (50 mM Tris-Cl, 300 mM NaCl, 1 M Urea, 1 mg/mL lysozyme, protease inhibitor cocktail; pH 8.0), incubated at 4° C for 30 min, lysed by sonication and centrifuged at 30000 x g for 20 min at 4° C. The supernatant was filtered using 0.2 µm filter and loaded into nickel-charged HiTrap Chelating HP columns that had been pre-equilibrated with lysis buffer without lysozyme. Unbound and weakly bound proteins were washed with lysis buffer followed wash buffer (20 mM imidazole, 50 mM Tris-Cl, 300 mM NaCl; pH 8.0). Fusion proteins were eluted with elution buffer (350 mM imidazole, 50 mM Tris-Cl, 300 mM NaCl; pH 8.0) followed by removal of imidazole on a desalting column. The final purity of the ZnT8-aa275-369 proteins was evaluated by Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) analyses and staining with Bio-Safe Coomassie Blue. Protein concentrations were determined by NanoDrop 2000 using molecular weights and extinction

coefficients calculated from aa sequences. Protein identity was verified on a Bruker Scout 384 Reflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany).

Coupled *in vitro* transcription translation ZnT8 (268-369) proteins

The C-terminal part (268-369) of the ZnT8R cDNA was subcloned into the pTnT vector. By site directed mutagenesis of the originally pThZnT8R plasmids, both the pThZnT8W and pThZnT8Q plasmids were generated and confirmed by sequencing analyses. To generate the ZnT8 proteins, the pThZnT8 plasmids (0.02 µg/µL) were added to a reaction mixture using the TnT® Coupled Reticulocyte Lysate System for *in vitro* transcription and translation with either radiolabeled ^{35}S-met or unlabeled methionine. The reaction mixture was incubated in 90 min at 30°C degrees by shaking followed by gel-separation on Illustra NAP-5 Columns. Incorporated radioactivity in radiolabeled ZnT8 proteins was determined in a 1450 MicroBeta Counter.

The radiolabeling with ^{35}S-met guided the labeling with unlabeled methionine and was therefore considered to be in similar concentration. Unlabeled methionine was used in parallel *in vitro* transcription translation using the same batch as the radiolabeled methionine. The rate incorporation was computed from the specific radioactivity supplied by Perkin Elmer.

Immunization of BALB/c mice

The BALB/c (NOVA-SCB, Sollentuna, Sweden) mice were immunized with either ZnT8R (n=4), ZnT8W (n=4) or ZnT8Q (n=4) short ZnT8 (318-331) peptides. Prior injection the peptides were conjugated to Keyhole Limpet Hemocyanin (KLH) to enhance the immune response. The immunization was performed at Innovagen AB and the mice were kept at the Innovagen AB animal house facility.

First, the mice received a 100 µg subcutaneous injection at three months of age. Thereafter, they were given four boosts (25-50 µg intraperitoneally) at 4-week intervals. Prior to the fourth booster injection serum was withdrawn and kept overnight at 4° C until antibody analysis the following day. The mice were exanguinated three days after the fourth booster.

Autoantibody assays

NPY autoantibody assay

Subcloning of NPY cDNA

Prepro-NPY cDNA covering the aa sequence of 1-97 was inserted in a de novo synthesized pJ201 vector (2759 bp) with customized restriction cloning sites of Xho1 and Not1 at the 5´ end and 3´ end of the insert. By cutting with the restriction enzymes, Xho1 and Not1, the NPY insert was excised and replaced into a high efficient vector pTnT™ vector (2871 bp) (Figure 6) and transformed using DH5α E.coli competent cells. The pThNPY plasmid DNA was extracted using the QiaPrep Spin Miniprep Kit according to manufacturer's instructions. The sequence of pThNPY insert was verified by sequencing using (3700/3730 BigDye Terminator 3.1 Cycle Sequencing Kit at the Region Skåne Competence Centre (RSKC) in Malmö, Sweden.

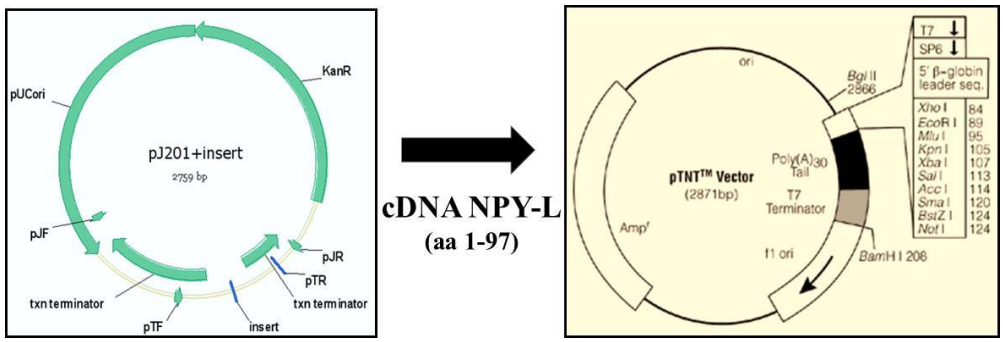

Figure 6. Subcloning of the cDNA of Neuropeptide Y-Leucine (NPY-L). The cDNA of amino acid 1-97 of the NPY gene was subcloned from the pJ201 vector to the pTnT vector using the restriction enzymes XhoI and NotI. The pThNPY-L was further subjected to generate NPY proteins by *in vitro* transcription and translation**.**

Site directed mutagenesis

Based on previously studies showing the association of the NPY SNP at rs16139 (T1128C) and the increased risk for T2D and related complications, we created the NPY-P (mutant) from the NPY-L (wild type).

Prior to the site directed mutagenesis, both forward and reverse primers were designed according to manufacturer's instructions (DNA Technology, Risskov, Denmark) with the Proline-variant (CTG (L) > CCG (P)) positioned in the primers. A Phusion™ site directed mutagenesis Kit was used to obtain Proline (P) in substitution for Leucine (L) (L7P) of the prepro-NPY insert. The pThNPY-P

variant was transformed in DH5α E.coli competent cells prior plasmid DNA extraction and sequencing as described for the pThNPY-L.

Radiolabeling

Various concentrations (0.5-2.0 µg per 100 µL reaction) of both pThNPY-L and pThNPY-P were tested to achieve maximal radioactive incorporation of ^{35}S-met in the NPY sequence by *in vitro* coupled transcription translation. For the continued IVTT experiments 1 µg (0.01/µL) of NPY plasmid DNA was used to generate ^{35}S-met NPY proteins using the TNT® SP6 Coupled Reticulocyte Lysate System in a 100 µL reaction mix. Following incubation of 90 min at 30 °C during shaking (300 rpm) the translation mixture was separated by gel filtration on Illustra™ NAP-5 Columns following equilibration of the columns with antigen buffer (150 mmol/L NaCl, 20 mmol/L Tris, pH 7.4, 0.15%, Tween20, 0.1% BSA). The separation of the protein products were performed by adding antigen buffer in various volumes from 100 to 1500 µL in eleven steps. The amount of ^{35}S-met incorporated in both ^{35}S-met NPY-L and ^{35}S-met NPY-P was determined (1450 MicroBeta Counter, Perkin Elmer). The ^{35}S-met NPY variant protein products were stored up to one month at −80° C.

Electrophoresis

To assure that the ^{35}S-met NPY protein products had been produced in the Coupled Reticulocyte Lysate System, they were verified by 16% SDS-PAGE. Briefly, the ^{35}S-met NPY protein samples were incubated with sample buffer and reducing agent for 3 min at 100° C degrees and loaded on the gel with a ^{14}C labeled molecular weight marker. The gel was dried for 72 h at room temperature using a Gel Drying Kit and was placed in contact with an X-ray sensitive film for 72 h at room temperature in the dark. The film was developed and fixed in DABdental developing and fixating solutions.

NPY autoantibody analyses

Serum samples were analyzed for autoantibodies against NPY-L and NPY-P using a standard RBA. In duplicates, 5 µL of serum sample was mixed with 60 µL of either ^{35}S-met NPY-L or ^{35}S-met NPY-P protein products to a final concentration of 425 ± 25 cpm/µL by dilution with antigen buffer in a 96-well plate. The reaction mix (antigen–antibody solution) was incubated overnight at 4 °C degrees constantly shaking. Washing buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 0.15% Tween 20) of 200 mL per plate was prepared on the first day and kept at 4° C degrees. Antigen buffer of 200 µL of antigen buffer was added to each well of a 96-well filter plate and incubated at room temperature overnight to block unspecific binding. The following day, the filter plates were emptied and 50 µL of the reaction mix was added to 20% v/v Protein A Sepharose (PAS, Invitrogen) in the filter plate and incubated for 1 h at 4° C with constant shaking. The filter plate was washed eight times with washing buffer using an ELx50 Microplate Strip

Washer. Antibody-bound radioactivity was analyzed in a β-counter (1450 MicroBeta Counter, Perkin Elmer).

Titration of standard curve

PAS-bound radioactivity was converted into in-house units (U) using a polyclonal rabbit IgG antibody against full length human NPY (Abcam, Cambridge Science Park, Cambridge, UK). Following optimization of the standard by testing various concentrations as maximal binding (starting concentration), the appropriate concentration corresponding to the maximal binding was found and diluted in seven doubling dilution steps as a standard for both NPY antigens.

Assay performance

The NPYA assays have not yet participated in any international standardization workshops such as the IASP. However, in our lab the mean value of the intra-assay coefficient of variation (CV) was 5% for duplicates for both the NPY-LA and NPY-PA assays. The inter-assay CV for NPY-LA and NPY-PA assays were 5%.

Major islet autoantibody assays

Previously, all major autoantibodies were analyzed as described in detail for the Skåne study [209]. In short, insulin autoantibodies were determined by non-competitive method using recombinant ^{125}I-insulin radiolabeling. The serum samples positive for IAA were further analyzed by competitive method using 0.072 IU (2IU/mL) of unlabeled insulin (Atrapid, Novo Nordisk, Denmark) [20, 246]. ICA were analyzed in a two color indirect immunofluorescence [247] while autoantibodies against GAD65 and IA-2 were analyzed by either commercially available kit (RSR Limited, Cardiff, UK) or by *in vitro* transcription and translation with ^{35}S-met for generation of radiolabeled protein products with sera from patients.

The RBA for ZnT8R and ZnT8W autoantibodies, respectively, has been described in detail elsewhere [211]. Briefly, the three pThZnT8 plasmids (pThZnT8R, pThZnT8W and pThZnT8Q) were labeled with ^{35}S-met by coupled *in vitro* transcription translation using the TnT® Coupled Reticulocyte Lysate System. The ^{35}S-met ZnT8 protein (^{35}S-met ZnT8R, ^{35}S-met ZnT8W and ^{35}S-met ZnT8Q) were purified on Illustra NAP-5 columns chromatography.

Validation in autoantibody standardization workshop

Our laboratory is participating in the ongoing IASP workshops. According to the recently presented IASP workshop of 2013, GADA showed 62% sensitivity at 98% specificity while IA-2A showed a sensitivity of 68% at the specificity of

100%. For ZnT8RA, ZnT8WA and ZnT8QA the sensitivity was 58%, 32% and 22%, respectively, at 100% specificity. In our laboratory the intra-assay CV for duplicates in the GADA assay was 7% and 11% in the IA-2A assay. The intra-assay CV for ZnT8RA was 6%, ZnT8WA 5% and ZnT8QA 4%. The inter-assay CV was 7%, 8% and 10% for ZnT8RA, ZnT8WA and ZnT8QA, respectively.

