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Characteristics of GADA in Type 1 Diabetes following
Immunomodulation with GAD₆₅

Mikael Chéramy

Division of Pediatrics
Department of Clinical and Experimental Medicine
Faculty of Health Sciences, Linköping University
581 85 Linköping Sweden



Linköping University

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During the course of the research underlying this thesis, Mikael Chéramy was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

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ABSTRACT

Type 1 diabetes (T1D) is a serious autoimmune disease which increases worldwide and affects children at a young age, but there still is no cure available. Clinical intervention trials in recent onset T1D patients are therefore very important, since even a modest preservation of β -cell function has proven to reduce end-organ complications. Glutamic acid decarboxylase 65 (GAD₆₅) is one of the major antigens in T1D, to which autoantibodies (GADA) are formed. Immunomodulation with aluminum-formulated GAD₆₅ (GAD-alum) has been considered both in the prevention and intervention of T1D. In a phase II trial using GAD-alum we showed clinical benefits in C-peptide preservation, but unfortunately a following larger European phase III trial failed to reach primary end-point. The general aim of this thesis was to study the characteristics and phenotypes of GADA following immunomodulation with GAD-alum in T1D patients during a phase II and III trial.

In the phase II trial, a transient increase of the GADA IgG3 and IgG4 subclasses, and a decrease in IgG1 was detected as part of the treatment-induced GADA levels after 2 GAD-alum doses, a result interpreted to be T helper (Th) 2-associated. This Th2-associated immune response was also observed, in parallel to increased GADA levels, during the following phase III trial including a larger group of patients. However, enhanced Th2-like IgG subclass distribution, reflected as increased IgG4 frequency, was in contrast only observed in the group treated with 4 doses of GAD-alum. In addition, the GADA fold-change was associated with *in vitro* GAD₆₅-stimulated cytokine secretion, but only in patients receiving 2 GAD-alum doses. Furthermore, a 4-year follow-up of the phase II trial showed that the effect of GAD-alum treatment was long-lasting as GADA titers remained elevated. Even though the phase III trial did not reach primary end-point, and was closed after 15 months, preservation of β -cell function was observed in the small sub-group of Swedish patients receiving 2 GAD-alum doses that completed the 30 months trial-period. During the trials, concerns were raised whether the elevated GADA titers might induce Stiff person syndrome (SPS), a disease affecting the nervous system, but *in vitro* analysis of GADA phenotypes showed that the GAD₆₅-enzyme activity and GADA epitope distribution differed from that detected in SPS patients.

Continued research to clarify how immunomodulation with autoantigens affects immune responses and also to identify which patients are suitable for treatment, is crucial for optimizing future T1D intervention- and prevention trials.

SUPERVISOR

Rosaura Casas, Associate Professor

Division of Pediatrics, Department of Clinical and Experimental Medicine,
Faculty of Health Sciences, Linköping University, Linköping, Sweden

CO-SUPERVISOR

Johnny Ludvigsson, Professor

Division of Pediatrics, Department of Clinical and Experimental Medicine,
Faculty of Health Sciences, Linköping University, Linköping, Sweden

OPPONENT

Mona Landin-Olsson, Professor

Division of Endocrinology and Diabetology, Department of Clinical Sciences,
Lund University, Lund, Sweden

COMMITTEE BOARD

Erik Kihlström, Professor

Division of Clinical Microbiology, Department of Clinical and Experimental Medicine,
Faculty of Health Sciences, Linköping University, Linköping, Sweden

Daniel Agardh, Associate Professor

Unit for Diabetes and Celiac Disease, Department of Clinical Sciences,
Faculty of Medicine, Lund University, Malmö, Sweden

Jorma Hinkula, Professor

Division of Virology, Department of Clinical and Experimental Medicine,
Faculty of Health Sciences, Linköping University, Linköping, Sweden

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Typ 1 diabetes (T1D) är en autoimmun sjukdom, vilket innebär att kroppens egna immunförsvar angriper och bryter ner de insulinproducerande β -cellerna i bukspottskörteln. På grund av det minskade antalet β -celler sjunker successivt produktionen av insulin, vilket är ett hormon nödvändigt för att reglera cellernas upptag av glukos från blodet, till den kritiska punkt då man tillslut drabbas av kliniska symptom. Flera typer av immunceller har visat sig vara inblandade vid T1D, T-hjälpar (Th) celler, cytotoxiska celler samt B-celler, dessutom riktar sig dessa immunceller mot specifika proteiner i β -cellerna, så kallade autoantigen. Ett av dessa autoantigen är Glutaminsyradekarboxylas (GAD_{65}), och flera studier har visat att de antikroppsproducerande B-cellerna utsöndrar autoantikroppar riktade mot både GAD_{65} och andra autoantigen i β -cellerna. Autoantikroppar riktade mot GAD_{65} (GADA) kan detekteras redan hos högriskindivider som senare utvecklar T1D, och upp emot 80 % av alla nydiagnostiserade T1D patienter har GADA i blodet.

Tidigare djur- och humanstudier har visat att injektioner med GAD_{65} har en positiv inverkan på bevarandet av insulinproduktionen hos de med T1D. Efter flera års studier kring GAD_{65} genomförde därför vår forskargrupp år 2005-2007 en klinisk fas II studie där 70 barn med T1D från 8 svenska kliniker behandlades med 2 injektioner av GAD_{65} , löst i adjuvantet aluminiumhydroxid (GAD-alum), eller placebo. Resultaten visade att de barn som fick GAD-alum hade bättre β -cellsfunktion jämfört med de som fick placeboinjektioner. För att ytterligare studera och säkerställa våra resultat från fas II studien genomfördes år 2008-2011 en europeisk fas III studie. Antalet deltagare ökades till 334 patienter, varav Sverige bidrog med 148 patienter från 20 olika kliniker, från vilka vi samlade in både blod- och serumprover. Den största skillnaden mellan fas II och fas III studien var att 1/3 av patienterna fick 4 GAD-alum injektioner mot tidigare 2 st. Tyvärr avbröts studien i förtid då det förväntade kliniska målet (primary end-point) inte uppfylldes vid 15 månader. Däremot har subgruppsanalyser visat att behandlingen fungerar hos vissa patientgrupper.

Det övergripande syftet med min avhandling var att studera hur behandling med GAD-alum påverkar nivåer och karaktär av GADA, för att på så sätt öka kunskapen om immunologiska mekanismer och identifiera biomarkörer som kan kopplas till klinisk effekt av behandling.

Resultaten från min forskning visar att behandling med GAD-alum inducerar högre GADA nivåer, samt att fördelningen av de olika GADA subtyperna (GADA IgG1-4) förändras. Under behandlingen ökar andelen av GADA IgG4 vilket antas representera ett mindre aggressivt immunsvär samt även vara kopplat till en mer skyddande Th2-profil. En 4-årsuppföljning av fas II studien visade även att behandlingen gav ett långvarigt immunsvär eftersom GADA nivåerna fortfarande var högre jämfört med placebo. Efter att vi visat att GAD-alumbehandlingen inducerade högre nivåer av GADA har det funnits farhågor att patienterna skulle kunna utveckla symptom liknande de som observeras hos Stiff person syndrome (SPS) patienter, vilket är en neurodegenerativ sjukdom som delvis definieras av höga GADA nivåer. Dock visar mina resultat att de GADA som induceras vid GAD-alum behandling inte uppvisar samma karaktäristika som de som detekteras bland SPS-patienter. Slutligen visas att även om fas III studien inte uppnådde primary end-point efter 15 månader, så detekterades positiva kliniska effekter vid 30 månader i den mindre svenska subgruppen som fullföljde studien.

Även om GAD-alum injektioner påverkar en rad faktorer i immunsystemet, så finns det ännu inte någon specifik biomarkör identifierad som direkt kan kopplas till klinisk effekt av behandling. Framtida studier får visa om behandling med GAD-alum kan ge positiva kliniska effekter vid preventionsbehandling av T1D, när det ges till specifika grupper av T1D patienter, eller som en del i kombinationsterapi med andra läkemedel.

LIST OF ORIGINAL PAPERS

Paper I

Chéramy M, Skoglund C, Johansson I, Ludvigsson J, Hampe CS, Casas R.

GAD-alum treatment in patients with type 1 diabetes and the subsequent effect on GADA IgG subclass distribution, GAD (65) enzyme activity and humoral response

Clin Immunol. 2010; 137(1):31-40

Paper II

Chéramy M, Hampe CS, Ludvigsson J, Casas R

Characteristics of GAD₆₅ autoantibodies (GADA) in high titer individuals

Manuscript

Paper III

Axelsson S*, Chéramy M*, Hjorth M, Pihl M, Åkerman L, Martinuzzi E, Mallone R, Ludvigsson J, Casas R

Long-lasting immune responses 4 years after GAD-alum treatment in children with type 1 diabetes

PLoS ONE. 2011; 6(12):e29008

Paper IV

Chéramy M, Axelsson S, Åkerman L, Pihl M, Ludvigsson J, Casas R

GAD₆₅ autoantibody (GADA) responses in Type 1 diabetes patients participating in a phase III GAD-alum intervention trial

Manuscript

ABBREVIATIONS

anti-Id	anti-idiotypic antibodies
AUC	Area under the curve
C-peptide	Connecting peptide
CSF	Cerebrospinal fluid
DASP	The diabetes autoantibody standardization program
ELISA	Enzyme-linked immunosorbent assay
GABA	γ -aminobutyric acid
GAD	Glutamic acid decarboxylase
GAD ₆₅	65 kDa isoform of GAD
GAD ₆₇	67 kDa isoform of GAD
GADA	Autoantibodies to GAD
GAD-alum	GAD ₆₅ formulated in aluminum hydroxide
HLA	Human leukocyte antigen
IA-2	Tyrosine phosphatase like protein islet antigen-2
IA-2A	Autoantibodies to IA-2
IAA	Autoantibodies to insulin
Ig	Immunoglobulin
IL	Interleukin
LADA	Latent autoimmune diabetes in adults
mAb	Monoclonal antibody
MMTT	Mixed meal tolerance test
NOD mouse	Non-obese diabetic mouse
PLP	Pyridoxal 5'-phosphate
RBA	Radiobinding assay
rFab	recombinant Fab
RIA	Radioimmunoassay
SPS	Stiff person syndrome
T1D	Type 1 diabetes
Th	T-helper
ZnT8	Zink transporter 8
ZnT8A	Autoantibodies to ZnT8

INTRODUCTION

Type 1 diabetes

Definition and Diagnosis

Type 1 diabetes (T1D) is a chronic autoimmune disease in which the pancreas produces little or no insulin, a hormone needed to allow glucose to enter cells to maintain a normal metabolism [1]. The lack of insulin is due to the active destruction of the pancreatic insulin-producing β -cells, found in the islet of Langerhans, by immune cells which have lost self tolerance. Although T1D usually appears in childhood and adolescence, it can develop at any age. Symptoms include: polyuria leading to increased thirst, hunger, weight loss and fatigue. Symptoms occur when glucose levels in blood increases (hyperglycemia), as the lack of insulin prevents glucose uptake by the cells in the body. If left untreated, hyperglycemia and disrupted cell metabolism may be followed by ketoacidosis, a life-threatening condition which ultimately leads to coma and death. Persisting hyperglycemia causes abnormal glycation of tissues which leads to long term microvascular complications such as retinopathy, nephropathy, neuropathy, and also macrovascular complications (e.g. stroke and heart infarction).

T1D diagnosis is based on glucose measurements and the criteria for diagnosis are, established by the American Diabetes Association (ADA), fasting plasma glucose ≥ 7.0 mmol/l (fasting is defined as no caloric intake for at least 8 h), or symptoms of hyperglycemia and a casual plasma glucose value ≥ 11.1 mmol/l, or a 2-h plasma glucose ≥ 11.1 mmol/l during an oral glucose tolerance test (OGTT) [2]. As hyperglycemia occurs, the hemoglobin molecules of the red blood cells are glycated by excess glucose. By measuring the percentage of glycated hemoglobin (HbA1c) the average blood glucose control during the past 8-12 weeks can be estimated. In 2009, an international expert committee including representatives of the ADA, the International Diabetes Federation (IDF), and the European Association for the Study of Diabetes (EASD) recommended the use of the HbA1c test to diagnose T1D, with a threshold of $\geq 6.5\%$ [3]. Although a high HbA1c supports a T1D diagnosis, a normal HbA1c will not exclude diabetes, especially not in children with a rapid disease-manifestation showing high blood glucose values, but still normal or near-normal HbA1c. Therefore it is not clear what role HbA1c will have for diagnosis, but its main importance is as a parameter for development of late vascular complications [4-5].

Classification of diabetes

The term diabetes mellitus describes a metabolic disorder of multiple etiologies, characterized by chronic hyperglycemia resulting from defects in insulin secretion and/or insulin action [6]. In contrast to the rapidly progressing T1D, which is dominant in children, there is also an autoimmune slowly progressive form referred to as latent autoimmune diabetes in adults (LADA). Type 2 diabetes (T2D) is more common than T1D and results from a progressive insulin secretory defect together with insulin resistance. Thus, in T2D the islet cells are still functioning, but insulin production is impaired or the target cells become resistant to insulin, or both. Gestational diabetes mellitus (GDM), which is not clearly overt diabetes, is diagnosed during pregnancy and blood glucose levels usually return to normal soon after delivery.

Incidence of T1D

Second to Finland, Sweden has the highest incidence of T1D in the world. During 1990-1999 the World Health Organization (WHO) began the Multinational Project for Childhood Diabetes (DIAMOND) [7]. The study reported the age-standardized incidence of T1D in children aged 14 years or under (per 100 000/year) from 112 centers in 57 different nations worldwide. The lowest incidence was found in China and Venezuela (0.1/100 000/year) and the highest was observed in Finland (40.9/100 000/year) and Sweden (30/100 000/year), representing a variation of over 300-fold in the incidence. Since then, the incidence has in fact continued to increase even more and is now reaching around 65 /100 000/year in Finland and approximately 45/100 000/year in Sweden. As T1D treatment has improved during the last decades, resulting in longer life expectancy, the prevalence of T1D will continue to increase. However, a recent Swedish nationwide study reported that the past stable increase in T1D might level off which is suggested to be due to changed lifestyle among children (causing rapid early growth and weight development) [8] or other environmental changes [9], but whether incidence really has reached maximum is still too early to conclude.

Pathogenesis and etiology

The underlying mechanisms causing T1D are still largely unknown, but it has been established that both genetic predisposition and environmental factors interplay. When the first symptoms of T1D occur, the sustained autoimmune process may have been ongoing for years (Fig. 1). For example, autoantibodies against islet specific antigens can be detected long before clinical onset of the disease [10], which occurs when 80–90% of the β -cell function has been lost [11].

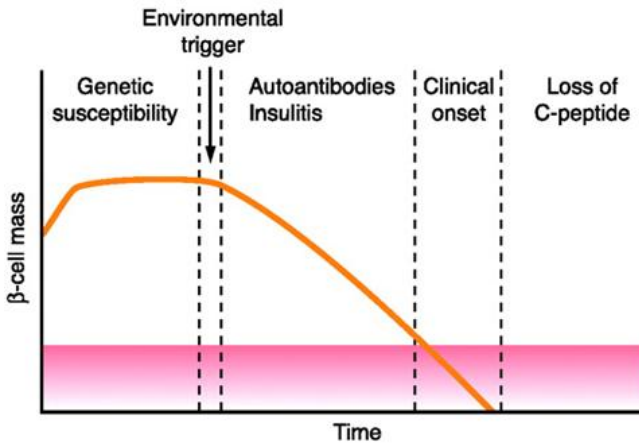


Figure 1. Model for the β -cell mass destruction. Illustration adopted from [11].

The vast time-period between the initial autoimmune process to the initiation of clinical symptoms possess a problem since traces of the specific triggering environmental factors may have disappeared. However, except for genetic susceptibility a number of possible factors have been proposed, including: viral infections, early introduction of cow's milk, exaggerated hygiene, as well as β -cell stress.

Genetic risk

Several studies have shown the inherited risk for T1D to be determined by the human leukocyte antigen (HLA) class II genes, conferring to 40-50% of the risk [12]. In addition, HLA class I genes [13], and several polymorphisms in other genes, including insulin (INS), cytotoxic T lymphocyte antigen-4 (CTLA-4) and protein tyrosine phosphatase N22 (PTPN22), also contributes to increased risk [14]. The highly polymorphic HLA class II immune recognition molecules DR and DQ are located on chromosome 6, and the protein

products are expressed on antigen presenting cells (APC) that capture and present processed peptide antigen to the T-cell receptor (TCR). Extensive studies have revealed a large number of high- and low-risk HLA alleles, for example 45% of the general population in the US expresses DR3 or DR4 whereas 95% of those who develop T1D express these haplotypes [15].

Although the role of genetic risk variants in the disease pathogenesis is not completely understood, some are thought to influence the initiation of β -cell autoimmunity whereas others seem to play a role during the later stages of the autoimmune process. However, concordance rates between monozygotic twins amount to 40-50% [16], and only about 10% of genetically susceptible individuals progress to T1D [17]. In addition, the majority (80–90%) of newly diagnosed T1D children do not have a first degree relative already affected by the disease [18], pointing out the substantial impact of environmental factors as key components in the pathogenesis of T1D.

Viral infections

The discovery of seasonal variation in T1D incidence, with higher diagnosis rate during the autumn and winter months [19], raised suspicion that viruses might be a possible triggering factor. A number of viruses have been associated with T1D, including enteroviruses such as Coxsackie B virus [20], but also rotavirus [21], mumps virus [22], and cytomegalovirus [23]. The study of enteroviruses has attracted much attention as higher neutralizing antibody titers were found in serum from recent-onset T1D patients compared to healthy controls [24], and remnants of virus have also been detected in β -cells [25] and blood [26] from T1D patients. The Diabetes Autoimmunity Study in the Young (DAISY) study found that the rate of progression from autoimmunity to T1D was significantly higher after enterovirus detection [27], and a Finnish prospective study revealed an association between enterovirus infection and disease progression [28-29]. Viruses may cause β -cell destruction either by direct cytopathic effects on the target cells or indirectly by triggering or potentiating the autoimmune response, as reviewed by Grieco *et al* [30]. The Coxsackie B virus is a prime candidate among enteroviruses as an amino acid segment of the β -cell restricted protein GAD₆₅ (aa 247-279) shares sequence similarity with the P2-C protein of Coxsackie B virus, which suggests molecular mimicry as a mechanism to mediate a β -cell-directed autoimmune response [31].

However, the correlation of T1D and exposure to viruses and other pathogens may be just one side of the coin. The hygiene hypothesis suggests that early exposure to microbial agents may be beneficial for the development of a balanced immune system and ability to maintain self tolerance [32]. While the incidence of various infectious diseases have decreased over the last few decades due to increased hygiene, widespread use of antibiotics and vaccination programs, the occurrence of autoimmune disorders has increased rapidly [33]. Thus, it has been hypothesized that exposure to a large number of infections early in life appropriately shapes the adaptive immune system, and failure of this process can result in autoimmunity or inappropriate immune responses to environmental triggers [33]. A comparative study of Finnish and Estonian children during their first year of life found that enterovirus infections inversely correlated with T1D risk. While Estonian children had a higher incidence of enterovirus infections than Finnish children, T1D incidence in Estonia was 5 times lower than in Finland [34].

Vitamin D deficiency

Yet another hypothesis to explain seasonal variation in T1D is that of variations in vitamin D levels and its postulated effect on both β -cells and immune cells. Vitamin D deficiency has been associated with T1D [35-36] and the use of cod liver oil as a vitamin D supplement during pregnancy [37] and the first year of life [38], has been associated with a lower risk of T1D. If true, it might also explain the high incidence of T1D in the Nordic countries as the short summers and long winters further increase the risk of vitamin D deficiency.

Early exposure to cow's milk

Early exposure to a cow's milk (CM)-containing diet has also been implicated as possible risk factors for T1D. An international intervention trial TRIGR (Trial to Reduce IDDM in the Genetically at Risk), was initiated to study administration of hydrolyzed infant formula compared to a conventional CM-based formula in children who carry risk-associated HLA genotypes and have a first-degree relative with T1D [39]. The study was aimed to decrease the risk of T1D and also to determine relationships between CM antibodies, a measure of CM exposure, and diabetes-associated autoantibodies. After a median observation period of 10 years in a pilot study, it was recently reported that feeding with the hydrolyzed formula was

associated with a decreased risk of seroconversion to islet-cell antibodies [40], but there was no difference in incidence of T1D.

β -cell stress

An early observation showed that children with T1D tend to grow slightly faster than population controls prior to diagnosis [41]. It has since then been hypothesized that not only rapid growth during puberty, but also psychological stress and infections, with a subsequent increased insulin demand, causes β -cell stress and thereby initiate the autoimmune process [42]. Already in 1994 it was shown that rapid growth during the first years of life was associated with increased incidence of T1D [43], and this has later been confirmed in many other studies. Thus, another study analyzing data from the DAISY trial suggested that height growth velocity in genetically susceptible pre-pubertal children was associated to islet autoimmunity and T1D development [44].

Treatment of T1D

In 1869, the German medical student Paul Langerhans found clusters of cells within the pancreatic tissue which were later identified as the insulin-producing β -cells, and the pancreatic islets were also named after him. When the Polish-German physicians Oscar Minkowski and Joseph von Mering in 1889 removed the pancreas from a healthy dog, the animal keeper observed swarms of flies feeding on the urine. When testing the urine, they found elevated sugar levels, thereby for the first time establishing a relationship between the pancreas and diabetes.

In 1916, the Romanian professor Nicolae Paulescu developed a pancreatic extract which, when injected into a diabetic dog, proved to have a normalizing effect on blood glucose levels. Unfortunately he had to interrupt his experiments due to the WWI, and was not able to publish his research until 1921. By then the Canadian doctor Fredrick Banting, inspired by Minkowski's research, was able to extract insulin and successfully keep a pancreatectomized dog alive by insulin injections. In 1922, the 14-year-old diabetic Leonard Thompson was saved from dying of T1D when he received the first insulin injection in history. The work published by Banting *et al* [45] earned him and John Macleod (head of the department) the 1923 Nobel prize in physiology or medicine for the discovery of insulin. Although Paulescu

discovered the principles of insulin treatment, he was not acknowledged by the Nobel prize committee.

Despite intensive research T1D still has no cure, although it can be managed with insulin treatment, and patients now expect to live longer healthier lives than in the past. Biosynthetic human recombinant insulin analogs are nowadays manufactured for widespread clinical use and can be administered by daily insulin injections or continuously released from insulin pumps. Maintaining a stable glycemic control ($\text{HbA1c} \leq 6.5\%$) is crucial in diabetes care, as elevated HbA1c is correlated to increased risk of long-term complications including microvascular (retinopathy, neuropathy and nephropathy) as well as macrovascular (cardiovascular) complications [4-5, 46-47].

C-peptide

Insulin is produced in a pre-form by the pancreatic β -cells as proinsulin. As proinsulin is cleaved to form insulin, equimolar concentrations of C-peptide is released (Fig. 2). In contrast to C-peptide, a significant portion of insulin undergoes hepatic extraction, resulting in a much shorter plasma half-life of 3-4 minutes while the plasma half-life for C-peptide is approximately 30 minutes [48].

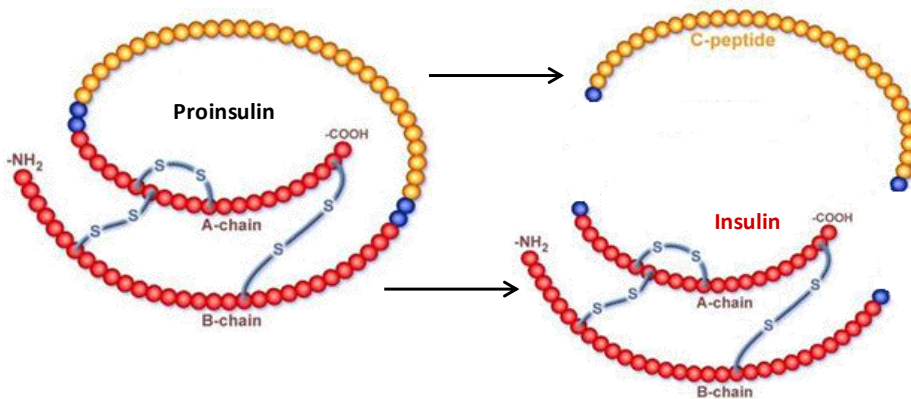


Fig 2. Schematic representation of human proinsulin. C-peptide is indicated in yellow, the insulin A- and B- chains in red and the cleaving points in blue. Illustration modified from [49].

There is now also increasing evidence that C-peptide should not be regarded as just a bi-product during insulin synthesis, but instead as a bioactive peptide which may have a positive effect in decreasing long-term complications when administered exogenously to T1D patients

[50]. To clinically establish the residual β -cell function in T1D patients, fasting and stimulated C-peptide is analyzed [51]. Stimulated C-peptide can be measured during a mixed meal tolerance test (MMTT), where blood samples are drawn at baseline and at 30, 60, 90 and 120 minutes after ingestion of a standardized liquid meal. The β -cells respond to the increased glucose, fat and protein levels by elevated secretion of insulin and equivalent amounts of C-peptide, usually peaking at 90 minutes, until returning to baseline levels after 2h. Area under the curve (AUC) is calculated by plotting the C-peptide values from each time-point during the MMTT and a lower AUC and/or peak value indicates a reduced β -cell mass.

Immunology of T1D

Before the appearance of classical clinical symptoms of T1D, a subclinical autoimmune destruction of the β -cells may have been ongoing for years. T cells play a major pathogenic role in islet cell infiltration and destruction, and a T helper (Th)1-dominated infiltration, secreting the cytokine IFN- γ , has been observed in patients with T1D [52]. Whereas T cells in T1D patients exhibit polarization toward a Th1-type response to islet autoantigens *in vitro*, non-diabetic control subjects display a Th2/Treg bias (secreting the cytokines IL-4, IL-5, IL-13) [53]. Although the cellular destruction is mediated by autoreactive T cells [54-55], a preceding triggering event probably leads to the release of β -cell specific antigens, thereby inducing a subsequent islet-specific autoantibody production by B-cells.

B-cell activation

B cells are an essential component of the humoral immune response as they upon activation generate antibody producing plasma cells. B cells are created in the bone marrow and migrate to the spleen or other secondary lymphoid tissues where they mature and differentiate into immune competent cells. To activate B cells, two distinct signals are required which results in B-cell differentiation into memory B cells or plasma cells [56-57] (Fig. 3). The first activation signal occurs upon antigen binding to B-cell receptors (BCRs) (Fig. 3A). After binding, the antigen is internalized by endocytosis, becomes fragmented, and peptides are complexed with MHC II molecules on the B-cell surface (Fig. 3B).

The second activation signal occurs via interaction with T helper (Th) cells. The B cell presents antigen peptides through the MHC II molecule to a T cell, in parallel to expression of cytokine receptors and the co-stimulatory CD40 molecule. The T-cell receptor (TCR) on the Th cell then binds to the antigen-complex class II MHC molecule on the B-cell surface, which results in T-cell activation. As a response, the activated T cell secretes cytokines which bind to the up-regulated B-cell cytokine receptors (Fig. 3C). The primary function of plasma cells is to secrete B-cell clone-specific antibodies targeted to a certain antigen-target (epitope). In the next stage, activated B cells proliferate and form germinal centers in lymphoid tissues where they can differentiate into memory B cells or plasma cells (Fig. 3D), and some B cells also undergo antibody isotype switching and hypersomatic mutation (affinity maturation). The memory B cells, which express high-affinity surface antibodies, can survive for a longer period of time to enable a rapid secondary response upon antigen re-challenge.

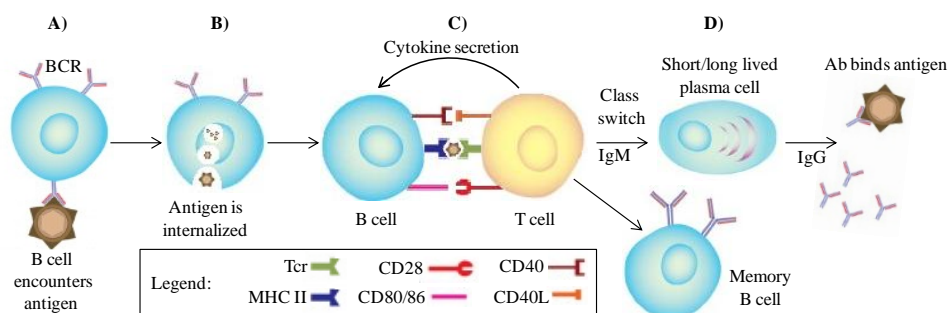


Figure 3. Overview of T-cell dependent B-cell activation. Schematic illustration of the process for B-cell activation by a T cell and maturation into antibody producing plasma cells and memory B cells.