Competitive displacement experiments

Competitive RBA displacement experiments were conducted to determine the autoantibody reactivity to the recombinant ZnT8 (318-331) peptides (Figure 7, panel A), ZnT8 (275-369) proteins as well as the unlabeled *in vitro* transcribed translated ZnT8 (268-369) proteins (Figure 7, panel B).

In the competitive displacement assays T1D patient sera specific for either the ZnT8RA (solely positive for R, and not for W or Q) or ZnT8W (solely positive for W, and not for either R or Q) were analyzed.

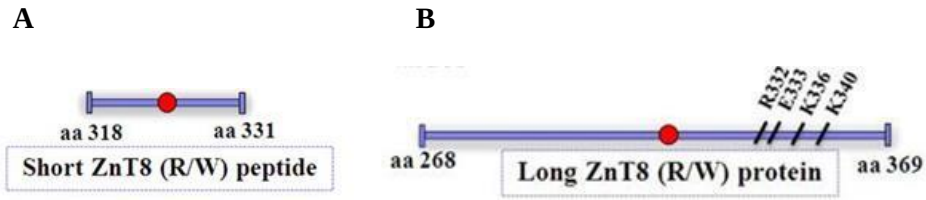

Figure 7. Schematic figures representing the variant recombinant ZnT8 (318-331) peptides with R, W or Q positioned (red filled circle) at the aa position 325 (panel A) as well as ZnT8 (268-369) *in vitro* transcription translation proteins (panel B). The ZnT8 peptides were used for immunization of BALB/c mice. Both short peptides and long proteins were used in competitive displacement studies in binding to the ZnT8 autoantibodies in human sera (Paper I).

Competitive displacements with synthetic ZnT8 (318-331) peptides

By using a reciprocal permutation design (cross-reactivity), either of the short (318-331) ZnT8R or ZnT8W peptides at various concentrations (1.5-100 µg/mL corresponding to approximately 0.98-62.5 µM/L) were incubated with radiolabeled ZnT8R or ZnT8W proteins and with autoantibody positive sera specific for either the ZnT8R or the ZnT8W variant (Figure 8, panel A).

In brief, a solution mixture of half a volume of the ZnT8 (318–331) peptide and half a volume of radiolabeled ZnT8 (268-369) protein (containing 850 ± 50 cpm/µL) were added to 5 µL of serum in a 96-well microtiter plate for incubation overnight at 4° C, shaking at 300 rpm. If autoantibodies were present in the sera, antibody-antigen complex was created during the incubation and the following day

separated by precipitation with protein A sepharose (binds IgG antibodies efficiently). Following one hour of incubation with protein A sepharose, the mixture was washed. Antibody-bound radioactivity was analyzed in a Beta-counter. The sepharose-bound radioactivity was converted into arbitrary units (U) using individual standard curves of T1D sera with high ZnT8A reactivity.

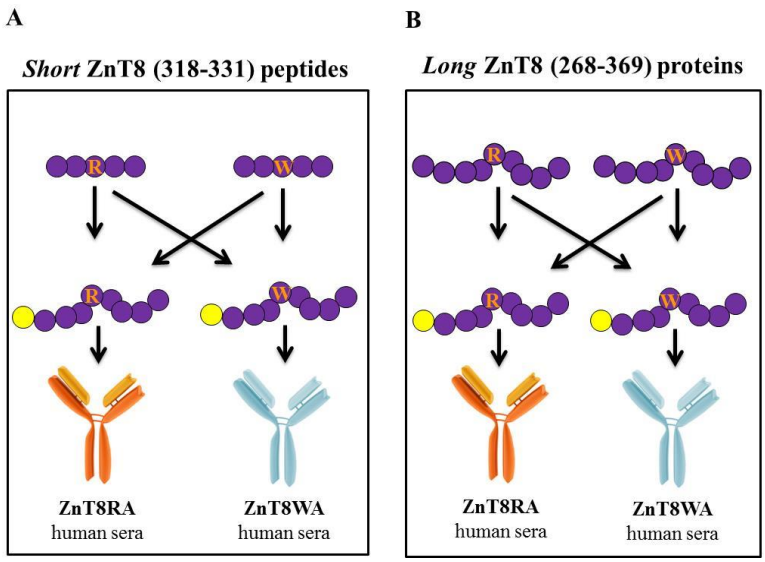

Figure 8. The design of the reciprocal permutation experiments in the competitive radioligand binding assay. The figure demonstrate the competition between the unlabeled short ZnT8 (318-331) peptides (panel A) or the unlabeled *in vitro* transcribed and translated ZnT8 (268-369) proteins with the ^{35}S-met ZnT8 proteins in binding to patient sera specific for either the ZnT8RA or the ZnT8WA.

Competitive displacements with recombinant ZnT8 (275-369) proteins

In an identical design as described for the ZnT8 (318-331) peptides, sera and different concentrations (0.001-100 µg/mL) of the recombinant MBP-ZnT8 (275-369) proteins or MBP-GFP were tested against radiolabeled ZnT8 (268-369) proteins in binding to ZnT8A human sera. Also, in these experiments a reciprocal permutation design was performed in patient sera specific for either ZnT8RA or ZnT8WA.

Competitive displacements with ZnT8 (268-369) proteins

As described for both recombinant ZnT8 peptides and ZnT8 proteins, different concentrations (pmol/L), but via dilution steps, of the long unlabeled *in vitro* translation ZnT8R and ZnT8W proteins were incubated with equally long

radiolabeled ZnT8R or ZnT8W proteins with ZnT8A sera specific for either the ZnT8R or ZnT8W were tested (Figure 8, panel B).

Assay performance

In the competitive displacement assays (paper IV) all samples were analyzed in duplicates. The intra-assay coefficient of variation was 4% for both the ZnT8RA and ZnT8WA serum samples. Each of the 24 patients was analyzed in three independent experiments and the inter-assay coefficient of variation was 4% for the ZnT8RA- and 4% for the ZnT8WA-specific patients.

HLA-DQ genotyping

HLA-DQ genotypes were typed by sequence-specific oligonucleotide probes described elsewhere [54, 246, 248] using a DELFIA hybridization assay (Perkin Elmer, Boston, MA, USA). The first set of probes defined the presence of HLA-DQB1*02, 0302, 0301, 0602, 0603 and 0604 alleles. The second set of probes defined the presence of additional HLA-DQB1 alleles. HLA-DQA1 probes defined the DQA1*0201, 03 and 05 alleles.

Statistical analyses

Papers I and IV

In both paper I and III, displacement experiments were carried out in three independent experiments using duplicate determinations. The mean and standard error of the mean (SEM) were calculated for these three independent experiments. In paper I, affinity was calculated as half-maximal (Kd) and maximal (Vmax) binding and expressed as pmol/l. Affinity differences between the R and W proteins were tested with two-tailed paired t-test. In paper IV, differences in ZnT8A titers were calculated with Mann Whitney U-test. Affinity was calculated as half-maximal (Kd) and expressed as ng/mL. Differences in % displacement were calculated by Student's t-test.

Papers II and III

Quantile-quantile (Q-Q) analysis was used for the quantiles of the autoantibody distribution against the quantiles of a standard distribution. Pearson Chi-square (and two-sided Fisher's exact test and Yate's correction for continuity value when applied) was used to assess differences of autoantibody frequency in un-paired

groups, and McNemar test in paired groups. Mann Whitney U-test was used for calculation of differences in autoantibody levels between patients and controls and Wilcoxon signed ranks test for change in autoantibody levels over time. Spearman two-tailed correlation test was used for correlation analyses. Agreement of islet autoantibodies was calculated with Kappa statistics. A value of one indicated perfect agreement, whereas a kappa of zero was what would be expected by chance and negative values a systematic disagreement between the observers. In paper III, odds ratio (OR) with 95% confidence interval (CI) were calculated from simple logistic regression models to assess the degree of association between categorical variables. Factors adjusted for were age at diagnosis, gender, multiple major autoantibodies and HLA-DQ genotypes.

Interpretation of kappa agreement:

<0 Less than chance agreement

0.01–0.20 Slight agreement

0.21–0.40 Fair agreement

0.41–0.60 Moderate agreement

0.61–0.80 Substantial agreement

0.81–0.99 Almost perfect agreement

In all papers, p-value <0.05 was considered to be statistically significant. The statistical analyses were performed using GraphPad Prism version 6.00, GraphPad Software (La Jolla, CA, USA) and IBM SPSS Statistics version 18.0, 20.0 and 22.0 (Chicago, IL, USA).

RESULTS AND DISCUSSION

Paper I – ZnT8 peptide antigenicity

Main findings

- ZnT8 peptides (318-331) were antigenic in mice but did not react with ZnT8A in humans.
- The affinity of the ZnT8 autoantibodies was higher for proteins with the same epitope variant as the patient sera.
- ZnT8 autoantibodies were dependent on the conformational 325-epitope of ZnT8.

Properties of the ZnT8 (318-331) peptides

To our knowledge, there are no available monoclonal antibodies directed against the 325-epitope of the human ZnT8 protein with specificity against the polymorphic residue of R, W or Q at the aa position 325. In an attempt to generate specific ZnT8R, W and Q monoclonal antibodies in mice, 15-mer ZnT8R, ZnT8W and ZnT8Q (318-331) peptides were synthesized and coupled to KLH prior immunization of 12 BALB/c mice at Innovagen AB. Eventually, five antibodies from originally ZnT8R or ZnT8W immunized mice only (not from ZnT8Q), were purified. The specificity of the five antibodies was tested against ZnT8QRWtriple (antigen mix of R, W and Q variants), ZnT8R only, ZnT8W only, GAD65 and IA-2 antigen in our RBA. In the following, only results from the ZnT8R and ZnT8W mice will be presented as mice immunized with the Q variant were not available for further investigation.

The difference between the ZnT8R and the ZnT8W (318-331) peptide sequences was the residue at the aa position 325. In a Hopp-Woods hydrophilicity scoring plot the ZnT8R peptide (R-score at 3.0) (Figure 9, panel A) seemed to have hydrophilic features compared to the ZnT8W peptide (W-score at -3.4) (Figure 9, panel B). The scores are based on the values given by the original Hopp-Woods scoring system, which was developed to predict potential antigenic sites. Regions with values above zero are considered to be hydrophilic and are therefore more likely to be exposed [249].

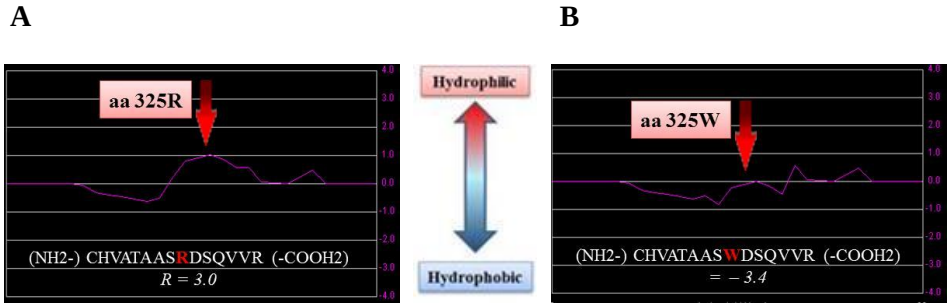

Figure 9. Hydrophilicity plots of the 15-mer (318-331) ZnT8R (panel A) and ZnT8W (panel B) peptides. The ZnT8R peptide (scoring for R= 3.0) showed hydrophilic characteristics compared to the ZnT8W peptide (scoring for W= -3.4) ranked by the Hopp-Woods scoring system. Values above zero were considered with hydrophilic characteristics.

The ZnT8R and ZnT8W (318-331) peptides were purified and analyzed by High performance liquid chromatography (HPLC) and mass spectrometry (MS) at Innovagen AB (Figure 10). The ZnT8R and ZnT8W peptides showed the molecular weight/charge (m/z) of 533.6 m/z and 543.6 m/z, respectively, and a purity of 70% after HPLC and MS analyses.

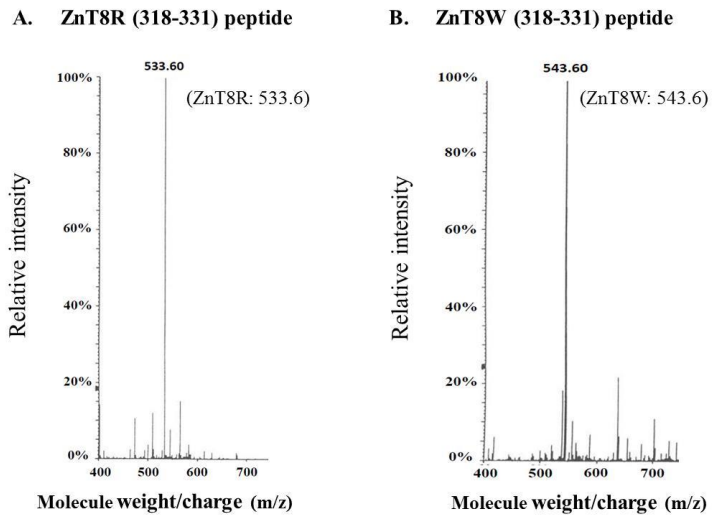

Figure 10. High performance liquid chromatography (HPLC) purification and mass-spectrometry (MS) analysis of the 15-mer ZnT8R and ZnT8W (318-331) peptides. At 70% purity of the ZnT8 peptides the MS analyses showed 533.6 m/z for the ZnT8R and 543.6 m/z for the ZnT8W peptide. The reactivity of human ZnT8-specific autoantibodies from newly diagnosed T1D patients were tested against the two ZnT8 peptides.

Mice are not Men

Following the third immunization, the reactivity of the mice sera was tested for ZnT8 peptide-antibodies as detected by ELISA. The 12 BALB/c immunized mice developed varying levels of peptide antibodies. However, the six mice (two from each group) with the highest response of peptide-antibodies failed to develop peptide-antibodies able to distinguish the three ZnT8 aa variants (Figure 11).

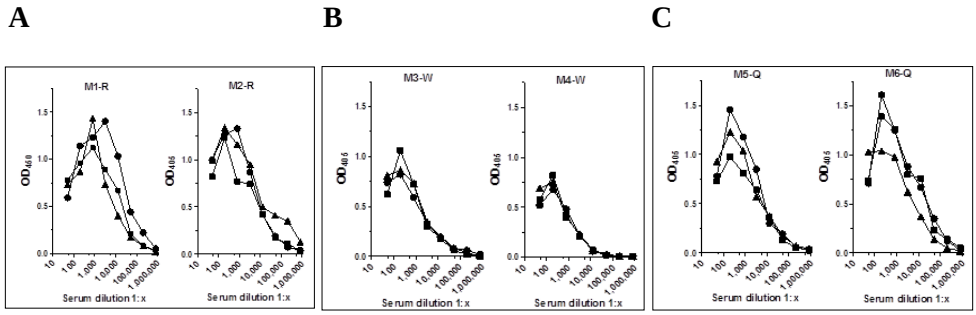

Figure 11. The reactivity against the ZnT8 (318-331) peptides in sera from immunized mice following the third booster injection**.** The six mice, two from each group of ZnT8R (panel A), ZnT8W (panel B) and ZnT8Q (panel C) displayed the highest titers of peptide-antibodies and were therefore tested for sequence-specificity against the ZnT8R, ZnT8W or ZnT8Q by ELISA (Paper I).

Next, the reactivity of human ZnT8-specific autoantibodies from newly diagnosed T1D patients were tested against the ZnT8 (318-331) peptides by RBA in a competitive displacement assay. Neither of the short ZnT8 (318–331) peptide variants were able to compete with the radiolabeled ZnT8R or ZnT8W (268–369) in binding to ZnT8RA or ZnT8WA in the human sera. The lack of recognition to the short ZnT8 peptides in the human ZnT8A sera supports previous studies that the ZnT8A most likely are conformation dependent [123, 208].

By using variant-specific *in vitro* transcription translation unlabeled ZnT8 (268-369) proteins tested on its variant-specific patient serum, we showed that the affinity (Kd50) was higher for proteins with the same epitope as the patient sera (Table 2), which has previously not been elucidated. This finding underscores the conclusion that a single aa is unable to solely control the epitope specificity.

Table 2. The reactivitys to the 325-epitope of the ZnT8R and ZnT8W (268-369) proteins demonstrated by affinity differences in binding to either ZnT8RA- or ZnT8WA-specific patient sera. Values are computed mean values of duplicate determinations of three independent experiments, expressed as pmol/L (Paper I).

Patient	ZnT8R-protein	ZnT8W-protein	*p-value*
P1-R	3.0	26.1	0.0003
P2-R	4.1	11.1	0.0023
P5-W	> 108.6	10.4	0.0016
P6-W	27.2	15.5	0.2193

Abbreviations: P1-2-R, Patient 1-2 with ZnT8RA-specific serum; P5-P6-W, Patient 5-6 with ZnT8WA-specific serum. P-value <0.05 was considered significant.

Monoclonal ZnT8 antibodies were not specific

The mouse anti-human ZnT8R or ZnT8W antibodies did not show specificity against the C-terminal part of the ^{35}S-met ZnT8 protein tested in the RBA. The mouse anti-human 325R:1 did not show reactivity against any of the ZnT8QRWtriple, ZnT8R, ZnT8W, GAD65 or IA-2 antigen (Figure 12, panel A) while mouse anti-human 325R:2 showed reactivity against all antigens, although to various extent (Figure 12, panel B). Even though, mouse anti-human 325R:2 showed highest reactivity (binding corresponding to 500 U/mL) against the ZnT8R antigen, it showed almost equally high response to the ZnT8QRWtriple and the ZnT8W antigen. Notice that the Y-axis shows 50% (500 U/mL) of maximal binding capacity (1000 U/mL) of the standard curve. In similar, the third anti-human ZnT8R antibody and the two anti-human ZnT8W antibodies lacked reactivity to all antigens tested (data not shown).

There may be several reasons why sequence-specific antibodies were not generated in the mice. It may depend on the design of the synthetic peptides, the immunization protocol or the fact that the mouse immune system is unable to present the polymorphic determinant the same way as in humans. It cannot be excluded that the association between ZnT8A and DQ6.4 or DQ8 may be important [206, 210] . Even though, mice mirror the human biology remarkably well, one should remember that there are important differences between mice and men regarding the development, activation and response of the immune system, both within the innate and adaptive type [250]. Mice are commonly used for monoclonal antibody production. Although, the mice in our study failed to produce sequence-specific antibodies the generation of single aa-specific antibodies have previously been successful [251, 252].

A **B**

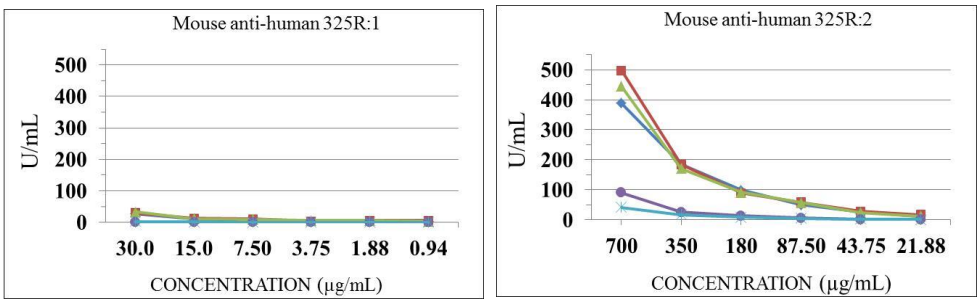

Figure 12. The reactivity of two mouse anti-human ZnT8R antibodies, 325R:1 (panel A) and 325R:2 (panel B), to the antigens, ZnT8QRWtriple (blue line), ZnT8R (red line), ZnT8W (green line), GAD65 (purple line) and IA-2 (turquoise line). Various concentrations (1:1 step dilution from the maximal concentration achieved) of the two antibodies were tested in RBA. The 325R:1 antibody lacked reactivity against all antigens (panel A) tested while the ZnT8R:2 antibody was not specific against the ZnT8R antigen (panel B).

In paper I, we showed that the variant-specific *in vitro* transcribed and translated ZnT8 (268-369) proteins primarily displaced the corresponding specific ZnT8A variant. These data suggest that the 325 variant is part of a conformation-dependent ZnT8A epitope, but one which may not exclusively controlled by the aa at this position. In addition, we demonstrated that the 15-mer ZnT8 peptide was insufficient to define the conformation-dependent epitope suggesting that the 325-epitope of ZnT8 may be broader than that of aa 318-331.

Paper IV – ZnT8A specificity and affinity

Main findings

- Recombinant human ZnT8 (275-369) proteins (R or W at position 325) were successfully produced, expressed in E.coli and purified to homogeneity for competitive reciprocal permutation experiments.
- Unlabeled ZnT8R and ZnT8W (275-369) protein displaced the binding of ^{35}S-met ZnT8R and ^{35}S-met ZnT8W, respectively, to epitope-specific sera in a concentration dependent manner.
- The ^{35}S-ZnT8R binding was inhibited to 98% by the unlabeled ZnT8R but only to 34% by the reciprocal unlabeled ZnT8W protein.
- The ^{35}S-ZnT8W binding was inhibited to 98% by the unlabeled ZnT8W but only to 8% by the reciprocal unlabeled ZnT8R protein.
- The ZnT8W-specific autoantibodies showed higher affinity compared to the ZnT8R-specific autoantibodies.
- The affinity of the variant-specific ZnT8A did not correlate to the autoantibody endpoint titers or age at diagnosis in the patients.

In paper IV, we followed up our ZnT8A 325-epitope investigation by analyzing 24 newly diagnosed T1D patients with HLA-risk genotypes (DQ2 and DQ8 or in combination with another haplotype) (Table 4). The T1D patients had high titter variant ZnT8-specific autoantibodies against either ZnT8R (3527-8236 U/mL) only (n=12) or ZnT8W (5218-11382 U/mL) only (n=12).

Successful production of recombinant C-terminal ZnT8 proteins

In this study, a major advantage was the successful generation and expression of the two recombinant ZnT8R and ZnT8W (275-369) proteins fused with the MBP-protein used in the competitive displacement experiments. MBP-ZnT8R and MBP-ZnT8W proteins (Figure 13) were confirmed at the expected size of 53 kDa by SDS-PAGE analyses. Also, the MBP-GFP protein used as a control in the subsequent competitive displacement experiments was confirmed at 70 kDa.