Tcr: T cell receptor, MHC II: major histocompatibility complex-II, CD40L: CD40 ligand.

Illustration modified from [57].

General structure and phenotype of antibodies

Antibodies are glycoproteins belonging to the immunoglobulin superfamily. As being the main component of the humoral responses, they are theoretically able to bind and neutralize an infinite number of agents. All antibodies consist of some basic structural units; the two large heavy chains and the two light chains, the latter holds the highly variable antigen binding site (Fig. 4). The specific sites on the surface of an antigen molecule, which are both

identified by immune cells and the antigen binding site of antibodies, are called epitopes. Antibodies that have a binding capacity directed towards self antigen epitopes are called autoantibodies and appear in a range of autoimmune diseases.

By screening which epitopes the antibodies recognize in patients sera (epitope mapping), differences in immune- and disease responses can be identified [58-59]. There are also antibodies that specifically bind to the antigen-binding site of other antibodies, called anti-idiotypic antibodies (anti-Id). It has been proposed that the network of anti-Id antibodies may be involved in preventing autoimmune diseases by neutralizing and inhibiting the secretion of autoantibodies [60-61]. Further, imbalances in- or lack of anti-Id antibodies has also been suggested as a possible promoter of autoimmunity.

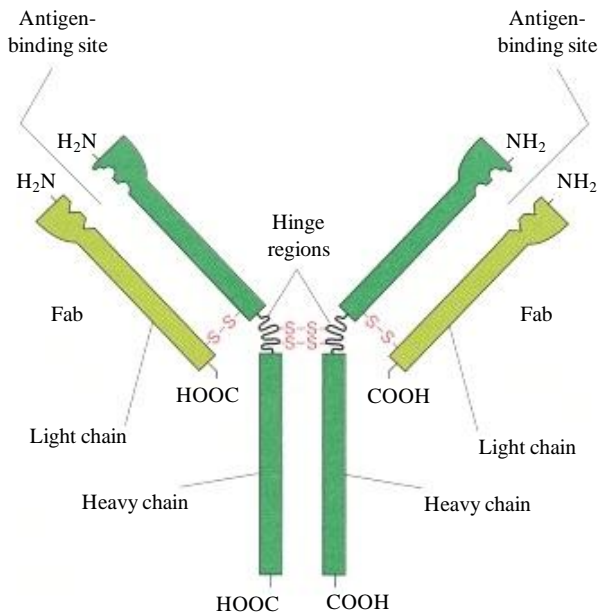


Figure 4. Schematic representation of the antibody structure. Antibodies are composed of four polypeptide chains; two identical heavy chains and two identical light chains. The Fab regions, containing the antigen-binding sites, are linked by hinge regions to the Fc part of the antibody. Adapted from [62].

Antibodies can be classified into different groups of isotypes depending on the constitution of the heavy chains. There are five different isotypes: IgG, IgE, IgM, IgA and IgD which are found in blood and mucosa, and when increased represent a specific type of immune response (Table I). For example, allergic reactions are associated to increasing IgE titers, specific for

the putative allergen, resulting in a subsequent release of histamine from activated basophils and mast cells.

Table I. Antibody isotypes and subclasses, molecular weight and function [56, 62-63].

Name/ subclass	Weight (kDa)	Description
IgA1	160	Found in mucosal areas, preventing colonization by pathogens. Also found in saliva, tears and breast milk. Expressed as a mono- or dimeric antibody.
IgA2	160	
IgD	184	Functions mainly as an antigen receptor on antigen unchallenged B cells.
IgE	188	Involved in allergy by triggering histamine release from mast cells and basophils.
IgG1	146	Four different subclasses that provide a majority of the antibody-based immunity against invading pathogens. The only antibody capable of crossing the placenta to give passive immunity to fetus.
IgG2	146	
IgG3	165	
IgG4	146	
IgM	970	B cell surface, or secreted as very high avidity pentamer ab in the early B-cell response.

During B-cell activation the genes encoding for the antigen binding site, or hypervariable region, undergo a high rate of point mutations (somatic hypermutation) resulting in antibodies with higher affinity for the antigen. The genes encoding for the heavy chain are also able to re-organize in a process called class switching, creating a different isotype of the antibody, from the same activated B cell, that retains the antigen specific variable region [64].

Some of the isotypes are divided in subgroups of antibodies called antibody subclasses. Within the IgG isotype are four different subclasses; IgG1, IgG2, IgG3 and IgG4, and the numeral indicates the general total frequency in which they appear in serum (% of total IgG); IgG1 (67%) >IgG2 (22%) >IgG3 (7%) >IgG4 (4%) [65]. The major structural differences between the IgG subclasses are found in the number of inter-heavy chain disulfide bonds in the hinge region (Fig. 5).

IgG antibodies are flexible molecules, and depending on the structure of the hinge region the light chains are able to rotate and bend in respect to the heavy chain, which affects the antibodies sterical binding capacity. The hinge region of IgG1 is more flexible than IgG2 which has a shorter hinge region containing 4 disulfide bonds, whereas the flexibility of IgG4 is intermediate between the other two [66]. The IgG3 subclass on the other hand differs from the other classes by its unique extended hinge region. As the Fab fragments are relatively far away from the heavy chain, the molecule has a greater flexibility than the other types (Fig. 5). The elongated hinge in IgG3 is also responsible for its higher molecular weight (Table I).

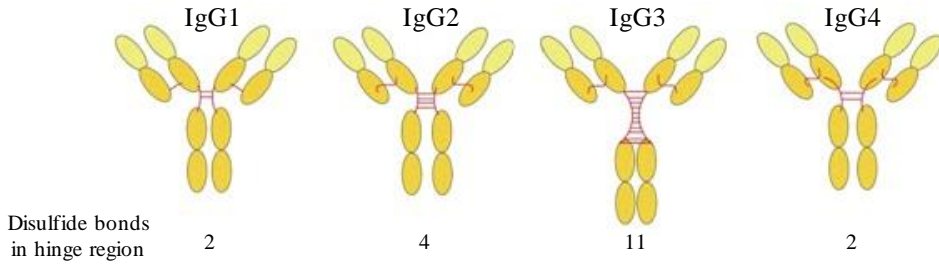


Figure 5. A schematic illustration of the four IgG subclasses. The difference between the IgG1-4 subclasses lies in the number of disulfide bonds within the hinge-region (marked in red), which connects the heavy and light chains. Modified from [67].

IgG antibodies are able to activate the complement system, neutralize toxins, viruses and bacteria and by opsonization facilitate phagocytosis. However not all IgG subclasses are able to bind complement; IgG1 and IgG3 are effective complement activators, while IgG2 is a weak activator whereas IgG4 is unable to activate complement [65-66].

The determinant for which isotype or subclass that is produced during a B-cell response depends on the cytokine milieu in which the B cell is activated by a T cell. The distribution of various subclass-specific antibodies may therefore reflect whether the immune response is Th1-or Th2-biased. This association has been extensively studied in various animal models, thus the relationship between certain cytokines and induction of various subclasses is much better defined in murine models than in humans. However, one must be cautious to draw conclusions from results originating from murine models as it has been shown that they may reflect opposite situation in humans. In mice, Th1 responses are associated with the generation of IgG2 and IgG3 subclass antibodies and Th2 responses with IgG1. Thus, the Th2 cytokine interleukin 4 (IL-4) enhances IgG1 and suppresses IgG3 in murine settings [68], while in humans the IL-4 and IL-13 are characteristic for IgG4 production [69-71].

Autoantibodies and autoantigens in T1D

The first identified pancreatic islet cell autoantibodies (ICA) were described in the 1970's [72-73], where it was shown that ICAs could be detected in newly diagnosed T1D patients. However, the assay used to analyze ICA depended on human pancreatic tissue as a substrate, which made it hard to standardize for laboratory use. As a consequence efforts were focused

to identify highly specific single T1D autoantibodies, in contrast to ICAs which instead represent autoantibodies capable of binding unspecified pancreatic antigens. Since then, four major T1D-specific autoantigens to which autoantibodies are formed have been identified; Insulin [74], Glutamic acid decarboxylase 65 (GAD₆₅) [75], Tyrosine phosphatase islet antigen 2 (also known as insulinoma-associated protein 2; IA-2) [76], and more recently Zinc transporter 8 (ZnT8) [77]. All of these islet autoantigens are located within the secretory pathway of the β -cell (Fig 6). However, beside insulin, the complete function of these proteins concerning β -cell homeostasis is still incomplete.

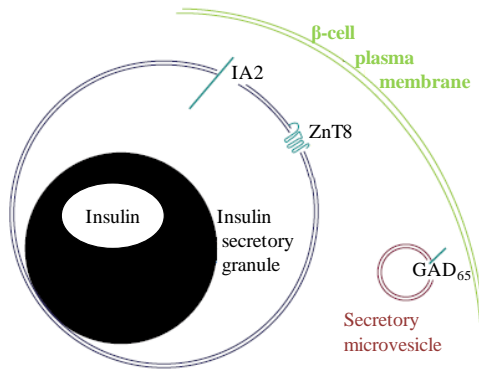


Figure 6. The predominant intracellular distribution of major T1D autoantigens in β -cells. The secretory granule contains primarily insulin, whereas the granule membrane is the primary site of ZnT8, a polytopic membrane protein, and IA-2 which is a single-spanning transmembrane protein with both extensive luminal and cytosolic domains. GAD₆₅ is localized away from these other autoantigens, residing primarily on the cytosolic side of the membrane of secretory microvesicles. Illustration modified from [78].

Next to insulin, the second specific T1D autoantigen to be identified was GAD [79], and it was also shown that a large majority of newly diagnosed T1D patients display immune reactivity towards this protein compared to healthy controls [75]. Later studies revealed that GAD is an enzyme required for the production of the neurotransmitter γ -amino butyric acid (GABA) and exerts its enzymatic capability through the decarboxylation of glutamic acid, together with the cofactor pyridoxal 5'-phosphate (vitamin B6), to produce GABA.

GAD exists in two isoforms which have different molecular weights, one at 67 kDa (GAD₆₇) encoded by the GAD1 gene and another at 65 kDa (GAD₆₅) encoded by the GAD2 gene, and the two isoforms share a 65 % homology at the primary amino acid sequence level [80]. Both isoforms have identical enzymatic activity and are expressed in the CNS and synaptic

vesicles of neurons where they act as the major producers of GABA. In contrary only GAD₆₅ is expressed in the secretory microvesicles of the β -cells (Fig. 6), where it is expressed as a dimeric molecule (Fig. 7).

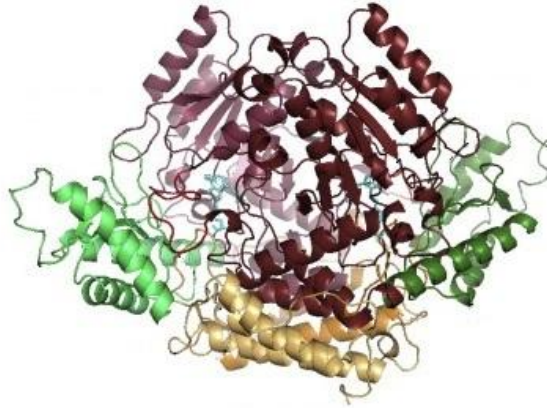


Figure 7. The dimeric structure of GAD₆₅. Illustration modified from [81].

The function of GAD₆₅ within β -cells remains unclear, but it has been shown that islets contain GABA stored in synaptic-like vesicles which might be involved in the regulation of insulin secretion [82]. Moreover, long term exposure of isolated islets to glucose increases the transcription and expression of GAD₆₅ [83-84].

IA-2 consists of a signal peptide and a transmembrane, extracellular and intracellular, domain which is located in the membrane of the secretion vesicles in endocrine and neuronal cells. The actual function within the islets is unknown, although it has been suggested that it may play a role in regulating insulin secretory granule content and regulate β -cell growth [85]. This is also supported by findings showing that IA-2 knock-out mice are glucose intolerant with reduced insulin secretion [86-87].

In 2007 the latest T1D-specific antigen was discovered, namely the membrane bound ZnT8 [77]. The β -cells maintain high levels of cellular zinc and they express several zinc transporters, but the most consistently expressed β -cell transporter is ZnT8. The molecule has a unique C-terminal end epitope at amino acid position 325 distinguished by specific autoantibodies against Arg (R), Trp (W) or Gln (Q), alone or in combination. For this reason, specific assays to analyze autoantibodies against ZnT8R, ZnT8W, ZnT8Q either alone or in

combination have been developed. The function of ZnT8 is also unclear, but studies from murine models suggest that ZnT8 is needed for optimal insulin storage and secretion [78].

At the clinical onset of T1D it is estimated that 98% of all patients show positivity for one or more autoantibodies to GAD₆₅ (GADA), IA-2 (IA-2A), ZnT8 (ZnT8A) and insulin (IAA) [77, 88]. While positivity for a single autoantibody may not represent progression to T1D, the

appearance of transient and/or sustained multiple islet autoantibodies at several time-points is a marker of a progressive autoimmune destruction of the β -cells and T1D (Fig. 8). Thus, in studies of first-degree relatives, 60–100% of individuals with three autoantibodies developed T1D within 5–6 years [89–91], and seroconversion with rapidly increasing autoantibody titers early in life strongly predicts progression to T1D before puberty [92–93].

After disease onset, autoantibody positivity and titers may fluctuate, and a recent prospective study showed a general decrease in GADA, IA-2A and ZnT8A in T1D children followed for 3–6 years post-diagnosis [94]. As insulin treatment induces the generation of insulin antibodies, analysis of IAA only have a value before T1D diagnosis when administration of exogenous insulin is initiated.

GADA IgG1-4 subclass frequencies

At the time of T1D diagnosis 70–80% of all patients have detectable serum GADA levels [15] and even though GADA titers may fluctuate over time, detectable levels persist for many

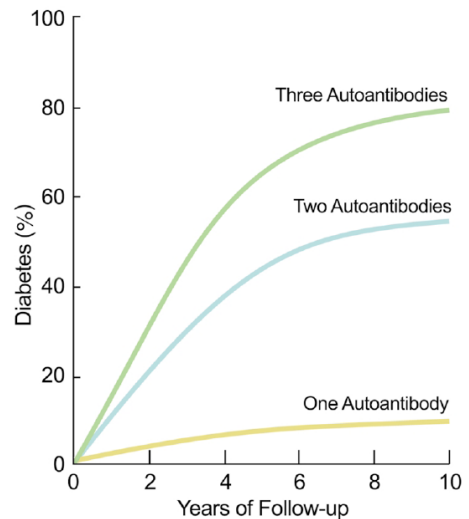


Figure 8. Model for association of autoantibody positivity and T1D.