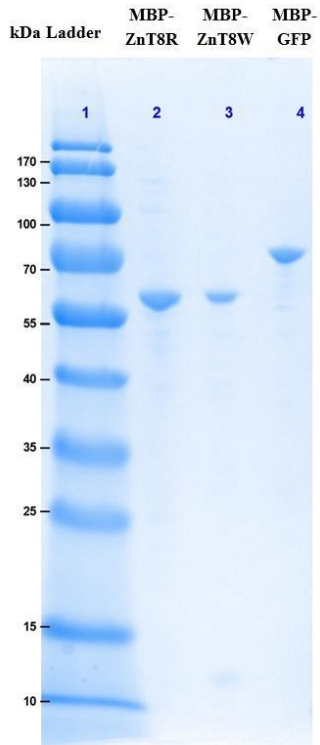

Figure 13. Identification of the MBP-ZnT8R, MBP-ZnT8W and MBP-GFP fusion proteins. The MBP-ZnT8R protein (lane 2), MBP-ZnT8W protein (lane 3) and MBP-GFP (lane 4) purified on nickel-charged columns were verified at the expected size of 53 kDa (MBP-ZnT8) and 70 kDa (MBP-GFP) by SDS-PAGE. The molecular weight marker is shown in lane 1. The gel was loaded with 1 µg of protein per lane (Paper IV).

ZnT8R and ZnT8WA may differ in specificity

The endpoint titers of the sera used in the displacement experiments were comparable for ZnT8RA (median 6532 U/mL) and ZnT8WA (median 7197 U/mL; p=0.083). Yet, all sera were diluted to represent a binding level of 50% of the reactivity in the standard curve, corresponding to about 500 U/mL.

In the reciprocal (cross-over) permutation design of our competitive RBA, we found that newly diagnosed T1D patients may have ZnT8A against either ZnT8R or ZnT8W and that these autoantibodies may have higher specificity for either R or W. In each of the 12 ZnT8RA and ZnT8WA specific sera, unlabeled ZnT8 (275-369) protein displaced the binding of ^{35}S-met ZnT8 with corresponding specificity in a concentration dependent manner. We showed that the median % inhibition of ^{35}S-met ZnT8R by the unlabeled ZnT8R (275-369) protein at the maximal concentration (100 µg/mL) tested was 98% (range 95-99%) compared to

34% (range 10-89%) of the reciprocal ZnT8W (275-369) protein in the ZnT8R-positive sera (Table 3). The median % inhibition of ^{35}S-met ZnT8W binding to the ZnT8WA-positive sera by 100 µg/mL of the unlabeled ZnT8W (275-369) protein was 98% (range 97-99%) compared to 8% (range 0-39%) in the reciprocal competition with the ZnT8R (275-369) protein. This may indicate that the ZnT8A specificity may differ depending on ZnT8A variant.

Table 3. The reactivity to the 325-epitope of the ZnT8R- and ZnT8W (275-369) proteins demonstrated by specificity expressed in % displacement at 100 µg/mL. The affinity differences (mean values of Kd50) in binding to either the 12 ZnT8RA- or the 12 ZnT8WA-specific patient sera expressed in ng/µL (Paper IV).

	ZnT8RA (n=12)	ZnT8WA (n=12)
Displacement (%) at 100 µg/mL	**Median (range)**	**Median (range)**
ZnT8R-protein	98 (95-99)	8 (0-39)
ZnT8WA-protein	34 (10-89)	98 (97-99)
Kd50 (ng/mL)	**Mean ± SEM**	**Mean ± SEM**
ZnT8R-protein	169 ± 60	N.D.
ZnT8W-protein	N.D.	68 ± 3

N.D. is none determined.

ZnT8RA and ZnT8WA may differ in affinity

Next, mean values of the half-maximal binding (Kd50) were computed for each of the 12 patients being specific for either ZnT8RA (Table 4A) or ZnT8WA (Table 4B). We found that the affinity of ZnT8WA (68 ± 3 ng/mL (mean ± SEM; n=12) was twice as high as that of ZnT8RA (169 ± 60 ng/mL (mean ± SEM; n=12) (Table 3).

Table 4. The half-maximal binding (Kd50) of the ZnT8R- and ZnT8W (275-369) proteins, expressed in ng/mL, in each of the T1D patients (n=12) specific for either the ZnT8RA (panel A) or the ZnT8WA (panel B), respectively. Gender, age at onset, HLA genotypes and ZnT8A endpoint titers (U/mL) in the patients are also shown (Paper IV).

A

PATIENT	GENDER	AGE ONSET	HLA GENOTYPE	ZnT8RA ENDPOINT TITERS U/mL	ZnT8R Kd50 ng/mL	ZnT8W Kd50 ng/mL
P-R1	F	1.5	DQ8/5.1	4232	88	7,000
P-R2	F	2.5	DQ2/9	6723	82	N.D.
P-R3	M	3.0	DQ2/8	4415	86	N.D.
P-R4	M	4.1	DQ2/8	3645	81	70,000
P-R5	F	5.7	DQ2/8	7898	84	82,000
P-R6	F	6.1	DQ8/8	7504	86	N.D.
P-R7	F	7.1	DQ2/8	8236	81	N.D.
P-R8	M	7.1	DQ2/5.1	5083	88	N.D.
P-R9	F	7.3	DQ2.2/04	3527	77	N.D.
P-R10	M	10.9	DQ8/6.3	6763	84	N.D.
P-R11	F	12.3	DQ6.4/2.1	7636	450	N.D.
P-R12	M	13.6	DQ2/8	6341	740	N.D.

B

PATIENT	GENDER	AGE ONSET	HLA GENOTYPE	ZnT8WA ENDPOINT TITERS U/mL	ZnT8W Kd50 ng/mL	ZnT8R Kd50 ng/mL
P-W1	F	4.7	DQ2/2	11382	72	N.D.
P-W2	F	5.7	DQ8/8	9128	61	N.D.
P-W3	F	6.2	DQ6.4/9	5445	64	N.D.
P-W4	M	6.4	DQ2/8	7529	45	N.D.
P-W5	M	8.5	DQ2/8	5218	73	N.D.
P-W6	F	8.7	DQ2/5.1	5638	60	N.D.
P-W7	M	8.7	DQ2/8	8016	76	N.D.
P-W8	M	9.8	DQ2/8	6450	73	N.D.
P-W9	F	10.3	DQ8/5.1	9664	66	N.D.
P-W10	F	11.2	DQ8/6.3	8677	79	N.D.
P-W11	F	12.3	DQ8/6.3	6866	69	N.D.
P-W12	M	14.9	DQ2/2.2	6127	80	N.D.

Abbreviations: F, female; M, male; ZnT8RA, Zinc transporter 8 arginine autoantibodies; ZnT8WA, Zinc transporter 8 tryptophan autoantibodies; Age-onset is expressed in years. N.D. is none determined and N.A. is not applicable.

In the reciprocal permutation experiment at 100 µg/mL, the ZnT8RA-specific sera were more efficiently displaced compared to the ZnT8WA-specific sera (p=0.009) (Figure 14). The ZnT8RA sera were inhibited 37 ± 7% (mean ± SEM; n=12) by the unlabeled ZnT8W (275-369) protein (Figure 14, panel B) compared to 14 ± 4% (mean ± SEM; n=12) when the ZnT8WA sera were inhibited by the unlabeled

ZnT8R (275-369) protein (Figure 14, panel A). Complete displacement was observed for the unlabeled protein corresponding to the autoantibody specificity.

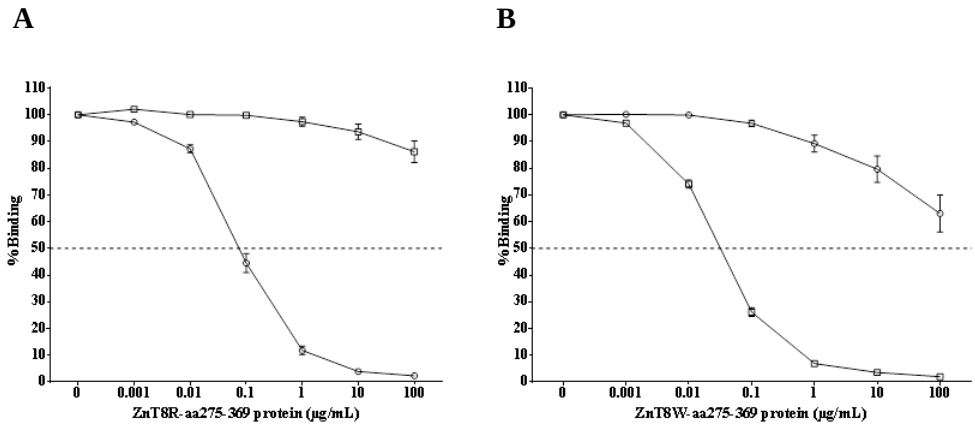

Figure 14. Effects of either 0.001-100 µg/mL unlabeled ZnT8R (275-369) (panel A) or unlabeled ZnT8W (275-369) (panel B) on the binding of either ^{35}S-met ZnT8R to ZnT8RA (opened circles) or ^{35}S-met ZnT8W to ZnT8WA (opened squares). The horizontal dotted lines indicate the half-maximal binding (Kd). Complete displacement was observed for the unlabeled protein corresponding to the autoantibody specificity. At 100 µg/mL, the specific ZnT8RA sera were more effectively displaced by unlabeled ZnT8W (275-369) (37 ± 7 % displacement) (panel B) compared to the reciprocal displacement by unlabeled ZnT8R (275-369) (14 ± 4 % displacement; p=0.009) (panel A). Mean values ± SEM for 12 patients with autoantibody specificity against either the ZnT8R or the ZnT8W variant are shown (Paper IV).

Neither of the specificity nor affinity seemed to be explained by the endpoint titers specific for either ZnT8RA (R^2=0.120; p=0.711) or ZnT8WA (R^2= -0.021; p=0.948) or age at diagnosis in ZnT8RA patients (R^2=0.539; p=0.446) or ZnT8WA patients (R^2=0.316; p=0.076).

In paper IV, recombinant C-terminal ZnT8 (275-369) proteins were produced and purified from *E.coli* sufficient for competitive displacement experiments to explore the ZnT8A binding to the 325-epitope in T1D patients. It should be emphasized that several attempts in collaboration with industry and academia to generate recombinant ZnT8 proteins failed until the present system was tested to obtain ZnT8 (275-369) as a fusion protein with MBP.

Adding to previous studies showing that ZnT8A have a remarkable specificity for the variant aa residue of R or W at position 325 [203, 208], we demonstrated that the specificity may vary depending on the residue of R or W in this position. The ability of the variant specific ZnT8A to discriminate between one aa in their epitope recognition may be unique in autoimmunity. Our findings that variant ZnT8A may differ in both specificity and affinity to the 325-epitope indicate that auto-immunization in humans may not always be polyclonal. This aa specificity may be unique in the autoantibody development during the prodromal stage of

autoimmune diabetes. In addition, it was demonstrated that low and high affinity as well as the specificity of IAA and GADA may have variable risk for T1D. Therefore, affinity and specificity of ZnT8A may be valuable to the prediction in children at risk for T1D. To our knowledge, this is the first report of ZnT8A affinity analysis using the C-terminal end of the ZnT8 protein.

Paper II – NPY autoantibodies in long-term diabetes

Main findings

- The NPY-LA and NPY-PA autoantibody variants (L7P) were present and often together in long-term T1D and T2D patients.
- NPY autoantibodies levels declined with time.
- NPY autoantibodies were not related to either peripheral or autonomic neuropathy but to long-term diabetes regardless of classification.