With increasing number of persistent and/or transient autoantibodies, the risk of developing T1D increases, adopted from [15].

years after the clinical onset of diabetes [95]. GADA subclass analysis in T1D patients have shown that the subclass distribution reflects that the disease is Th1-associated as IgG1 and IgG3 are commonly detected, while the Th2-related IgG2 and IgG4 are less frequently found [96], resulting in the following rank order; IgG1>IgG3>IgG2>IgG4 [97]. It has been suggested that the hierarchical order of GADA subclasses in T1D may be due to their ability to activate complement [96]. While IgG1 and IgG3 are complement fixing and promote binding of leukocytes via Fc gamma receptors, IgG4 on the other hand have low or no complement fixing and opsonizing activities and thereby may be considered as poor mediators of autoimmune pathology. Indeed, previous studies have shown that individuals with a susceptibility to T1D, which display a higher frequency of GADA IgG2 [96] and/or IgG4 [98], stay non-diabetic longer than those with a broader subclass response lacking the emergence of IgG4. In addition, LADA patients, which are considered to have less aggressive β -cell autoimmunity, display a hierarchical distribution dominated by IgG4 (IgG1>IgG4>IgG2>IgG3) [97, 99].

GADA epitope distribution

Autoantibodies to GAD₆₅ are not only found in T1D, LADA and individuals at risk for developing T1D, but also in patients suffering from the rare neurological disease Stiff Person Syndrome (SPS) which is characterized by muscular rigidity occurring as a result of deficient synthesis of the inhibitory neurotransmitter GABA. T1D and SPS are both autoimmune diseases with cellular and humoral immune responses to GAD₆₅ [100]. The shared immunological etiology is reflected by the coexistence of both diseases since as many as 30% of SPS patients also develop T1D [81, 101], however, only one in ten thousand individuals diagnosed with T1D is affected by SPS [102].

There are however differences in the GADA phenotypes present in these two diseases as the majority of GADA in T1D are directed to the smaller isoform GAD₆₅ in serum [103], while SPS patients also show high levels of GADA directed to GAD₆₇ [104-105] in both serum and cerebrospinal fluid (CSF). GAD₆₅-specific monoclonal antibodies and their recombinant Fab (rFab) have previously been used to map GADA epitopes associated with T1D and SPS. Results from these studies have shown that the GADA epitope defined by monoclonal antibody b96.11 is located in the middle region of GAD₆₅, and appears to be associated with progression to T1D [58, 106-107]. In contrast, SPS patients recognize a GADA epitope

defined by the monoclonal antibody b78 which is located in the C-terminal region [59], and has been associated with the inhibition of GAD₆₅ enzyme activity [108], a phenomenon only rarely observed for GADA positive sera from T1D patients [109].

GADA anti-idiotypic antibodies

It has been hypothesized that the increased GADA titers found in T1D may be due to an impaired GADA anti-Id function, and that even healthy individuals and first degree relatives (FDRs) to T1D patients may present GADA which is masked by GADA specific anti-Id [61]. The study also concluded that GADA positive T1D and SPS patients show a specific lack of anti-Ids to disease-associated epitopes, b96.11, or b78, and that purified anti-Ids from healthy individuals and FDRs inhibited the binding of GADA from T1D patients to GAD₆₅. Furthermore, induction of b96.11-specific anti-Ids has been shown to efficiently block the binding of b96.11 to GAD₆₅, and this inhibition was accompanied by a significant reduction of insulinitis incidence and diabetes in non-obese diabetic (NOD) mice [110]. Yet another study revealed that at clinical onset T1D patients presented no or low b96.11 anti-Id levels. Furthermore, during a follow-up of the same study, increasing anti-Id levels marked patients who experienced a temporary increase in C-peptide levels [60]. Anti-Id levels also correlated significantly with glycated hemoglobin and C-peptide levels.

Immunotherapy in T1D

During the last decades a wide range of immunotherapies aimed to prevent β -cell destruction in T1D patients with residual C-peptide or in individuals at risk for developing T1D have been evaluated. Even though a large proportion of the β -cell function is impaired at time of diagnosis, the pancreas is still able to produce a significant amount of insulin [111-112]. Even if a potential immunotherapy is unable to revert autoimmunity close after disease onset, a limited effect resulting in a modest preserved residual insulin secretion, with stimulated C-peptide levels > 0.2 nmol/l, has been reported to provide clinically meaningful benefits in terms of reducing long-term complications [113]. Early intervention trials included plasmapheresis- [114], immunosuppressant- (cyclosporine) [115] and corticosteroid treatment (prednisone) [116]. The major intervention- and treatment strategies for T1D currently

involve antigen-specific immunomodulation, specific immune inhibition by monoclonal antibodies and cytokine modulators.

Monoclonal anti-CD3 antibody (Teplizumab, Otelixizumab)

CD3 is a protein complex located on the surface of T cells and is fundamental to the initiation of T-cell activation. It has been hypothesized that administration of monoclonal antibodies against CD3 might restore immune self-tolerance by targeting pathogenic T cells and stimulating the amplification of regulatory T cells, thereby attenuating autoimmunity [117-118]. Anti-CD3 treatment was originally developed for treatment of organ transplant rejection, but early clinical trials in a small number of T1D patients showed positive results during the first year after treatment (i.e. preserved insulin secretion, lower HbA1c and insulin requirement) [119]. A second study revealed that anti-CD3 administration improved insulin preservation, measured as change in C-peptide from baseline up to 18 months after initiation of treatment, and that short disease duration improved the effect [120]. However, side effects were observed in both trials including: lymphopenia, cytokine release and reactivation of Epstein Barr virus (mononucleosis).

Still, the encouraging clinical effects lead to the initiation of two clinical phase III trials including new-onset T1D patients: the Protégé Study using Teplizumab and the DEFEND Study using Otelixizumab. Unfortunately, the Protégé Study recently failed to reach primary outcome (HbA1c level of <6.5%, and insulin dose of <0.5 units/kg/day) [121], as did the DEFEND Study which showed no differences in C-peptide between treated and placebo 1 year after initiation [122]. However, the international consortium TrialNet is currently recruiting autoantibody positive first-degree relatives to participate in a phase II trial to evaluate anti-CD3 for the prevention of T1D [123]. Future studies with anti-CD3 monoclonal antibodies need to evaluate the clinical efficacy regarding dosage contra side effects, as the earlier studies showing clinical efficacy used higher doses.

Monoclonal anti-CD20 antibody (Rituximab)

Even though T1D is considered as a T cell-mediated disease, the B cell-depleting anti-CD20 monoclonal antibody (Rituximab), commonly used in rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus and autoimmune hemolytic anemia [124], has been evaluated

in recent onset T1D patients. A randomized, double blind, phase II study using infusions of anti-CD20 showed a 20% higher stimulated C-peptide after one year in treated patients compared to placebo [125]. Treated patients also had significantly lower levels of HbA1c and required less insulin to maintain glycemic control. The original study reported reduced IgM levels whereas IgG levels were unaffected, suggesting that anti-CD20 is more effective in reducing the B-lymphocyte population than in reducing the number of cells that secrete IgG. Later analysis showed that the treatment significantly reduced IAA titers, whereas GADA, IA-2A and ZnT8A were unaffected [126]. The possible clinical effect observed in this trial however needs to be confirmed in larger future phase III trials.

Anti-CTLA-4 (Abatacept)

Cytotoxic T lymphocyte antigen 4 (CTLA-4) is a homologue of CD28, a high-affinity receptor that down-regulates T cells. T-cell activation requires binding of the TCR to the antigen-MHC complex on the APC, and secondly, a co-stimulatory signal provided by the binding of CD28 to the B7 protein on the APC. Anti-CTLA-4 (Abatacept) is a fusion protein composed of the Fc region of the immunoglobulin IgG1 fused to the extracellular domain of CTLA-4 which binds the APC B7 molecule, thereby inhibiting the co-stimulation of T cells [127-128]. The effect of anti-CTLA-4 in recent-onset T1D was recently evaluated in a multicenter, double-blinded, randomized controlled trial [127]. After two years stimulated C-peptide was 59% higher and HbA1c was lower in treated patients compared to placebo whereas insulin requirement did not differ. However, after six months, C-peptide declined in the same rate as placebo, even though anti-CTLA-4 infusions continued throughout the two-year study period. The authors speculate this might be due to that T-cell activation lessens with time.

Anti-IL1 receptor antagonist (Anakinra)

Interleukin 1 (IL-1) is a proinflammatory signal molecule that mediates the acute phase response in infection, inflammation, tissue trauma and stress as well as in autoinflammatory disorders [129]. Anakinra is an IL-1 receptor antagonist which blocks the biological activity of naturally occurring IL-1 by competitively inhibiting the binding of IL-1 to the Interleukin-1 type receptor [130], which is expressed in many tissues and organs. Anti-IL1 receptor

antagonists have been evaluated in a randomized trial including T2D patients [131]. Patients received daily doses of Anakinra or placebo during 13 weeks, and actively treated patients showed lowering of HbA1c, IL-6, C-reactive protein as well as higher C-peptide secretion compared to placebo. The currently ongoing European anti-interleukin-1 in diabetes action (AIDA) trial will evaluate safety, tolerability and potential efficacy of anti-IL-1 therapy in maintaining or enhancing β -cell function in people with new-onset T1D [129], however, it was recently communicated that the trial unfortunately failed to reach primary outcome [132]. Another recent NOD mouse study also suggested that combining IL-1 antagonists with FcR nonbinding anti-CD3 monoclonal antibody resulted in a synergistic effect, reversing T1D in NOD mice [133].

Anti-TNF- α (Etanercept)

Etanercept is a recombinant soluble tumor necrosis factor (TNF)- α receptor fusion protein that binds to TNF- α . It acts by clearing TNF- α from the circulation, thereby blocking the biological activity of the pro-inflammatory cytokine [134]. A small pilot study recently evaluated the effect of anti-TNF- α in newly diagnosed T1D patients. The study showed that treatment was well tolerated and after 24 weeks of treatment C-peptide had increased by 39% in the Etanercept group and had decreased by 20% in the placebo group. HbA1c and insulin dose were also both lower in treated- compared to placebo patients.

IL-2 (Proleukin)

Another approach to inhibit autoreactive T cells is to induce regulatory T cells (Treg) and thereby halt β -cell destruction. Interleukin 2 (IL-2), an inducer of Treg, in combination with the T- and B-cell immunosuppressant drug rapamycin, has been evaluated in a group of ten T1D patients during a recent phase I trial [135]. Despite a marked increase in Treg and improved T-cell signaling, C-peptide unexpectedly dropped at three months. Luckily the decrease was transient and C-peptide levels subsequently increased in almost all subjects. These findings highlight the importance of broadly interrogating the immune system to evaluate the effects of therapy in early trials.

Heat Shock protein 60 (DiaPep277)

DiaPep277 is a stable peptide isolated from heat shock protein 60 (Hsp60) [136], a widely expressed protein also located in mature insulin-secretory granules of pancreatic β -cells. Treatment with Hsp60 is thought to have immune modulatory effects as T cells reactive to Hsp60 have been shown to be of the Th2 type and that this response was accompanied by activation of adhesion, downregulation of chemokine receptors, and chemotaxis and inhibition of Interferon (IFN)- γ secretion [137]. Hsp60 treatment has been evaluated in several phase II trials including adult T1D patients [138-141]. Though considered safe and achieving the primary endpoint of stimulated C-peptide secretion preservation, no major effect has been observed on diabetes control parameters (i.e. HbA1c, insulin requirement). However, phase II trials that tested the efficacy of Hsp60-treatment in T1D children reported that there was no beneficial effect in improving metabolic control or preserving β -cell function [141-142]. A recently performed global phase III study, including 457 newly diagnosed T1D patients aged 16-45 years, has reported encouraging initial results [143]. A significant preservation of C-peptide levels was observed in patients treated with Hsp60 compared to the placebo arm, and the difference reflected a relative preservation of 23.4%. Additional analyses of clinical, efficacy and safety data from this study are ongoing.

Insulin

Administration of oral and nasal insulin has been tried for both the prevention and intervention of T1D in clinical trials [10, 144-145]. The North American Diabetes Prevention Trial-Type 1 (DPT-1) evaluated the effect of both parenteral and oral insulin administration in relatives of T1D patients who were at risk for T1D, without any clinical success [10]. However a subgroup of patients with very high IAA titers demonstrated up to a four-year delay in T1D onset in those given oral insulin as compared to placebo [146]. A TrialNet study is currently recruiting autoantibody positive relatives to T1D patients to further investigate oral insulin in the prevention of T1D [147].

GAD₆₅ AS AN IMMUNOMODULATOR IN T1D

Since the identification of GAD₆₅ as a major component in β -cell autoimmunity, several attempts to inhibit the progression of disease by GAD-immunizations, and thereby tolerizing GAD-reactive T cells, have been performed in both animal models and human trials.

Preclinical studies using GAD₆₅

Early studies using NOD mice showed that intravenous treatment with GAD₆₅ protected the animals from T1D and that the effect might be mediated through induction of GAD-specific T-cell tolerance [148-150]. Other administration routes such as intrathymic, intraperitoneal and intranasal GAD₆₅ treatment [151-154], have also proven to prevent T1D not only by inducing T-cell tolerance but also through upregulation of GAD₆₅-specific Th2 responses and decreased IFN- γ production. Other attempts to induce GAD-specific T-cell tolerance using different GAD-derived peptides injected emulsified in incomplete Freund's adjuvant resulted in disease protection at the onset of insulinitis, but were ineffective when given to prediabetic mice [150]. One study even suggested that certain GAD₆₅ peptides, in contrast to the whole GAD₆₅ protein, even might accelerate T1D in NOD mice [152]. Regarding the safety of treatment, it has been shown that mice not prone to diabetes receiving GAD₆₅ immunization, did not provoke onset of diabetes or induce islet cell pathology even though high GADA levels were detected [155].

Clinical trials using GAD₆₅

Phase I trial

Before initiation of clinical trials with GAD₆₅, skin prick tests were carried out in young newly-onset T1D patients and healthy controls to test whether T1D was associated with immunological sensitization to exogenous recombinant human GAD₆₅ [156]. At inclusion, none of the eight healthy controls were GADA positive whereas six out of seven T1D patients were positive, and the skin prick test to GAD₆₅ was found to be negative in all subjects. A phase I study was thereafter carried out by treating healthy male individuals negative for the T1D risk genotypes (non-DR3-DQ2 and non-DR4-DQ8), with a single

subcutaneous injection of unformulated recombinant human GAD₆₅ or placebo [156-157]. The doses were ascending from 20 to 500 µg GAD₆₅ and the treatment was concluded to be safe and well tolerated as no significant treatment related adverse clinical events were seen at any dose level and neither GADA, IAA or IA-2A were induced in any subject.

Early Phase II trials

Two subsequent Swedish phase II trials were thereafter initiated; the first included LADA patients and the second children and adolescents with T1D. The LADA study was designed as a randomized, placebo-controlled, dose-escalating trial [158]. Patients were assigned subcutaneous injections of placebo or 4, 20, 100, and 500 µg GAD₆₅ formulated in aluminum hydroxide (GAD-alum) at weeks 1 and 4. The 20 µg dose was found to preserve both fasting and stimulated C-peptide over the 24 week study period without any serious study-related adverse events. However, only patients receiving 500 µg GAD-alum showed a significant increase in GADA titers in response to treatment. Analysis of b96.11 anti-Id levels displayed a decline in seven out of nine placebo patients, whereas four out of five patients receiving 20 µg GAD-alum showed increasing anti-Id levels, which also were closely correlated to C-peptide levels [60]. In addition, a five year follow-up of the study suggested that the 20 µg dose may be suitable for future clinical trials on safety and efficacy, since this group maintained their fasting C-peptide over 5 years compared with placebo [159].

The second phase II trial was a randomized, placebo-controlled, double-blinded study, performed 2005-2007, including 70 children and adolescents with T1D [160]. Participants were assigned either two doses of 20 µg GAD-alum or placebo on day 1 and four weeks later, and blood and serum samples were collected at eight time-points during the study period. A significant preservation in stimulated C-peptide was observed in GAD-alum treated patients at 15 months, and at 30 months both stimulated and fasting C-peptide was higher compared to placebo patients [160]. Subgroup analysis revealed that C-peptide preservation was most evident in GAD-alum treated patients with a shorter (<6 months) disease duration. A 4-year follow-up of the trial also showed that patients receiving GAD-alum treatment within 6 months of diagnosis, still had a better preservation of fasting C-peptide levels than those treated after a 7-18 month disease duration [161].

Recent Phase II and III trials

Encouraged by the clinical results from the prior trials, two phase III trials, one in Europe (NCT00723411) and one in the US (NCT00751842) with identical designs were initiated. Furthermore, yet another phase II trial, with a different design, was initiated in the US by TrialNet (NCT00529399), in parallel to a Swedish phase II prevention trial (NCT01122446).

The phase III trials were designed as randomized, placebo-controlled, double-blinded studies, aimed to include 320 recent onset (≤ 3 months duration) GADA positive T1D patients (aged 10-20 years) [162-164]. The trials included three different treatment arms and patients were assigned 20 μg GAD₆₅ as: i) 4 doses of GAD-alum (4D), or ii) 2 doses of GAD-alum followed by 2 doses of placebo (2D), or iii) 4 doses of placebo. The subcutaneous injections of GAD-alum and/or placebo were administered at baseline, 1 month, 3 months and 9 months, and MMTT was performed at 6 times during the 30 months study periods. The primary outcome was change in stimulated serum C-peptide between baseline and 15 months. The European trial recruited the first patients in august 2008, and in November 2009 the last patient of 334 was included. Although patients treated with GAD-alum had on average 16.4 % more remaining C-peptide at 15 months compared to placebo patients [165], the results unfortunately showed that stimulated C-peptide levels declined to a similar degree in all three treatment arms at 15 months [162]. In addition, GAD-alum treatment did not affect the insulin dose, glycated hemoglobin level, or hypoglycemia rate compared to placebo. However, adverse events were infrequent and mild in the three groups, with no significant differences. Since the trial did not fulfill primary end-point, it was closed after 15 months, and the 30 months follow-up period was completed only for a minority of the patients. However, exploratory analysis revealed significant clinical effects in the 4 doses (4D) regimen, alone or combined with 2 doses (2D), in four subgroups; i) males, ii) patients with baseline Tanner puberty stage 2 or 3, iii) patients with baseline insulin dose 0.398-0.605 IU/Day/kg, and iv) patients from non-Nordic countries. As a consequence of the cancelled European phase III trial, the ongoing American phase III trial (DIAPREVENT), which had not yet reached primary end-point, changed focus to instead only collect safety data for at least 6 months following the last GAD-alum injection.

In parallel to the phase III trials, the TrialNet consortium initiated a placebo-controlled, double-blinded phase II study including 145 GADA positive T1D patients aged 3-45 years with disease duration of < 100 days [166-167]. Patients were randomized to one of three

treatments arms receiving 20 µg GAD-alum as; i) 3 doses of GAD-alum, or ii) 2 doses of GAD-alum followed by 1 dose of placebo, or iii) 3 doses of placebo. Injections were given subcutaneously at baseline, 1 month and 3 months and MMTT was performed at baseline and at 3, 6, 9, and 12 months. The primary outcome was baseline-adjusted stimulated C-peptide secretion at 1 year. Unfortunately, results showed that treatment with 2 or 3 subcutaneous GAD-alum injections, had no effect on the decline in insulin production after 1 year, compared to placebo [166].

The Swedish phase II prevention trial (DIAPREV-IT) [168-169], initiated in 2010, is designed as a double-blinded randomized study to determine the safety and effect of GAD-alum on the progression to T1D in 50 children aged 4-18 years, positive for multiple islet cell autoantibodies. Participants have been randomized to 2 injections of 20 µg GAD-alum or placebo at baseline and 1 month, and will thereafter be followed-up every 3rd month for 5 years. The primary aim of the study is to evaluate safety of the drug and secondary to analyze β -cell capacity after GAD-alum injections to evaluate if treatment may prevent β -cell destruction before clinical onset of T1D. The trial is currently fully recruited and no serious adverse events have been reported.

AIMS OF THE THESIS

The general aim of this thesis was to study the characteristics and phenotypes of GADA following GAD₆₅ immunomodulation in T1D patients.

The specific aims were:

- I.** To analyze changes in GADA IgG subclass distribution, GAD₆₅ enzyme activity and if the humoral response was antigen-specific in T1D patients treated with GAD-alum.
- II.** To determine phenotypical differences in GADA titers, IgG subclass distribution, GADA epitopes and ability to inhibit GAD₆₅ enzyme activity in individuals with high GADA titers: new onset T1D, T1D treated with GAD-alum, children and adolescents with high risk for T1D and SPS patients.
- III.** To evaluate long-lasting GAD-specific memory T- and B-cell responses in T1D children treated with GAD-alum.
- IV.** To characterize GADA and IA-2A levels, GADA IgG1-4 subclass distribution, cytokine secretion and B-cell characteristics in relation to clinical outcome and further to assess β -cell preservation at 21 and 30 months in a phase III clinical GAD-alum trial.

MATERIAL AND METHODS

Study populations

The GAD-alum phase II trial (Paper I, II)

One hundred and eighteen patients who were between 10 and 18 years of age and diagnosed with T1D within the previous 18 months were screened at eight pediatric clinics in Sweden for the presence of GADA and fasting C-peptide levels above 0.1 nmol/l [160] (Table II). A total of 70 patients were eligible and were randomly assigned to a double-blind treatment with either 20 µg of GAD-alum (35 patients) or placebo (i.e. alum alone) (35 patients) at day 0 and four weeks later (Fig. 9). Blood and serum samples were collected and sent to the pediatric laboratory in Linköping within 24h.

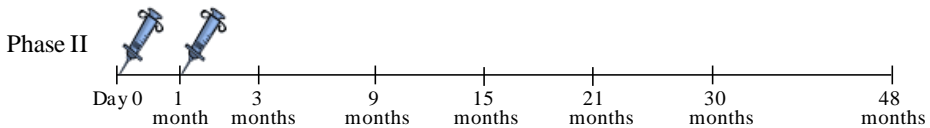


Figure 9. Overview of sample collection in the phase II trial and 4-year follow-up.

GAD-alum or placebo injections were given at day 0 and 1 month. Blood and serum samples were collected at 7 time-points during the study period and at 48 months during the extended follow-up.

Results from a study of latent autoimmune diabetes in adult patients [158] suggested that including 35 patients in each treatment group would provide the study with a statistical power of 80 to 90% for assessing differences in fasting C-peptide levels, with a significance level of 5%. Screening took place over a 2-week period in January and February 2005. The first injection of GAD-alum or placebo took place in February 2005, and the last patient completed the 30-month visit in October 2007. One patient in the placebo group was withdrawn from the study after 1 week, due to mononucleosis. A total of 69 patients, 35 in the GAD-alum group and 34 in the placebo group, were included in the analyses. All patients were treated with multiple daily injections of insulin with a target glycated hemoglobin level of less than 6.5%. At baseline, two patients in the placebo group but none in the GAD-alum group were using an insulin pump.

Table II. Baseline characteristics according to study group in the phase II trial (*Paper I*).

Characteristic	GAD-alum n=35	Placebo n=34
Age (years)	13.8±2.3	12.8±1.9
Months since diagnosis	9.9±5.3	8.8±5.5
Gender distribution, n (%)		
Female	23 (66)	18 (53)
Male	12 (34)	16 (47)
HLA risk classification, n (%)		
High	18 (51)	16 (47)
moderate	9 (26)	7 (21)
Low	8 (23)	11 (32)
Tanner puberty stage, n (%)		
1	4 (11)	7 (21)
2+3	8 (23)	10 (29)
4+5	23 (66)	17 (50)
C-peptide (nmol/l)		
Fasting C-peptide	0.33±0.19	0.35±0.23
Stimulated C-peptide AUC	0.62±0.28	0.71±0.43
Glycated hemoglobin (%)	6.3±1.3	6.2±1.0
Insulin dose (IU/Day/kg)	0.66±0.30	0.66±0.28
Fasting plasma glucose (mmol/l)	9.4±4.0	8.8±3.3
Median GADA (Units/ml)	601	861
Median IA-2A (Units/ml)	125	552

Values are mean±SD unless stated otherwise. HLA, human leukocyte antigen; AUC, area under the curve; GAD-alum, alum formulated glutamic acid decarboxylase; GADA, Glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies. The Tanner puberty stage ranges from 1 to 5, with higher stages indicating more developed genitalia. HLA risk classification was based on HLA-DQ-A1* and -B1* alleles.

4-year follow-up of the GAD-alum phase II trial (Paper III)

To evaluate long-term efficacy and safety of GAD-alum treatment, patients and when necessary their guardians, were asked in 2009 whether they were willing to participate in a 4-year follow-up of the study. Of the 70 patients included in the original phase II study, 59 agreed to participate, of whom 29 had been receiving GAD-alum and 30 had received placebo [161] (Table III), (Fig. 9). Blood and serum samples were collected and sent to the pediatric laboratory in Linköping within 24h.

Table III. Baseline characteristics according to study group, for patients that participated in the 4-year follow-up of the phase II trial (*Paper III*).

Characteristic	GAD-alum n=29	Placebo n=30
Age (years)	13.6±2.4	12.8±1.9
Months since diagnosis	9.4±5.4	8.5±5.4
Gender distribution , n (%)		
Female	19 (65.5)	15 (50)
Male	10 (34.5)	15 (50)
C-peptide (nmol/l)		
Fasting C-peptide	0.3±0.2	0.4±0.2
Stimulated C-peptide AUC	0.6±0.3	0.7±0.4
Glycated hemoglobin (%)	6.2±1.3	6.2±0.9
Insulin dose (IU/Day/kg)	0.7±0.3	0.6±0.3
Fasting plasma glucose (mmol/l)	9.5±4.1	8.7±3.4
Median GADA (Units/ml)	539	786
Median IA-2A (Units/ml)	125	552

Values are mean±SD unless stated otherwise. AUC, area under the curve; GAD-alum, alum formulated glutamic acid decarboxylase; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies.

The GAD-alum phase III trial (Paper IV)

The phase III trial was a multicenter, randomized, double-blind trial performed at 63 clinics in nine European countries (Finland, France, Germany, Italy, the Netherlands, Slovenia, Spain, Sweden, and the United Kingdom). The recruitment ratio was 1:1:1 across the three study groups, with an intended total enrollment of 320 patients. Screening was performed between August 2008 and November 2009 and lead to the inclusion of a total of 334 newly diagnosed T1D patients (<3 months duration) who were 10 to 20 years of age. Inclusion criteria included detectable serum GADA levels and a fasting C-peptide level above 0.1 nmol/l. Patients received either four doses of 20 µg GAD-alum on days 0, 30, 90 and 270 (4D regimen), two doses of GAD-alum on days 0 and 30 followed by two doses of placebo on day 90 and 270 (2D regimen) or four doses of placebo on days 0, 30, 90, and 270 (Fig. 10). The placebo and active drug product were both suspensions of alum in a buffer.

Among the European countries, Sweden was the first to commence screening of patients, as a consequence the Swedish cohort constituted a large proportion of the total European cohort (148 of 334 recruited patients). The 148 Swedish patients underwent randomization to 4D

(n=49), 2D (n=49) or placebo (n=50) (Table IV). A group of 45 patients completed the 30 months visit before the trial was closed (4D; n=14, 2D; n=15 and placebo; n=16).

Table IV. Baseline characteristics according to study group in the phase III trial. Characteristics are given for the entire Swedish cohort and for the subgroup of patients who completed the 30 month visit (*Paper IV*).