Hydrophobicity of NPY proteins

The signal peptide region of NPY has hydrophobic features based on the Kyte-Doolittle hydrophobicity analysis ranging between 4.6 (most hydrophobic) and -4.6 (least hydrophobic). The scores are based on the values given by the original Kyte-Doolittle scoring system, which is applied to define hydrophobic characters of a protein [253]. Regions with values above zero are considered to be hydrophobic. In the signal peptide, the SNP at rs16139 at the aa position 7 causes a drastic change from a hydrophobic aa (leucine) at scoring of 3.8 to hydrophilic aa (proline) at -1.6. The mature protein (aa 29-64) and the C-peptide (aa 68-97) displayed overall less hydrophobic characteristics ranging between -0.1 and -1.8 compared to the signal peptide (Figure 15).

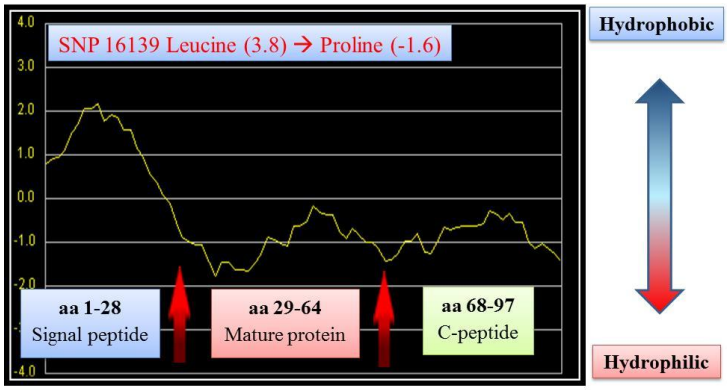

Figure 15. Hydrophobicity plot of the prepro-NPY (1-97) protein sequence**.** The NPY protein consists of the signal peptide region, which shows hydrophobic properties compared to the mature protein (aa 29-64) and the C-peptide flanking region (aa 68-97) showing less hydrophobic characters. The scores are based on the values given by the original Kyte-Doolittle ranging between 4.6 (hydrophobic) to -4.6 (hydrophilic). The SNP rs16139 at aa 7 causing a change in the hydrophobicity score from 3.8 (leucine) to -1.6 (proline).

Despite that hydrophobic signal peptide the radiolabeling with ^{35}S-met of the two pThNPY-L and pThNPY-P plasmids by *in vitro* transcription and translation were plausible. The ratio between the radiolabeled NPY-proteins (eluated in tube 1) and the free radiolabel (eluated in tube 4) were calculated to achieve the % incorporation rate indicating 34% for the NPY-L protein and 27% for the NPY-P protein (Figure 16).

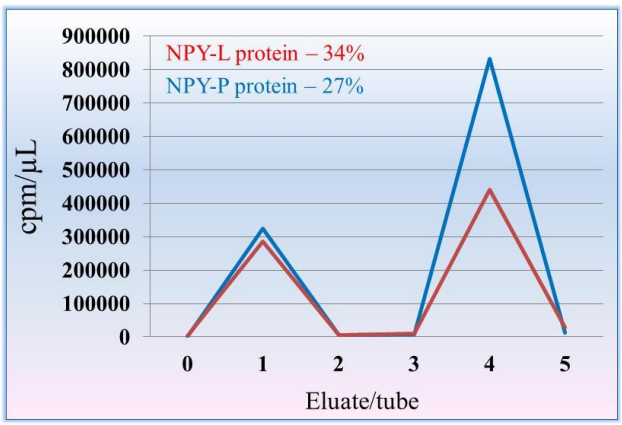

Figure 16. The ^{35}S-met NPY-L (red line) and NPY-P (blue line) eluated in tube 1 was separated from the free ^{35}S-met in tube 4 by gel filtration**.** The incorporation rate (free label: incorporated label) of ^{35}S-met, expressed in percentage (%), of NPY-L and NPY-P were 34% and 27%, respectively.

Both ^{35}S-met NPY protein products were verified at the expected molecular weight at Mr 10.8 by SDS-PAGE. To validate the radiolabeled NPY protein products, the binding of both ^{35}S-met NPY-L and ^{35}S-met NPY-P to the standard (polyclonal rabbit IgG antibody against full length human NPY) were displaced in a concentration dependent manner by unlabeled NPY-L and NPY-P proteins (Figure 17) in a competitive RBA described elsewhere [254].

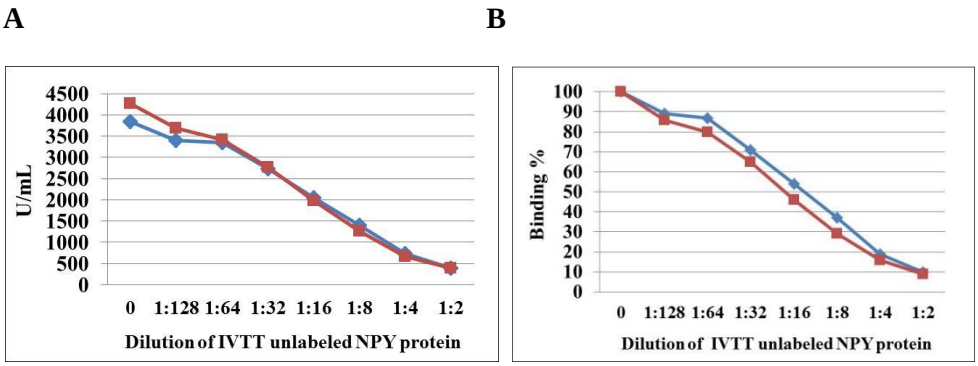

Figure 17. Displacement of ^{35}S-met *in vitro* transcription and translation NPY-L (blue line) and NPY-P (red line) proteins by unlabeled *in vitro* transcription and translation NPY-L and NPY-P proteins. The unlabeled NPY proteins displaced the binding to rabbit polyclonal anti-human NPY antibodies (concentration 25 µg/mL) in a concentration dependent manner, expressed in U/mL (panel A) and in binding % (panel B).

In order to distinguish the autoantibody positive individuals from the autoantibody negative, cut-offs at the 97.5th percentile for NPYA, GADA, and the ZnT8A variants and IA-2A at the 99th percentile were determined by Q-Q plot analyses of the 398 healthy controls. The cut-off levels at the 97.5th percentile were 44 U/mL for NPY-LA and 32 U/mL for NPY-PA (Figure 18).

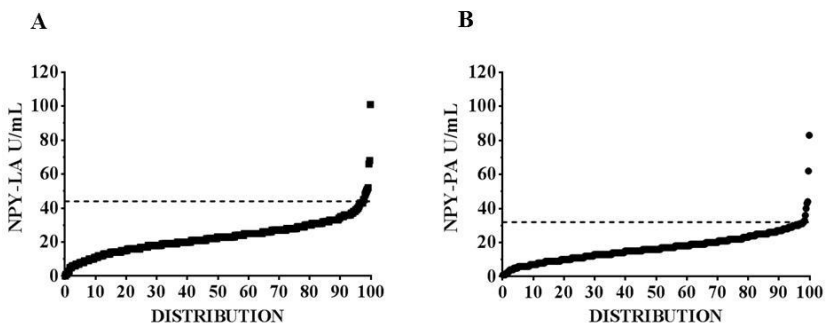

Figure 18. Quantile-quantile (Q-Q) plot analyses of the minor autoantibodies NPY-LA and NPY-PA in 398 healthy controls**.** The healthy control (n=398) levels showed that the cut-off at 97.5th percentile corresponded to 44 U/mL for NPY-LA (panel A) and 32 U/mL for NPY-PA (panel B) (Paper II).

The corresponding percentile (97.5th) resulted in cut-offs at 35 U/mL for both GADA (Figure 19, panel A) and ZnT8RA (Figure 19, panel C), 27 U/mL for ZnT8WA (Figure 19, panel D) and 55 U/mL for ZnT8QA (Figure 19, panel E). The 99th percentile was used for IA-2A corresponding to the cut-off level at 5 U/mL (Figure 19, panel B).

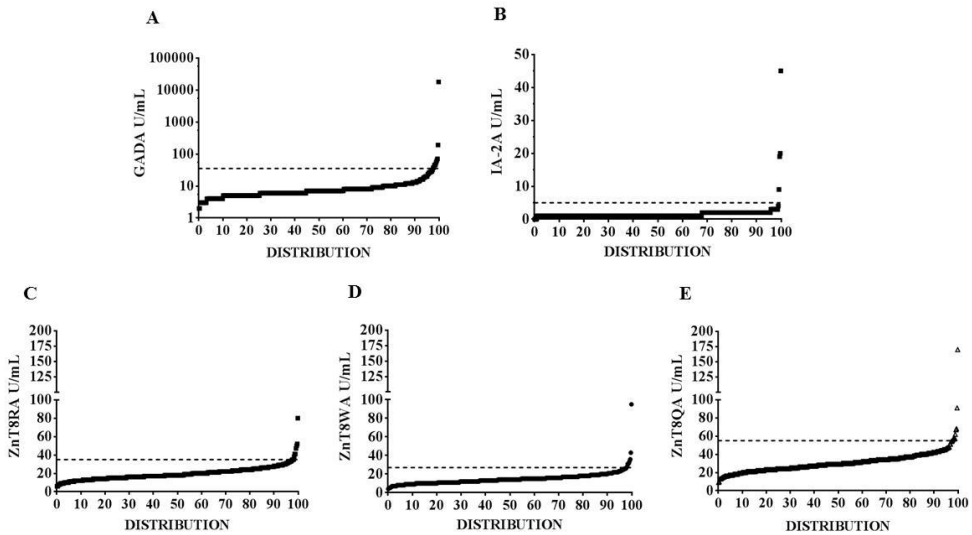

Figure 19. Q-Q plot analyses of the five major autoantibodies GADA, IA-2A and the three ZnT8A variants in 398 healthy controls**.** The healthy control levels showed that the cut-off at 97.5th percentile was 35 U/mL for GADA (panel A) while the cut-off for IA-2A at the 99th percentile was 5 U/mL (panel B). Similarly to GADA, the 97.5th percentile was used for the ZnT8A levels corresponding to 35 U/mL for ZnT8RA, 27 U/mL for ZnT8WA and 55 U/mL for ZnT8QA. The cut-off levels (dotted line) were used to distinguish the autoantibody positive individuals from the negative.

NPYA present in long-term T1D and T2D patients

The long-term T1D (n=48) patients were diagnosed at 15-26 years of age compared to the T2D (n=26) patients diagnosed at 31-50 years of age. Duration of the disease (26 and 23 years, respectively) was comparable between the two groups of patients. A total of 32 T1D patients were re-examined (5-8 years later) after 21-50 years of disease.

The frequency at baseline in the long-term T1D patients was 23% for NPY-LA ($p<0.001$) and 19% for NPY-PA ($p<0.001$) compared to controls (Table 5). There were 12.5% NPY-LA and NPY-PA double positive T1D patients. The corresponding frequency in the patients with T2D was 12% for NPY-LA

(p=0.039) and 23% for NPY-PA (p<0.001) as compared to the controls (Table 5). NPY-LA and NPY-PA double positive T2D patients were 11.5%.

Table 5. Frequency of autoantibodies against NPY-L, NPY-P, ZnT8R, ZnT8W, ZnT8Q, GAD65 and IA-2 in long-term T1D (n=48) and T2D (n=26) patients at baseline compared to the controls (n=398) (Paper II).