Characteristic	Swedish Subgroup			Entire Swedish cohort		
	4D n=14	2D n=15	Placebo n=16	4D n=49	2D n=49	Placebo n=50
Age (years)	13.2±2.4	13.3±2.4	13.4±2.7	13.3±2.1	13.3±2.2	13.4±2.5
Days since diagnosis	76.4±26.1	67.7±22.8	67.6±23.5	74.6±20.3	73.3±20.0	71.0±20.6
Gender distribution, n (%)						
Female	9 (64)	6 (40)	8 (50)	25 (51)	26 (53)	20 (40)
Male	5 (36)	9 (60)	8 (50)	24 (49)	23 (47)	30 (60)
HLA risk classification, n (%)						
very high	3 (21.3)	7 (47)	6 (38)	14 (29)	18 (37)	16 (32)
High	6 (43)	6 (40)	5 (31)	21 (44)	23 (47)	23 (46)
Moderate	3 (21.3)	2 (13)	4 (25)	8 (17)	4 (8)	8 (16)
Low	2 (14.3)	0 (0)	1 (6)	5 (10)	4 (8)	3 (6)
Tanner puberty stage, n (%)						
1	2 (14.3)	4 (27)	0 (0)	6 (12)	10 (20)	5 (10)
2+3	2 (14.3)	2 (13)	8 (50)	17 (35)	12 (25)	16 (32)
4+5	10 (71.3)	9 (60)	8 (50)	26 (53)	27 (55)	29 (58)
C-peptide (nmol/l)						
Fasting C-peptide	0.37±0.19	0.23±0.09	0.26±0.10	0.30±0.17	0.25±0.11	0.26±0.14
Stimulated C-peptide AUC	0.71±0.33	0.61±0.23	0.71±0.21	0.71±0.35	0.66±0.26	0.64±0.24
Glycated hemoglobin (%)	7.07±0.72	7.15±0.91	7.04±1.11	6.84±0.65	6.81±0.91	6.87±1.04
Insulin dose (IU/Day/kg)	0.64±0.26	0.71±0.27	0.51±0.23	0.59±0.29	0.62±0.31	0.55±0.23
Fasting plasma glucose (mmol/l)	6.81±1.92	6.32±2.15	5.87±1.24	6.28±2.16	5.95±1.74	5.69±1.19
Median GADA (Units/ml)	193	440	219	204	312	190
Median IA-2A (Units/ml)	408	667	943	472	620	736

Values are mean±SD unless stated otherwise. HLA, human leukocyte antigen; AUC, area under the curve; 4D, four dose regimen, 2D, two dose regimen; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies.

Data regarding HLA classification were missing for one patient in the 4D group. The Tanner puberty stage ranges from 1 to 5, with higher stages indicating more developed genitalia. HLA risk classification was based on HLA-DQ-A1* and -B1* alleles.



Figure 10. Overview of sample collection and the phase III trial. GAD-alum or placebo injections were given at day 0 and at 1, 3 and 9 months. The 4 dose regimen received only GAD-alum, whereas the 2 dose regimen received 2 GAD-alum injections followed by 2 placebo injections, while the placebo regimen received 4 placebo injections. Blood samples were collected at day 0 and after 1, 3, 9, 15, 21 and 30 months.

High GADA titer groups (Paper II)

Four groups with high GADA titers were included: T1D patients treated with GAD-alum, children with high risk for T1D, SPS patients and new-onset T1D (Table V).

Samples from the GAD-alum group (n=9) were selected among T1D patients participating in the phase II trial [160]. The treatment significantly increased GADA levels (up to 57 times) compared to patients receiving placebo, with the highest levels detected 3 months after initiation of treatment. Serum samples from the 3 month visit were selected based on the highest quartile of GADA levels within the treated group.

The High-risk group (n=6) was selected from the ABIS (All Babies in Southeast of Sweden) cohort, where 17,055 children born 1997-1999 have been prospectively followed with regular biological sampling [170]. From this cohort, children testing positive for several T1D-associated autoantibodies at \geq two time points (n=23), have been classified as having high risk for developing the disease [171]. Here we included 6 of the children with the highest GADA levels, of which three developed manifest T1D after sample collection.

Serum from the SPS group (n=12) were chosen exclusively based on sample availability, all SPS patients were GADA positive. Serum samples from ten patients were kindly donated by Mohammed Hawa and David Leslie at the Queen Mary University of London, UK, while two samples were collected from patients recruited from the Östergötland county council, Sweden. Eight out of twelve SPS individuals were also diagnosed with T1D.

Samples from the T1D group (n=7) were obtained from patients participating in a Swedish nationwide prospective cohort study, Better Diabetes Diagnosis (BDD), involving newly diagnosed T1D patients \leq 18 years recruited from 40 pediatric clinics [172]. Samples with the highest GADA titers ($>95^{\text{th}}$ percentile of GADA positive patients) were selected from BDD patients recruited at the Linköping University Hospital pediatric clinic.

Table V. Characteristics of SPS- and T1D patients, GAD-alum treated T1D patients and healthy individuals at High-risk for T1D (*Paper II*).

Patient	Age at sampling	Sex	Age T1D	Age SPS
SPS 1	53	M	15	51
SPS 2	48	F	25	N/A
SPS 3	45	M	24	42
SPS 4	48	F	32	47
SPS 5	65	F	60	63
SPS 6	71	F	no	69
SPS 7	37	F	no	34
SPS 8	33	F	28	31
SPS 9	61	F	no	60
SPS 10	71	F	31	68
SPS 11	64	M	29	49
SPS 12	56	F	no	47
T1D 1	10	F	10	no
T1D 2	17	M	17	no
T1D 3	10	F	10	no
T1D 4	5	M	5	no
T1D 5	12	F	12	no
T1D 6	4	F	4	no
T1D 7	17	M	13	no
High-risk 1	5	F	no	no
High-risk 2	8	M	13	no
High-risk 3	8	F	no	no
High-risk 4	8	F	11	no
High-risk 5	8	M	no	no
High-risk 6	8	M	13	no
GAD-alum 1	18	F	17	no
GAD-alum 2	15	F	15	no
GAD-alum 3	17	F	16	no
GAD-alum 4	12	M	11	no
GAD-alum 5	15	F	14	no
GAD-alum 6	18	M	18	no
GAD-alum 7	17	F	17	no
GAD-alum 8	11	F	10	no
GAD-alum 9	15	F	15	no

SPS, Stiff-person syndrome; T1D, Type 1 diabetes; GAD-alum, aluminum-formulated glutamic acid decarboxylase

C-peptide analysis

In the phase II trial and subsequent 4-year follow-up, laboratory analyses were performed at the Pediatric Research Laboratory, Linköping University, Sweden. C-peptide levels were measured in serum samples with a time-resolved fluoroimmunoassay (AutoDELFIA™ C-peptide kit). Results in each assay were validated by inclusion of a C-peptide control module containing a high, a medium and a low-level control (Immulite). A 1224 MultiCalc®

program (Wallac) was used for automatic measurement and result calculation, with measurements expressed in pmol/ml.

In Paper I, the clinical effect of treatment was determined by changes in stimulated C-peptide AUC from baseline to 15 months (main study period). The GAD-alum treated patients were stratified into three groups; Responders (loss of AUC < 10 %), Intermediate responders (loss of AUC between 10 % and 65 %) and Non-responders (loss of AUC > 65 %).

In Paper III, the clinical effect of treatment was defined by changes in stimulated C-peptide AUC from baseline to 48 months. GAD-alum-treated patients were divided in two subgroups; patients with a loss of C-peptide AUC \leq 60 %, and patients with a loss of AUC > 60 %.

For the phase III trial (Paper IV), serum C-peptide analysis was performed by BARC Laboratories (Ghent, Belgium) using an Immulite 2000 C-peptide kit on an Immulite 2000 analyzer using calibration standards based on the World Health Organization's National Institute for Biological Standards and controls (Reference Standard 84/510). In Paper IV, the clinical effect of treatment was determined by changes in stimulated C-peptide AUC from baseline to 30 months (i.e. the subgroup completing the main study period).

GADA analysis (Paper I, II, III, IV)

Serum GADA titers were determined using a radioimmunoassay (RIA) employing ^{35}S -labelled recombinant human GAD₆₅ produced by *in vitro* transcription/translation (pEx9 vector kindly supplied by Prof. Åke Lernmark, University of Washington, Seattle, WA, USA). Sepharose protein A was used to separate free from antibody bound labelled GAD₆₅ [173].

A standard curve, consisting of serial dilutions of GADA positive serum, was included on each plate. Wells containing only buffer were included as blanks. Positive and negative controls were also included in each plate. The immunoprecipitated radioactivity was counted in a Wallac 1450 Microbeta Liquid Scintillation Counter (Perkin Elmer Life and Analytical Sciences), and the results were expressed as units/ml in relation to the standard curve. The cut-off for positivity was regarded as 67.3 u/ml (corresponding to 23.1 WHO units) based on the 95th percentile of measurements from 1700 children, aged 5-6 years, participating in the ABIS study.

In the phase III trial (*Paper IV*), serum GADA titers were centrally analyzed by BARC Laboratories (Ghent, Belgium), using an enzyme-linked immunosorbent assay (GAD₆₅ Antibody ELISA, RSR) [174], and results were measured on a microtiter plate reader (PowerWave HT).

IA-2A assay (Paper I, II, III, IV)

Measurements of IA-2A were performed in serum as described for GADA. A plasmid (pSP64poly A, kindly supplied by Prof. Åke Lernmark) containing cDNA for the cytoplasmic portion of islet antigen 512 was used.

ZnT8A assay

Serum samples were analyzed for each single ZnT8A variant: arginine (ZnT8-RA), tryptophan (ZnT8-WA), and glutamine (ZnT8-QA) using the radioligand binding assay as previously described [175]. Briefly, the COOH-terminal constructs of ZnT8 were prepared using a Phusion site-directed mutagenesis kit. The produced [³⁵S]-methionine-labeled antigens were then incubated overnight at 4°C with duplicate serum samples to allow antibody-antigen complex formation. The immune complexes were then precipitated with protein A-Sepharose (PAS) and the antibody-bound radioactivity was counted in a β -counter (1450 MicroBeta Tri-Lux Microplate Scintillation-Luminescence Counter). The concentrations of ZnT8 antibodies were related to a known standard curve.

The DASP workshops

The Diabetes Autoantibody Standardization Program (DASP) is a collaboration of the Immunology of Diabetes Society (IDS) and the US Centers for Disease Control and Prevention. The major goals of DASP are to assist laboratories in improving methods by providing technical support, training and information, to organize workshops for harmonization of antibody testing among laboratories, and to provide reference materials for the development of new measurement technologies [176]. Our GADA and IA-2A radio-binding assays (RIA) have been validated through the DASP workshops since 2001 and ZnT8A since 2010. Specificity and sensitivity for assays included in this thesis, see Table VI.

Table VI. Sensitivity and specificity for GADA, IA-2A and ZnT8A validated by DASP workshops during 2007-2010.

	2007		2009		2010	
	Sens.	Spec.	Sens.	Spec.	Sens.	Spec.
GADA	82 %	94 %	66 %	99 %	80 %	100 %
IA-2A	65 %	98 %	64 %	99 %	66 %	100 %
ZnT8-RA	-	-	-	-	52 %	97 %
ZnT8-QA	-	-	-	-	28 %	100 %
ZnT8-WA	-	-	-	-	46 %	91 %

GADA IgG 1-4 subclass assay (Paper I, II, III, IV)

The GADA IgG 1, 2, 3 and 4 subclasses were measured using a modification of the conventional GADA assay and all samples were analyzed in duplicates. Briefly, 5 µl serum and 60 µl human-recombinant ³⁵S-labeled GAD₆₅ (hr³⁵S-GAD₆₅) were incubated in a 96-well plate under vigorous shaking at 4°C over night. In parallel, biotinylated monoclonal mouse anti-human IgG1, IgG2, IgG3 and IgG4 were incubated with streptavidin agarose beads under vigorous shaking at 4°C over night. After incubation, 50 µl of the hr³⁵S-GAD₆₅-GADA complex was incubated with 50 µl of the biotinylated anti-human IgG antibody coupled to streptavidin agarose beads in a 96-well filtration plate. After incubation under vigorous shaking at 4°C for 2 h, samples were washed 8 times with 150 µl/well assay buffer using a vacuum device. Then, scintillation liquid was added to the wells, and the activity was measured in a liquid scintillation counter (1450 Microbeta Trilux). The cut-off value for each subclass was determined using a GADA negative control, which was run in duplicate in each assay. Results were expressed as cpm, and positivity of each sample was calculated by subtraction of the mean cpm value plus three times the standard deviation (SD) obtained for the negative control.

GAD₆₅ enzyme activity assay (Paper I, II, III)

GAD₆₅ enzyme activity was measured by a ¹⁴CO₂-trapping method based on the enzymatic reaction of glutamate to GABA. GAD₆₅ works as an enzyme together with the coenzyme pyridoxal 5-phosphate (PLP). GADA-positive serum from SPS patients have been shown to inhibit this reaction [59]. Briefly, GAD₆₅ (Diamyd Diagnostics AB) dissolved in a buffer solution was mixed with 15 µl serum in duplicates, and incubated for 1 hour at room temperature. Subsequently, 28 µl of a mix containing L-glutamic acid and radioactive labeled

L-[^{14}C -(U)]-glutamic acid, was added to each serum tube. All tubes were sealed with a rubber stopper attached to a center well in which a NaOH-soaked filter paper was placed. The tubes were incubated in a water bath at 37°C for 1 hour with gentle agitation, during which $^{14}\text{CO}_2$ is released as a bi-product through the decarboxylation of glutamic acid by GAD₆₅. The reaction was stopped by placing the tubes on ice for 10 minutes. The radioactivity of $^{14}\text{CO}_2$ captured on the filter papers was measured in a Wallac Microbeta Liquid Scintillation Counter. Serum from one SPS patient was included in all the assays as a positive control for inhibition.

GADA epitope assay (Paper II)

Monoclonal antibodies b96.11 and b78 were derived from a patient with Autoimmune Polyendocrine Syndrome - type 2 [177], and recognize conformational epitopes formed by the 3D structure of amino acid residues 308-365 and 451-585 of GAD₆₅, respectively. Both mAbs recognized GAD₆₅ in its native conformation and do not bind GAD₆₇. The capacity of their rFab to inhibit GAD₆₅ binding by human serum GADA was tested in a competitive ES-RBA as previously described [107]. The two rFab were added to separate wells at a concentration sufficient to compete binding of the originating intact mAb to GAD₆₅ by at least 80%. Non-competitive GAD₆₅ binding was established by no addition of rFab. The cut-off for specific competition was determined as >15% by using a negative control rFab CG7C7 specific to insulin, at 2 µg/ml. Each sample was measured in triplicates, and the mean value was calculated. A control serum was included on each plate to correct for inter-plate variations. Binding of GADA to GAD₆₅ in the presence of rFab was expressed as follows: Ratio = GADA cpm in the presence of rFab (competed) / GADA cpm in the absence of rFab (non-competed). A higher binding to GAD₆₅ in the presence of an rFab indicates a lower proportion of GADA binding to the respective epitope. Cases where the rFab competed sample resulted in higher cpm than the non-competed sample, were regarded to have a 100% binding capacity (i.e. no GADA with the epitope specificity in question).

GADA anti-idiotypic antibodies

The assay is based on the detection of GADA-specific anti-Id which requires the initial removal of anti-Id from sera using protein A sepharose (PAS) immobilized monoclonal GAD antibodies, followed by detection of the subsequently exposed GADA. As for the epitope

assay, human mAbs b96.11 and b78 specific to GAD₆₅ were derived from a patient with autoimmune polyendocrine syndrome type 2 [61]. B96.11 recognizes an epitope that is specifically bound by patients with T1D. B78 recognizes an epitope that is specifically bound by patients with SPS.

Briefly, they recognize epitopes located at independent clusters that are positioned on opposing faces of the molecule's C-terminal domain. The antibodies were purified from supernatants of the respective B cell line by using protein G Sepharose (PGS). Purified antibodies were cross-linked to PAS by using the dimethylpimelimidate method. One milligram of antibody was cross-linked to 1 ml of PAS beads. The efficiency of the coupling was 50% (0.5 mg/ml). Any uncoupled antibody was removed by washing beads with 0.05M glycine (pH 11). Serum samples (100 µl) were incubated with antibody-PAS beads (25 µl of 50% slurry) for 1 h. The bead volume was previously titrated for optimal assay conditions (data not shown). The bound fraction was separated from the unbound serum by gravity flow. To dissociate complexes of GADA and their bound inhibitors in serum samples, the heat dissociation method was used. Briefly, samples (100 µl) were heated to 55°C for 10 min in the absence or presence of antibody-PAS beads (25 µl of 50% slurry). The mixture was then incubated at 37°C for 30 min and at room temperature for a final 10 min and analyzed for GADA using RIA.

B-cell flow cytometry (Paper IV)

For the analysis of B cells, freshly separated PBMC from the 4D- (n=11); 2D- (n=15) and placebo (n=13) groups, collected at baseline, 1, 3, 9 and 15 months controls, were cultured for 24h with or without 5 µg/ml of GAD₆₅. After incubation, 1×10^6 PBMC were stained with APC-Cy7-conjugated CD19, Alexa-700-conjugated anti-CD3, PE-conjugated anti-CD20, FITC-conjugated anti-CD27, APC-conjugated anti-CD38, PE-Cy5-conjugated anti-CD45RA. Isotype controls (BD Biosciences) were included to estimate the amount of non-specific binding. Flow cytometry was performed with a Becton Dickinson FACSaria. To analyze cells in the CD19+ B cell lineage, lymphocytes were gated by FSC and SSC and CD3+ cells were excluded and data was analyzed using Kaluza™ version 1.1 (Beckman Coulter).

Cytokine secretion assay (Paper III, IV)

PBMC were isolated from sodium-heparinised venous fasting blood samples using Ficoll Paque density gradient centrifugation according to the manufacturer's instructions. One million PBMC diluted in 1 ml AIM-V medium supplemented with 20 μ M β -mercaptoethanol were cultured for 72h (*Paper III*) or 7 days (*Paper IV*) in the presence of 5 μ g/ml GAD₆₅, or in medium alone at 37°C in 5% CO₂. Interleukin (IL)-1 β , IL-2, IL-5, IL-10, IL-13, IL-17, tumour necrosis factor (TNF)- α and interferon (IFN)- γ were measured in cell culture supernatants using a Bio-Plex Pro Cytokine Panel (Bio-Rad) according to the manufacturer's instructions. Due to reagent incompatibilities, TGF- β 1 was assayed separately from the other analytes, using a Bio-plex Pro TGF- β 1 assay (Bio-Rad). Data was collected using the Luminex 200 (Luminex Corporation) and analyzed using MasterPlex QT software. The specific antigen-induced cytokine secretion was calculated by subtracting the spontaneous secretion (i.e. secretion from PBMC cultured in medium alone).

Tetanus toxoid antibody assay (Paper I)

Determination of tetanus toxoid antibodies in serum was performed using an Immunozyne Tetanus Ab ELISA according to manufacturer's instructions (IBL). The optical density was measured at 450 nm using a VersaMax microplate reader (MDS). Cut-off levels were determined at > 0.070 IU/ml.

Total IgE assay (Paper I)

Total serum IgE was quantified using the ImmunoCap100^C system (Phadia AB). The measuring range for the assay was 2-50000 kU/l, and calibrators were run in duplicate to obtain a full calibration curve. Levels of total IgE \geq 85 kU/l were regarded as positive.

Statistics

As data sets were determined to be significantly different from a Gaussian distribution using Shapiro-Wilk test, non-parametric tests corrected for ties were used. Unpaired analyses were performed using the Kruskal-Wallis test followed by Mann-Whitney U-test, and correlations were analyzed with Spearman's rank correlation coefficient test. Differences within groups

were calculated by Friedman's test followed by Wilcoxon signed rank test. A probability level of <0.05 was considered statistically significant. Calculations were performed using SPSS version 17.0 for Windows (SPSS Inc) in Paper I, and PASW statistics version 18 for Windows (SPSS Inc) in Papers II, III and IV.

Ethics

The phase II trial and 4-year follow-up study, as well as the phase III trial was approved by the Research Ethics Committee at the Faculty of Health Sciences, Linköping University, Sweden. Written informed consent was obtained from all patients, and for those <18 years old also their parents, in accordance with the Declaration of Helsinki. For the cohorts included in Paper II, informed consent from the participants, or their guardians, was obtained as part of previous clinical- and epidemiological studies according to the Helsinki Declaration.

RESULTS AND DISCUSSION

GADA responses following GAD-alum immunomodulation

Between 2005 and 2007 we performed a phase II clinical trial with GAD-alum in 70 T1D children. In our first publication from this study in 2008 [160], we reported clinical benefits of GAD-alum injections which were accompanied with GAD₆₅-specific effects on the immune system. Patients receiving 20µg GAD-alum injections twice increased serum GADA titers, FOXP3 and TGF-β gene transcription, as well as IFN-γ, IL-5, IL-10, IL-13, IL-17, and TNF-α expression in peripheral blood mononuclear cells (PBMC), as compared to placebo. Further analysis revealed an early and sustained increase in GAD₆₅-specific IL-5 and IL-13 responses [178], and increased GAD₆₅-specific CD4⁺CD25^{hi}FOXP3⁺ T cells [179]. The treatment-induced GADA titers peaked at three months, and then remained elevated throughout the study period (Fig. 11).

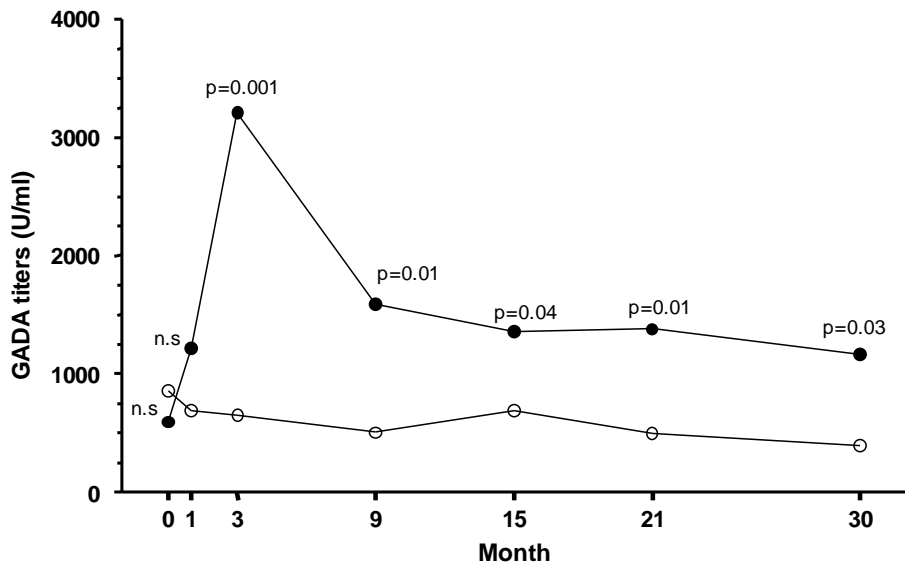


Figure 11. Median GADA levels in patients participating in the phase II trial. Median GADA levels (U/ml) in patients treated with GAD-alum (n=35, black circles) or placebo (n=34, empty circles).

We next analyzed if GADA titers in the phase II trial were associated to the clinical outcome of the study. The clinical effect of treatment was determined by changes in stimulated C-peptide AUC from baseline to 15 months (main study period). The GAD-alum treated

patients were stratified into three groups; Responders (loss of AUC < 10 %), Intermediate responders (loss of AUC between 10 % and 65 %) and Non-responders (loss of AUC > 65 %). We found that patients classified as Responders, entered the trial with significantly higher baseline GADA levels as compared to Intermediates and a tendency was found towards Non-responders (Fig. 12). Even if these results were based on smaller subgroups, we hypothesized that individuals with higher baseline GADA might have the possibility to mount a more effective GAD₆₅-specific response, through B-cell antigen presentation, associated to a better clinical response. Similar to this finding, the Diabetes Prevention Trial (DPT-1) using oral insulin showed clinical efficacy only in individuals with high levels of insulin autoantibodies pre treatment [146].

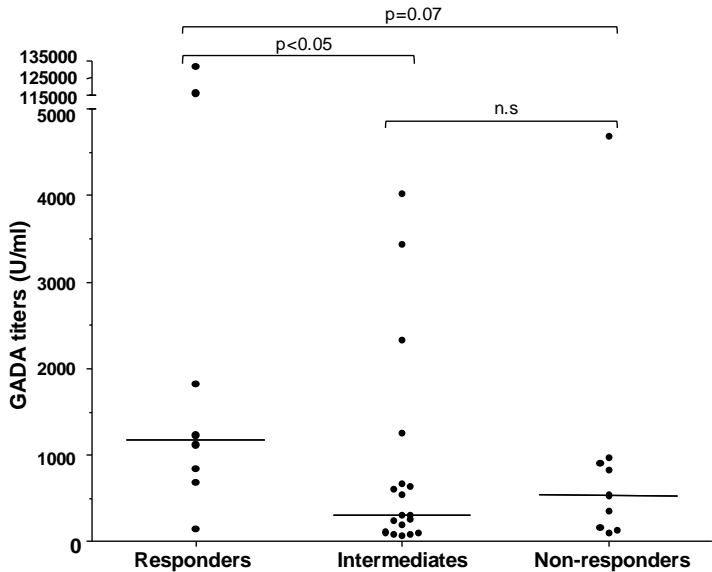


Figure 12. Baseline GADA levels in serum samples from GAD-alum treated. Patients were stratified as Responders, Intermediate and Non-responders, according to the preservation of β -cell function, defined as changes in the stimulated C-peptide (AUC) from baseline until 15 months. Horizontal lines indicate median levels.

In a previous dose-finding phase II trial including LADA patients, treatment-induced GADA levels were only observed in the group receiving two injections of 500 μ g GAD-alum, whereas no increase was observed in the 20 μ g group [158]. This inconsistency might be due to the different disease processes in LADA and T1D patients, and that the different types mainly affect adults and children, respectively. A subsequent 4-year follow-up of our phase II

trial revealed that GAD-alum treated patients, in addition to better preserved insulin secretion [161], still displayed higher GADA titers compared to placebo [180] (Fig. 13). We also showed a GAD₆₅-induced T-cell activation accompanied by secretion of Th1, Th2 and T regulatory cytokines in parallel to an induction of T-cell inhibitory pathways important for regulating the GAD₆₅ immunity. Taken together these results suggest that GAD-alum treatment induces a long-lasting memory T- and B-cell response, still detectable 4 year after the first injection.

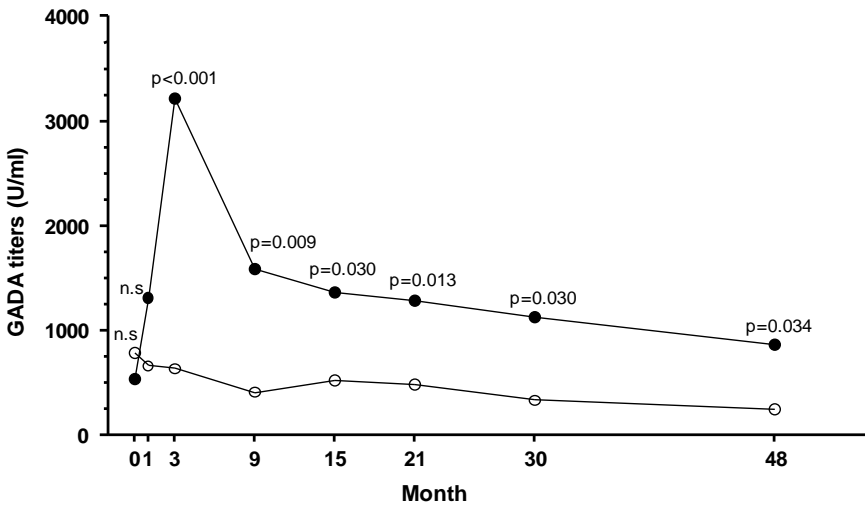


Figure 13. Median GADA levels in patients participating in the phase II trial 4-year follow-up.

Median GADA levels (U/ml) in GAD-alum- (n=29, black circles) and placebo patients (n=30, empty circles).

The following phase III trial, initiated in 2008, included three treatment arms to study if additional GAD-alum doses would affect the clinical outcome. Even though the trial did not reach the primary end-point [162], a strong GADA response was observed in the 2D and 4D GAD-alum groups, both in the whole European cohort [162] as well as in the Swedish cohort (Fig. 14A). Already at 1 month (i.e. after the first GAD-alum injection) GADA titers significantly increased compared to placebo, and after peaking at 3 months remained elevated until 21 months. GADA titers also remained significantly elevated in 2D and 4D patients compared to placebo in the subgroup of Swedish patients who completed the whole trial period of 30 months (data not shown). When calculating the GADA fold-change from baseline for each patient, the 4D group, after receiving the last injection at 9 months, displayed a significant fold-change increase at 15 and 21 months (Fig. 14B). We also

searched for a possible correlation in high baseline GADA and C-peptide preservation, as observed in the phase II trial; however a similar association could not be observed (data not shown). It is however important to note that the efficacy found in the phase II trial was smaller and not significant in the phase III trial.