	T1D n=48 (%)	T2D n=26 (%)	Controls n=398 (%)	*p-value*
NPY-LA	11 (23)	3 (12)	10 (3)	*p<0.001 **p=0.039
NPY-PA	9 (19)	6 (23)	8 (2)	*p<0.001 **p<0.001
NPYA (1 ≥)	14 (29)	6 (23)	12 (3)	*p<0.001 **p<0.001
NPY-LA only	1 (2)	0 (0)	1 (0.03)	*N.A. **N.A.
NPY-PA only	0 (0)	3 (12)	1 (0.03)	*N.A. **N.A.
ZnT8RA	19 (40)	4 (15)	8 (2)	*p<0.001 **p=0.004
ZnT8WA	19 (40)	3 (12)	9 (2)	*p<0.001 **p=0.031
ZnT8QA	17 (35)	3 (12)	9 (2)	*p<0.001 **p=0.031
ZnT8A (1 ≥)	25 (52)	6 (23)	14 (4)	*p<0.001 **p<0.001
GADA	25 (52)	0 (0)	8 (2)	*p<0.001 **N.A.
IA-2A	15 (31)	0 (0)	4 (1)	*p<0.001 **N.A.
Autoantibody negative	7 (15)	18 (69)	367 (92)	*p<0.001 **p<0.001

P-values are shown as *T1D patients compared to controls and **T2D patients compared to controls. P-value <0.05 was considered significant.. Abbreviations: NPY-LA, Neuropeptide Y-Leucine autoantibodies; NPY-PA, Neuropeptide Y-Proline autoantibodies; N.A. is not applicable.

The follow-up subgroup of 32 T1D patients was analyzed for NPYA after duration of 21-50 years, 5-8 years after baseline. The median levels of NPY-LA had declined from 34 U/mL to 21 U/mL (p<0.001) (Figure 20, panel A) and of NPY-PA from 20 U/mL to 12 U/mL (p<0.001) (Figure 20, panel B).

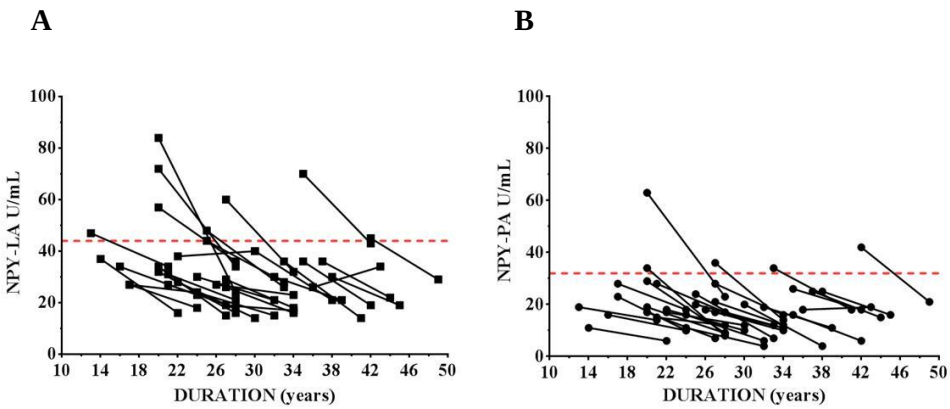

Figure 20. Median levels of the NPY autoantibodies in the subgroup of long-term T1D patients (n=32) analyzed during follow-up (duration 21-50 years), 5-8 years after baseline**.** The median levels of NPY-LA (panel A) declined from 34 U/mL to 21 U/mL ($p<0.001$) and of NPY-PA (panel B) from 20 U/mL to 12 U/mL ($p<0.001$). The dotted line represents the cut-off for each NPYA variant which distinguishes the autoantibody positive patients from the negative (Modified from paper II).

NPYA did not predict neuropathy in T1D patients

The relationship between NPY autoantibodies and peripheral or autonomic neuropathy were investigated in the long-term follow-up patients with T1D. Although, there were not an association between the presence or levels of NPYA and the neuropathy, the peripheral neuropathy was related to cardiac autonomic neuropathy ($R^2=0.328$; $p<0.001$). In addition, males (86%) had more often an abnormal cardiac autonomic neuropathy (cut-off at −1.64) compared to females (14%) ($p=0.007$).

Previously, it was indicated that the structure of the mature NPY protein is dependent on the aa residue at SNP rs16139 [148] resulting in an altered packaging and secretion of NPY [149]. The genetic polymorphisms of the patients in this study were unknown. It would be of interest to determine if autoantibodies against NPY are related to the L7P genetic polymorphism. Our kappa analysis suggests that there was substantial agreement between the two NPYA variants, which suggest that NPY L7P polymorphisms may not be as strong as the ZnT8 R325W polymorphism [254] to determine a crucial epitope. In order to detect specific serum autoreactivity against either NPY-P or NPY-L protein, displacement assays with an unlabeled inhibitor was used. However, specific sera were rare and further analysis of large number of sera will be needed to search for possible isoform-specific sera. It cannot be excluded that individuals may have such autoantibodies.

The presence of ZnT8A in 23% of the T2D patients (Table 5) is complicated due to the fact that our T2D patients had had the disease for 10-31 years. It cannot be decided at this time whether the ZnT8A had been present early on in the disease or appeared later during the years of treatment for T2D. Also, it may be discussed whether the T2D patients were misdiagnosed during the time between 1968 and 1989 when they first visit the clinic. Among the 26 T2D patients, 20 (78%) were overweight or obese, which would be consistent with their classification as type 2 (Table 6A). We noted that 6/26 (23%) T2D patients had at least one of the NPYA variants and 5/6 of these were overweight or obese (Table 6B). Similarly, ZnT8A were also positive in 5/6 overweight or obese T2D. Although there was no relation between NPYA (or ZnT8A) and neuropathy in the T1D patients, it cannot be excluded that there may be such a relation in T2D. Unfortunately, we did not have any neuropathy data of the T2D patients.

Furthermore, the majority (65%) of the T2D patients was treated with insulin while 27% was treated with orally hypoglycemic agents and 8% with dietary change (Table 6A). The patients with NPYA (n=6) were found among the insulin treated (n=4) and diet (n=2) and not among oral hypoglycemic agents (n=0) treated patients. Similarly, the patients with ZnT8A (n=6) were found only among the insulin (n=4) and diet (n=2) treated.

Table 6. Descriptive data of the T2D patients including height, weight, BMI and treatment strategies (Table A) and the frequency of NPY and ZnT8 autoantibodies in the T2D patients in relation to BMI (Table B).

A

	T2D PATIENTS n=26
Height, cm, median (range)	179 (154-196)
Weight, kg, median (range)	92 (65-138)
BMI, median (range)	28.8 (22.3-39.5)
Ideal weight - BMI 19.0-25.9, n (%)	6 (22)
Overweight - BMI 26.0-30.0, n (%)	10 (39)
Obesity - BMI > 30.0, n (%)	10 (39)
Insulin treatment, n (%)	17 (65)
Oral hypoglycemic agents, n (%)	7 (27)
Diet, n (%)	2 (8)

B

	IDEAL WEIGHT BMI 19.0-25.9	OVERWEIGHT BMI 26.0-30.0	OBESE BMI >30.0
AUTOANTIBODIES			
NPY-LA, n (%)		2 (8)	1 (4)
NPY-PA, n (%)	1 (4)	4 (15)	1 (4)
NPYA, n (%)	1 (4)	4 (15)	1 (4)
ZnT8RA, n (%)	1 (4)	2 (8)	1 (4)
ZnT8WA, n (%)	1 (4)	2 (8)	
ZnT8QA, n (%)	1 (4)	2 (8)	
ZnT8A, n (%)	1 (4)	4 (15)	1 (4)

Abbreviations: NPY-LA, Neuropeptide Y-Leucine autoantibodies; NPY-PA, Neuropeptide Y-Proline autoantibodies; BMI, Body mass index calculated by; Weight (kg) / Height $(m)^2$.

In paper II, we established an autoantibody radiobinding assay to assess the levels and frequency of two NPY autoantibody variants (NPY-LA and NPY-PA) in patients with T1D and T2D compared to controls. The major findings were that 23% and 19% of the long-term T1D patients had NPY-LA and NPY-PA, respectively, compared to 12% and 23%, respectively in the patients with T2D. The frequency of autoantibodies was based on using the control 97.5th percentile as cut-off. Previously, NPY-LA was reported in 9% of T1D patients newly diagnosed below the age of 19 years [84]. It was therefore of interest that the present group of patients not only were diagnosed as adults but also had disease duration of median 26 years at the time when the first samples were obtained (referred to as baseline). The observation that there was a difference of the

abnormal autonomic neuropathy between genders was not explained by any of the islet autoantibodies, including NPY-LA or NPY-PA. The results of our study demonstrate that NPY is not necessarily a minor autoantigen restricted to T1D. NPY may rather be related to autoimmunity in general as NPYA were also found in T2D patients. It is well accepted that autoimmunity in general is increasing with increasing age.

To our knowledge this is the first study to investigate two variants of NPY autoantibodies in patients with long-term T1D and T2D. Although the appearance of islet autoantibodies prior to the clinical onset of T1D have made it possible to predict disease, it is still of interest to establish to what extent islet autoimmunity may be related to late diabetes complications.

Paper III – NPY autoantibodies at clinical onset

Main findings

- NPY autoantibodies were identified in 26% of the newly diagnosed T1D patients.
- Autoantibodies against the NPY-P variant (24%) were more common in the patients compared to the NPY-L variant (17%), although the levels correlated (R^2=0.631; p<0.001).
- The risk of having NPYA at clinical onset was increased with older age at diagnosis (≥10 years), being a female and having multiple autoantibodies.
- NPY was a minor autoantigen in the newly diagnosed T1D patients.

NPYA present in newly diagnosed T1D patients

In paper III, we followed up our findings of NPYA in long-term T1D by investigating NPYA in 673 newly diagnosed T1D children from the Skåne study.

By comparing the two NPYA variants, we observed that the NPY-PA were more common than NPY-LA in the newly diagnosed patients (p<0.001). Out of 673 T1D patients, 26% (n=175) were positive for at least one of the NPY variant autoantibodies compared to 1.5% (n=6) controls. The major autoantibodies (IAA, GADA, IA-2A and the ZnT8A variants R, W, Q as well as ICA) combined together encompassed 94.5% (n=636) of the patients with one or several of the major autoantibodies. By including the NPY-LA and NPY-PA with the major autoantibodies the autoantibody negative T1D patients were reduced from 5.5%

(n=37) to 4.8% (n=32). This indicated that 5 T1D patients were positive for NPYA solely resulting in an increase of the diagnostic sensitivity with 0.7%. The median levels of both NPYA variants were higher in the T1D patients compared to the controls, although the levels of NPY-LA were higher than that of NPY-PA in both patients and controls (Figure 21, panel A). The levels of the two NPYA in the patients were correlated (R^2=0.631; p<0.001) (Figure 21, panel B).

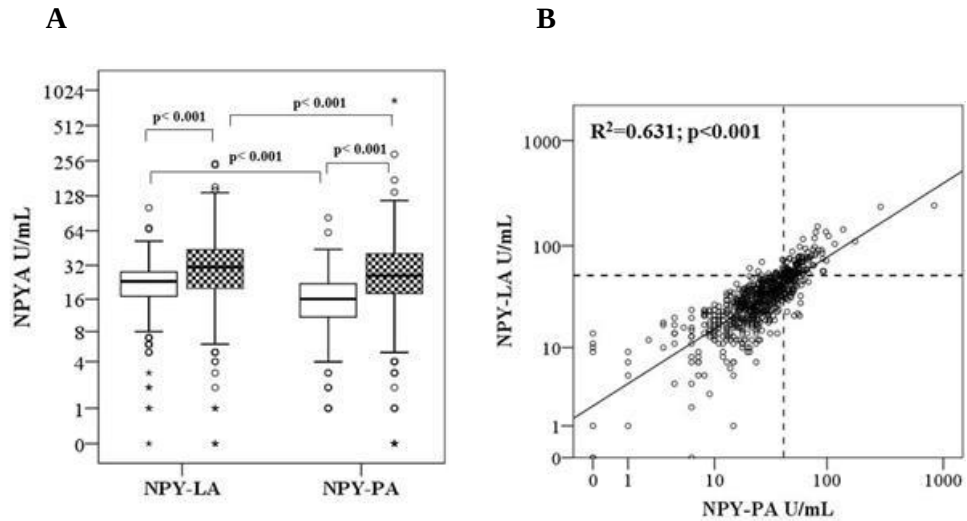

Figure 21. Median levels of NPY-L and NPY-P autoantibodies (panel A) in 673 newly diagnosed T1D patients (▩) compared to 398 controls (□) as well as the correlation between the NPY-LA and NPY-PA levels (panel B) are shown. The median levels of NPY-LA in the controls was 23 U/mL compared to 31 U/mL in the patients (p<0.001). The corresponding values for NPY-PA were 16 U/mL and 26 U/mL, respectively (p<0.001). In panel B, the dotted lines represent the cut-off values for NPY-LA at 52 U/mL and at 42 U/mL for NPY-PA, respectively. The coefficient of correlation was R^2=0.373 (p<0.001) above and R^2=0.466 (p<0.001) below the cut-off levels (Paper III).

Are the NPY autoantibodies valuable in the prediction of T1D?

To investigate whether the NPYA may be useful as diagnostic markers for T1D, the sensitivity and specificity were analyzed and plotted in receiver operating characteristic (ROC) curves. The cut-off values for NPY-LA and NPY-PA at the specificity of 99% and sensitivity of 17% and 24% corresponded to 52 U/mL and 42 U/mL, respectively, in the patients (Figure 22). In order to evaluate how well the NPYA may distinguish between patients and controls, the area under the curve (AUC) was calculated. AUC for NPY-LA (Figure 22, panel A) was 0.68 (95% Cl 0.66-0.72) and 0.74 (95% Cl 0.71-0.77) for NPY-PA (Figure 22, panel B). An AUC value of >0.70 may be considered to be of diagnostic value.

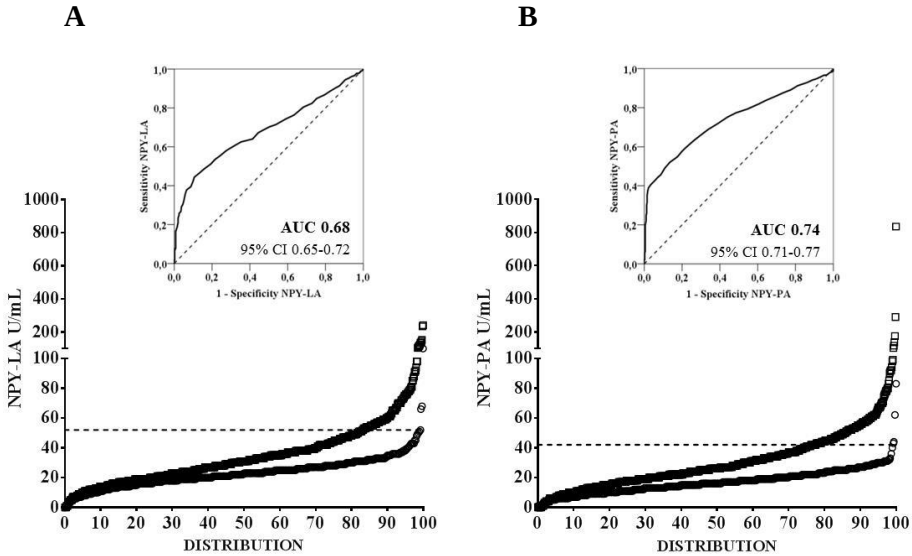

Figure 22. Q-Q plot analyses of NPY-LA (panel A) and NPY-PA (panel B) levels in T1D patients (□) and adult controls (○). The levels of NPY autoantibody binding in healthy adult controls (n=398) showed that the cut-off at 99th percentile was 52 U/mL for NPY-L and 42 U/mL for NPY-P autoantibodies (dotted lines). The 99th percentile in the controls corresponded to 83rd percentile for the NPY-LA and 76th percentile for NPY-P autoantibodies in the T1D patients. Inserts demonstrate ROC curve analyses of the NPY-LA (panel A) and NPY-PA (panel B) in the T1D patients compared to the controls. In the ROC analyses, the cut-off values for NPY-LA and NPY-PA at a specificity of 99% and sensitivity of 17% and 24% corresponded to 52 U/mL and 42 U/mL, respectively, in the patients (Paper III).

Age at diagnosis and gender – related to the odds of NPYA

We next investigated whether NPYA were related to the major autoantibodies, age at diagnosis, gender or the HLA genotypes. In patients diagnosed after 10 years of age HLA-DQ2/2 was negatively associated with the NPYA (OR=0.20; 95th Cl 0.05-0.87; p=0.016) primarily explained by the NPY-PA variant (OR=0.23; 95th Cl 0.05-0.98; p=0.029) and not by the NPY-LA (OR=0.36; 95th Cl 0.82-1.58; p=0.272). However, in the regression analyses adjusted for autoantibody status, age at diagnoses and gender, the HLA-DQ2/2 association disappeared. Moreover, we observed that major autoantibody status (p=0.032), age at diagnosis after 10 years of age (p<0.001) and female gender (p=0.023) increased the odds of being diagnosed with NPYA (NPY-LA and NPY-PA taken together) (Table 7).

Table 7. The risk of having any or both of the NPY autoantibody variants at the diagnosis of T1D. Both unadjusted and adjusted values in all T1D patients (n=673) are shown (Paper III).

FACTORS	UNADJUSTED			ADJUSTED*		
T1D patients (n=673)	OR	95% CI	*p-value*	OR	95% CI	*p-value*
Positivity for major autoantibodies	12.04	1.63-88.80	0.002	2.92	1.10-7.80	0.032
Age at diagnosis ≥ 10 years	2.14	1.50-3.05	<0.001	2.31	1.60-3.33	<0.001
Female	1.45	1.02-2.04	0.036	1.51	1.06-2.15	0.023
HLA-DQ genotypes [a]						
DQ2/X	1.31	0.77-2.23	0.327	1.21	0.59-2.47	0.599
DQ2/8	1.20	0.83-1.72	0.331	1.15	0.64-2.05	0.645
DQ8/8	0.99	0.58-1.70	0.970	0.91	0.44-1.86	0.789
DQ8/X	0.82	0.55-1.21	0.315	0.83	0.45-1.52	0.537
DQ2/2	0.45	0.19-1.09	0.071	0.47	0.17-1.29	0.142

* Logistic regression adjusted for positivity for major autoantibodies, age at diagnosis, gender and the HLA-DQ genotypes (X was a haplotype not tested for and was neither DQ2 nor DQ8). Major autoantibodies included GADA, IA-2A, IAA, ZnT8R, ZnT8WA, ZnT8QA and ICA.

[a]The following abbreviations were used for the HLA-DQ haplotypes: DQ2/8, DQ A1*05:01-B1*02:01/A1*03:01-B1*03:02; DQ8/X, DQ A1*03:01-B1*03:02/X (X is any haplotype except for DQ2 or DQ8); DQ8/8, DQ A1*03:01-B1*03:02/ A1*03:01-B1*03:02; DQ2/X, DQ A1*05:01-B1*02:01/X (X is any haplotype except for DQ2 or DQ8); DQ2/2, DQ A1*05:01-B1*02:01/A1*05:01-B1*02:01.

In the first series of experiments the control samples were from 398 adult (19-81 years of age) blood donors. It was found that the most efficient cut-off for positivity was at the 99th percentile (Figure 22). The frequency in the T1D patients was found to be 26% for NPYA. However, the T1D children represented 1-19 year olds. Therefore, an alternative control group was tested to exclude a possible effect of age. In the second series of experiments we obtained control sera from 608 healthy children (9-12 years) in the CiPiS study [245]. The levels of NPY autoantibody binding in the healthy control children (n=608) showed that the cut-offs for the NPYA levels at the 97.5th percentile was 52 U/mL for NPY-LA and 50 U/mL for NPY-PA (Figure 23). At the 99th percentile the cut-offs for NPY-L and NPY-P autoantibodies were 65 U/mL and 70 U/mL, respectively. The median levels of NPY-LA were 29 U/mL in the control children compared to 23 U/mL in the adult controls and for NPY-PA the median levels were 27 U/mL compared to 16 U/mL, respectively. The levels of both NPY-LA and NPY-PA between the control adults and control children differed (NPY-LA, $p<0.001$; NPY-PA, $p<0.001$) (Figure 24).

At the 97.5th percentile the frequency of NPY-LA was 17% (115/676) and 14.8% (100/676) for the NPY-PA compared to 2.8% (17/608) in the control children. The double NPYA positive patients were 11.2% (76/676) compared to 1.5% (9/608) in the controls. By using the 99th percentile corresponding to 1% (6/608) positive control children, we identified 8.7% (59/676) positive NPY-LA patients and 4.1% (28/676) positive NPY-PA patients. There were 3.7% (25/676) patients who were double positive for the NPYA.

It may be debated what percentile to be used as it determines the relation between the specificity and sensitivity of the assay. The percentile is important as a cut-off at a low percentile such as the 97.5th percentile would increase the risk to include false positive individuals. On the other hand, a high percentile cut-off may increase the risk for false negative subjects. The percentile used may have a significant impact on the NPYA frequency especially as they may be regarded as low-level binding autoantibodies.

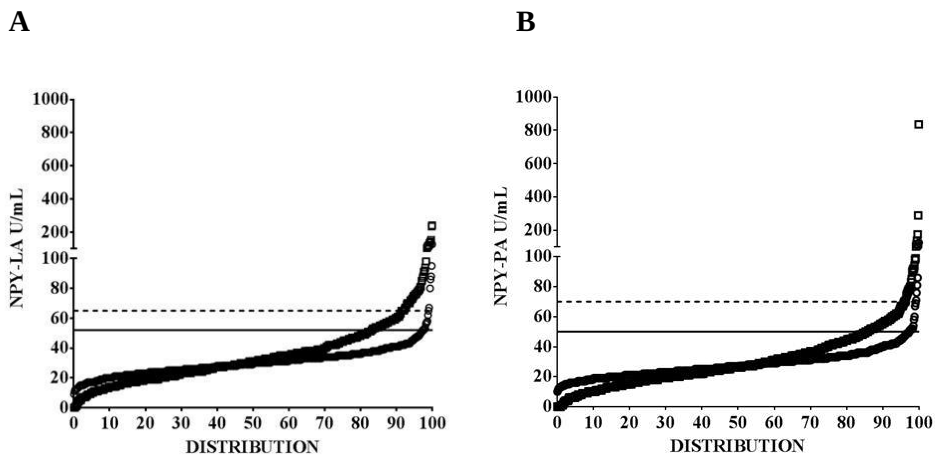

Figure 23. Q-Q plot analyses of NPY-LA (panel A) and NPY-PA (panel B) levels in newly 673 diagnosed T1D patients (□) compared to 608 control children (○). The levels of NPY autoantibody binding in the control children showed that the cut-offs at 97.5th percentile were 52 U/mL for NPY-L and 50 U/mL for NPY-P autoantibodies (solid lines). At the 99th percentile the levels of NPY-LA corresponded to 65 U/mL and 70 U/mL for NPY-PA (dotted line).

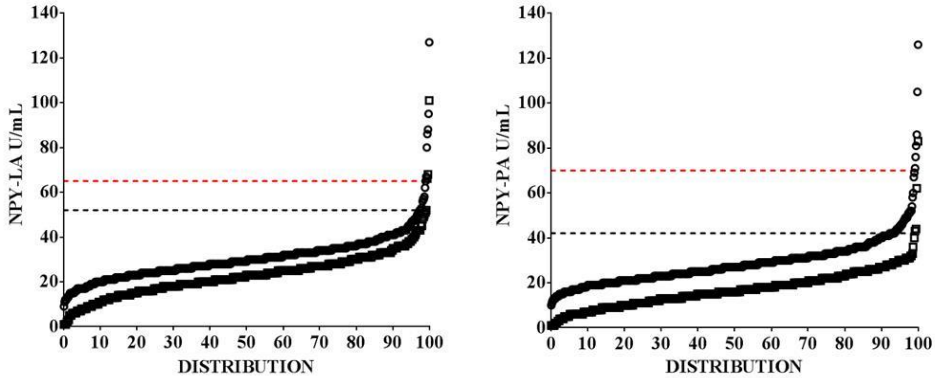

Figure 24. Q-Q plot analyses of NPY-LA (panel A) and NPY-PA (panel B) in 398 healthy adult controls (□) compared to 608 healthy control children (○). The levels of NPY autoantibody binding in the adult controls showed that the cut-off at 99th percentile were 52 U/mL for NPY-L and 42 U/mL for NPY-P autoantibodies (black dotted lines) compared to 65 U/mL for NPY-LA and 70 U/mL for NPY-PA in the control children (red dotted lines).

In paper III, we used the 99th percentile as cut-off in the adult controls. It was demonstrated that 26% of the newly diagnosed T1D patients had autoantibodies against NPY. Our data indicated that there was a strong interrelationship between the two NPYA variants and would therefore support the view that the autoantibody response tend to be polyclonal rather than being monoclonal (or oligoclonal) against the L or P polymorphic site. This is consistent with our observation that 15% of the T1D patients had autoantibodies against both variants. In addition, patients with a clinical onset after 10 years of age were shown to have a 2.3-fold increased risk for NPYA at onset.

Whether NPYA develop in patients with an extensive prodromal stage of autoimmunity is unknown. NPYA have not been studied longitudinally in subjects at risk as have been done for the major autoantibodies IAA, GADA and IA-2A [54, 255, 256]. There is a reason to believe that NPYA develops secondary to the major autoantibodies as we found that NPYA were more often present in older patients. This would support the hypothesis that NPYA may be recognized as a consequence of ongoing β-cell destruction due to epitope spreading.

SUMMARY AND GENERAL CONCLUSIONS

Papers I and IV

The epitope-specificity and affinity of the autoantibodies against the major autoantigen, ZnT8, in T1D had previously not been investigated. Therefore, we set up a competitive ZnT8 autoantibody displacement assay to study the 325-epitope to which ZnT8 variant-specific autoantibodies in human T1D sera bind. This assay was used in a reciprocal permutation design in both papers I and IV. In Paper I, we studied the binding to synthetic (15 aa) ZnT8R and ZnT8W peptides of the ZnT8A. In paper IV, we also used patient-specific ZnT8A to study recombinant (95 aa) ZnT8R and ZnT8W proteins.

In addition, in paper I, we tested whether the C-terminal part (aa 268-369) of the unlabeled *in vitro* transcription and translation ZnT8 could displace the binding of the equally long but radiolabeled ZnT8 proteins in binding to ZnT8A. As these ZnT8 proteins were produced by *in vitro* transcription and translation and were not concentration determined, serial dilution of the ZnT8 proteins had to be used. Consequently, we were not able to determine at which concentration the ZnT8A were displaced, even though we observed that the ZnT8A were displaced in a concentration dependent manner and estimated in pmol/L. In contrast to paper I, the successful production of recombinant ZnT8 proteins in paper IV allowed us to determine the concentration dependent displacement of ^{35}S-met ZnT8 in binding to epitope-specific ZnT8A. This made it possible to determine the ZnT8A affinity expressed as half-maximal (Kd50) binding.

As we found that the ZnT8-specific autoantibodies did not bind to the ZnT8 peptides, we concluded that the ZnT8A may need a longer peptide extending the aa sequence of 318-331 for recognition of the 325-epitope.

In the competition experiments with both *in vitro* transcription and translation ZnT8 proteins (paper I) and recombinant ZnT8 proteins (paper IV) the ZnT8A affinity was remarkably higher for the proteins with the same epitope variant as the patient sera. We noted that three patients with ZnT8RA were partly displaced by the ZnT8W protein while none of the ZnT8WA positive patient sera were displaced by the ZnT8R protein. Therefore, we concluded that the autoantibody affinity to the respective variant may be different. Also, the specificity for either R

or W in subjects with ZnT8A may be higher than previously appreciated. Although our data clearly demonstrate that T1D patients may have single amino acid specific autoantibodies, further studies will be needed to test whether the conformational epitope is dependent on some neighboring amino acids as previously suggested [238].

Future perspectives

The RBA used in both studies had high precision and reproducibility and should prove useful for extensive analyses of ZnT8A affinity in studies of T1D prediction as well as in clinical trials with ZnT8 immunomodulation. Further studies of epitope specificity as well as affinity of ZnT8 variant autoantibodies are needed to establish to what extent an affinity measure during follow up will be useful to predict the risk for clinical onset of diabetes among islet autoantibody positive children. We suggest that the epitope-analysis should be combined with affinity determinations to better define ZnT8A-positive subjects at risk of diabetes such as in Diabetes Prediction in Skåne (DiPiS) [255], Type 1 Diabetes Prediction and Prevention study (DIPP) [224] or the TEDDY study [54]. Also, it may be of advantage to improve the enrollment of subjects in intervention trials or to design distinct immune therapies [257].

Papers II and III

In papers II and III, we developed and optimized a new autoantibody RBA to assess the levels and frequency of two NPYA variants (L and P) in patients with long-term T1D and T2D as well as in newly diagnosed T1D patients. Previously, NPY was proposed as a minor autoantigen in patients newly diagnosed with T1D [84]. In addition, the NPY SNP rs16139 was associated with increased risk for T2D and diabetes-related complications. Therefore, it was of interest to study autoantibodies against NPY in both T1D and T2D and its relation to neuropathy.

Consistent with one (and only) previous report about the existence of NPYA, we proposed that NPY fulfills the criteria for a minor autoantigen in T1D. This is for the reason that we found autoantibodies against NPY in both long-term and newly diagnosed T1D patients. We identified two variants of NPYA variants (NPY-LA and NPY-PA), which were hypothesized to be directed against the polymorphic L7P region. The findings of 12.5% (paper II) and 15% (paper III) NPY autoantibody (L and P) double-positive T1D patients and that NPYA variants often appeared together support the notion that the NPYA response may rather be polyclonal than monospecific as seen for the ZnT8A variants. The NPY

polymorphic L7P region may not represent the exclusive autoantibody binding site.

We speculated that the autoantibodies against NPY develop secondary to the major autoantibodies (IAA, GADA, IA-2A or ZnT8A) as we observed that NPYA were more often present in older newly diagnosed T1D patients compared to younger. NPYA may be a result of long-term β cell autoimmunity as a consequence of an ongoing autoimmune destruction of the β cells. NPYA were also present in patients who have had their diabetes diagnosis up to 40 years. In these long-term T1D patients, there was no relation between their neuropathy and the presence or levels of NPYA (or any of the major autoantibodies). However, it cannot be excluded that there may be such relation to T2D.

By including the NPY autoantibodies to the analyses of the major autoantibodies the diagnostic sensitivity was increased from 94.5% to 95.2%. Therefore, NPY autoantibodies may be important to classify T1D at diagnosis of diabetes, particularly in older children. However, the importance of NPYA as a predictive marker should be weighed against the costs of screening subjects at risk for T1D or at clinical onset. We suggest that the absence of an association with HLA-DQ may either be due to a haplotype not tested for (e.g. HLA-DR subtypes) or that epitope spreading to NPY is independent of HLA-DQ.

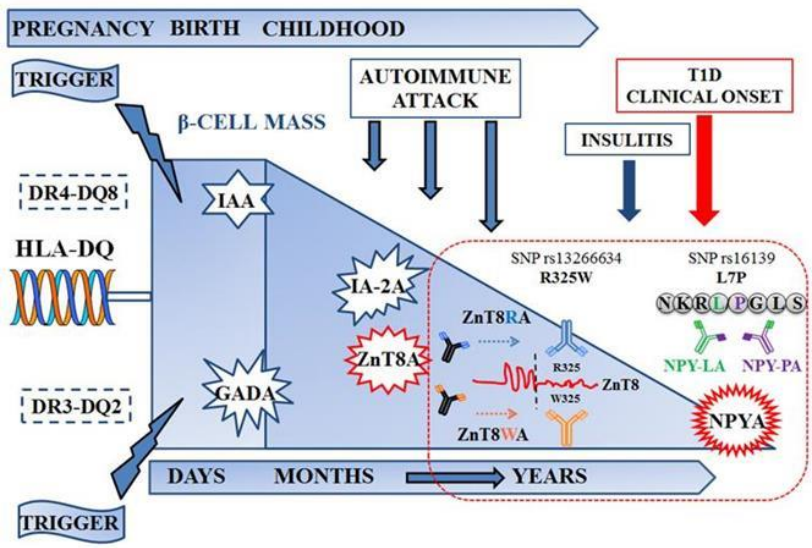

Figure 25. Schematic figure of possible factors contributing to the progression to clinical onset of T1D. The T1D susceptible HLA-DQ genes in combination with an environmental trigger such as a virus are crucial for the prodromal stage of islet autoimmunity to appear. During the ongoing β-cell destructive phase major autoantibodies as well as minor autoantibodies such as NPYA seem to develop in a sequential pattern. Also, autoantibodies such as the ZnT8A are likely to undergo affinity maturation due to a frequent autoantigen re-exposure. These factors may contribute to the progression of clinical onset of autoimmune T1D.

Future perspectives

Further studies are warranted to investigate the possibility that NPYA may be associated with diabetic neuropathy. Furthermore, it would be of interest to determine if autoantibodies against NPY are related to the L7P genetic polymorphism as such relation has been identified between the ZnT8A and the SLC30A8 SNP 13266634 (R325W). Next step would include validation of the NPYA to predict T1D in long-term by follow up from birth such as being done in the Diabetes Autoimmunity Study in the Young (DAISY) [159], DIPP [224], BABYDIAB [164] and TEDDY study [54].

Final conclusions

Taken together, the prodromal stage of islet autoimmunity seems to be reflected by autoreactive T cells followed by B cells producing islet-autoantibodies directed against β-cell specific proteins in the islets of Langerhans (Figure 3). During the ongoing β-cell destructive phase the major autoantibodies as well as minor autoantibodies seem to develop in a sequential pattern. Also, autoantibodies such as the ZnT8A are likely to undergo affinity maturation due to a frequent autoantigen re-exposure (Figure 25). Taken together, the studies in the present thesis have revealed novel insights of the ZnT8A affinity to the 325-epitope and the possible importance of autoantibody affinity in T1D. Novel insights also include NPY as a minor autoantigen of significance to diabetes etiology and pathogenesis. Both epitope-specific ZnT8A and NPYA may be included in future attempts to identify islet autoimmunity and to predict the clinical onset of autoimmune T1D.

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