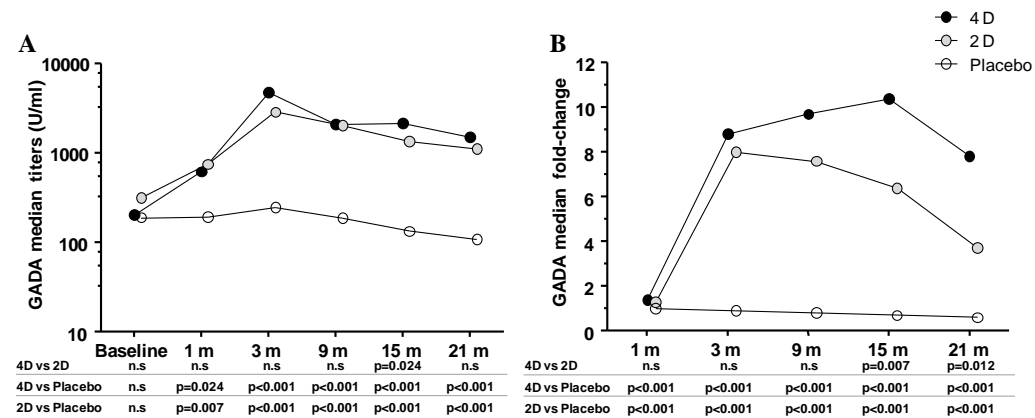


Figure 14. GADA titers and GADA fold-change over time during the phase III trial. GADA median titers (A) and GADA median fold-change from baseline (B) in 4D (n=49, black circles), 2D (n=49, grey circles) and placebo (n=50, empty circles).

Since there was a discrepancy between the phase II and III trial concerning when GADA titers became significantly increased compared to placebo patients (i.e. after two injections versus one injection, respectively), we next compared the GADA fold-change increase between the 2D groups from each trial. The reason for not comparing absolute GADA is due to the fact that GADA levels in the phase II trial were analyzed at our own facilities using a RIA-assay, whereas titers in the phase III trial was analyzed centrally at BARC-laboratories using ELISA. However, the analysis showed that the fold-change was similar in the 2D groups from the two trials (Fig. 15). The explanation for the different time-points at which GADA titers starts to increase between the phase II and III trials, might be due to the different GADA screening assays or to the changed inclusion criteria (i.e. T1D duration <18 months as compared to <3 months, respectively).

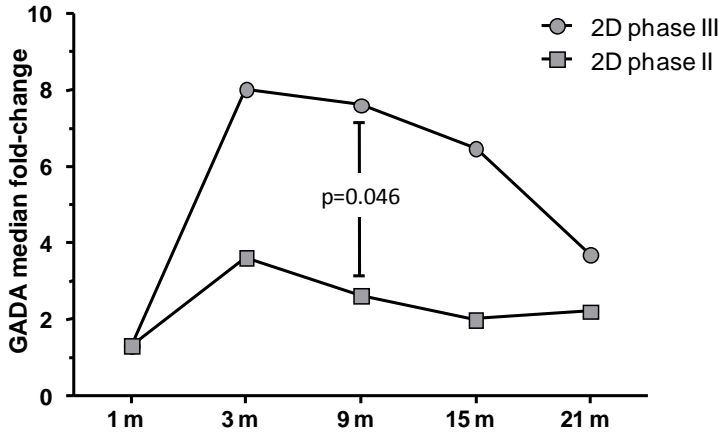


Figure 15. The GADA median fold-change from baseline in the 2D groups from the phase II- and III trial. The GADA fold-change in 2D patients from the phase II trial (n=30, grey squares) and from the phase III trial (n=49, grey circles).

GADA and cytokine response

To further elucidate if the induced GADA response observed in the two GAD-alum groups from the phase III trial was associated to a cellular response, the GAD₆₅-induced *in vitro* cytokine secretion in PBMC supernatants was correlated to the GADA fold-change (*Paper IV*). A correlation between GADA fold-change and cytokine secretion was found at 15 and 21 months both for Th1 and Th2 type cytokines (Interleukin (IL)-1 β , IL-2, IL-5, IL-10, IL-13, IL-17, tumour necrosis factor (TNF)- α , interferon (IFN)- γ and transforming growth factor (TGF)- β 1), suggesting an association between the GAD-alum induced humoral and cellular responses. However, this correlation could only be observed in 2D group, which might indicate that the two extra GAD-alum doses administered to 4D patients further enhanced the GAD₆₅-specific humoral response without simultaneously affecting the specific cellular response.

B-cell frequencies and phenotypes

Even though GADA titers increased in the 4D and 2D group, no differences in B-cell frequencies or phenotypes (CD19, CD20, CD27, CD38, CD45RA) were detected throughout the phase III trial. This might indicate that increased titers arise from specific activation of existing plasma B cells, or that the flow cytometric setup used was not sensitive enough to detect the infrequent GAD₆₅-specific B cells.

GADA anti-idotypic antibodies

During the phase II trial we aimed to investigate a possible effect of GAD-alum treatment on GADA anti-Id antibody frequencies. Our goal for analyzing anti-Id was to test if a part of the treatment-induced GADA titers might be due to unmasking of previously masked GADA by decreasing anti-Id levels. In addition, we were also interested to analyze if part of the decrease in GADA titers, after peaking at 3 months, might be due to increased anti-Id levels. For this purpose we adopted an assay developed by Ass. Prof Christiane Hampe (University of Washington, Seattle, USA) [61]. All samples from the phase II trial were analyzed at baseline, 3 and 15 months for anti-id directed to GADA with a b96.11 and b78 defined epitope specificity. Unfortunately, data analysis showed inconsistencies, which might be attributed to the dramatic increase in GADA levels after GAD-alum treatment, since the method previously had been developed using sera at lower titers. Since then, other methods have been further developed by Hampe *et al* and there now exists two additional assays to detect anti-id [181]. Thus, our material would need to be re-analyzed before any conclusions can be drawn.

GADA IgG 1-4 subclass distribution following GAD-alum treatment

Since GADA titers significantly increased after GAD-alum treatment, we wanted to analyze whether these changes influenced the GADA IgG 1-4 subclass distribution, as the subclass frequencies can be associated to Th1/Th2 responses. Frequencies were calculated with respect to the combined sum of all four subclasses in each sample (i.e. total IgG). In the phase II trial a transient increase in IgG3 and IgG4, and decrease in IgG1 was observed from baseline to 15 months in GAD-alum treated (Fig. 16).

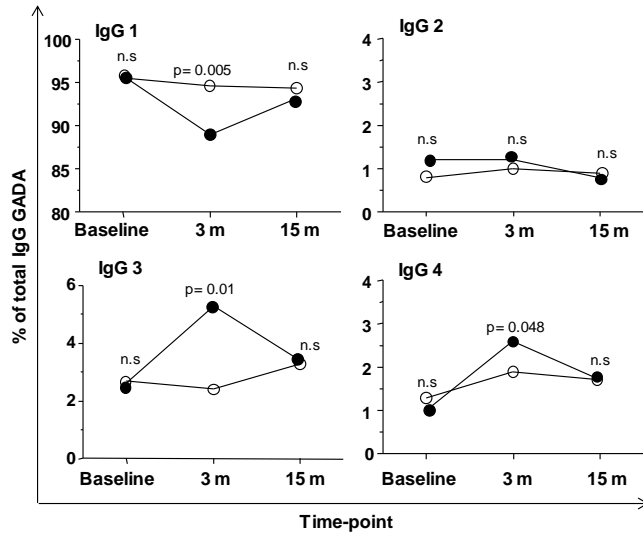


Figure 16. The effect of GAD-alum in GADA IgG subclass distribution during the phase II trial.

GADA IgG 1, 2, 3, 4 subclasses in GAD-alum treated (n=35, black circles) and placebo patients (n=34, empty circles). Frequencies were calculated with respect to the combined sum of all four subclasses in each sample (i.e. total IgG). The median percentage of total IgG is shown for each respective subclass.

The significant difference between the groups was observed at 3 months, when total GADA peaked in treated compared to placebo. At baseline, the hierarchical distribution within GAD-alum patients was IgG1>IgG3>IgG2>IgG4, however, at 3 months the distribution changed to IgG1>IgG3>IgG4>IgG2 and remained similar also at 15 months. Even though the subclass distribution in GAD-alum treated returned to that observed for placebo individuals at 15 months, we analyzed GADA IgG frequencies during the 4-year follow-up to evaluate the long-term effect. However, the GADA subclass frequency did not differ between the treatment groups 4 years after initiation of the trial.

During the phase III trial we analyzed the GADA IgG distribution at all time-points. At baseline, GADA IgG1 subclass was the most frequent in all groups, followed by IgG3>IgG4>IgG2. Even though some transient changes in each subclass between the groups were observed, this hierarchy remained unchanged for the 2D and placebo groups throughout the trial. The 4D group however displayed significantly increased proportions of IgG4 levels at 9, 15 and 21 months, compared to 2D and placebo, which resulted in a changed subclass hierarchy (IgG1>IgG4>IgG3>IgG2) for this group (Paper IV). Further, the proportion of IgG4 at 9, 15 and 21 months was also significantly higher within the 4D group compared to

baseline, while in contrast the proportions of IgG1 decreased from baseline to 3, 9 15 and 21 months (Fig. 17). In addition, a transient increase in IgG3 was observed at 3 months in the 4D group, when total GADA levels peaked and IgG1 started to decrease.

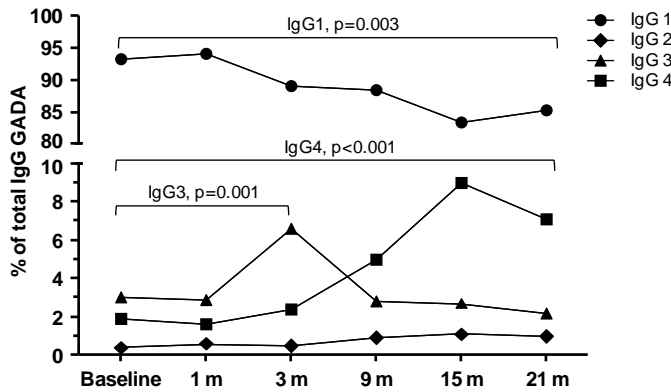


Figure 17. GADA IgG1-4 subclass distribution within the 4D GAD-alum group during the phase III trial. The median GADA IgG subclass distribution presented as a percentage of total IgG for the 4D group. Results were expressed as cpm, and positivity of each sample was calculated by subtraction of the mean cpm value plus three times the standard deviation (SD) obtained for the negative control.

The transient increase of IgG4 observed in the phase II trial (Fig.16), and the increased expression of IgG4 by additional GAD-alum doses in the phase III trial (Fig. 17), supports the notion of an enhanced humoral Th2-like response to treatment. Indeed, previous studies have reported that higher levels of GADA IgG4 are associated to slower progression to clinical onset of disease in at-risk individuals [96, 98]. Also, immunization with insulin has been shown to promote the IgG4 subclass in T1D patients, suggesting an association with a Th2 like response [182]. In addition, LADA patients which present a much slower and less aggressive disease progression have been found to express higher levels of GADA IgG4 than T1D patients [97, 99].

In the previous phase II trial we detected decreased IgG1 levels and a transient increase in IgG3 and IgG4 in the 2D group compared to placebo. Surprisingly, in the phase III trial a similar effect was observed only in the 4D group, and not in 2D patients. This inconsistency might be due to considerably shorter disease duration at inclusion in the phase III trial, requiring additional GAD-alum injections to affect the transforming humoral immune response observed close to disease onset [98, 183].

The specific effect of GAD-alum treatment on humoral responses

Since GAD-alum treatment was shown to impact the humoral responses, reflected as increased GADA levels, we further investigated the specificity of this response.

The T1D associated autoantibodies IA-2A and ZnT8A

To investigate if GAD-alum injections affected T1D associated autoantibodies other than GADA, we analyzed IA-2A in the phase II and III trials, and ZnT8A in patients participating in the phase II trial, including the 4-year follow-up. In the phase II trial, analysis of IA-2A levels revealed no longitudinal changes in either GAD-alum or placebo patients (data not shown). Pre treatment levels of IA-2A were higher in placebo- compared to GAD-alum treated (Fig.18A), a difference that persisted at 3 and 9 months.

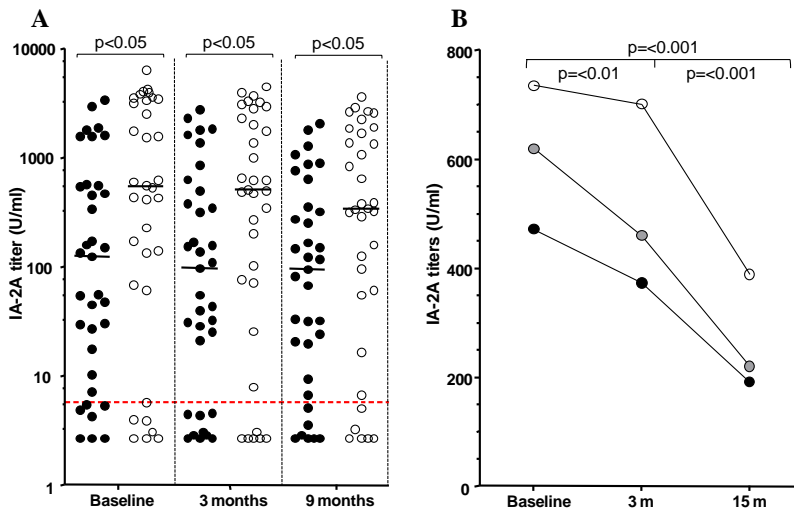


Figure 18. IA-2A titers in the phase II and III trial. (A) IA-2A titers from GAD-alum treated (black circles) and placebo (empty circles) participating in the phase II trial. Horizontal lines indicate median levels and dotted line indicate the cut-off limit. (B) Median IA-2A titers in the 4D (grey circles), 2D (black circles) and placebo (empty circles) groups participating in the phase III trial.

After correcting for the IA-2A baseline level, no significant difference in IA-2A titers was observed between the groups at 3 and 9 months (data not shown). No association between IA-2A levels and C-peptide secretion was observed. IA-2A levels were also measured during the 4-year follow-up, but no difference in titers was found between the two groups.

In the phase III trial, IA-2A was analyzed at baseline, 3 and 15 months without detecting any differences between the 4D, 2D and placebo groups (Fig. 18B). However, a longitudinal decrease was observed between all time points within each group. This observation might further support the notion of T1D patients with shorter disease duration (as included in the phase III trial compared to the phase II trial) in general have a still transforming humoral immune response compared to those with a long standing disease.

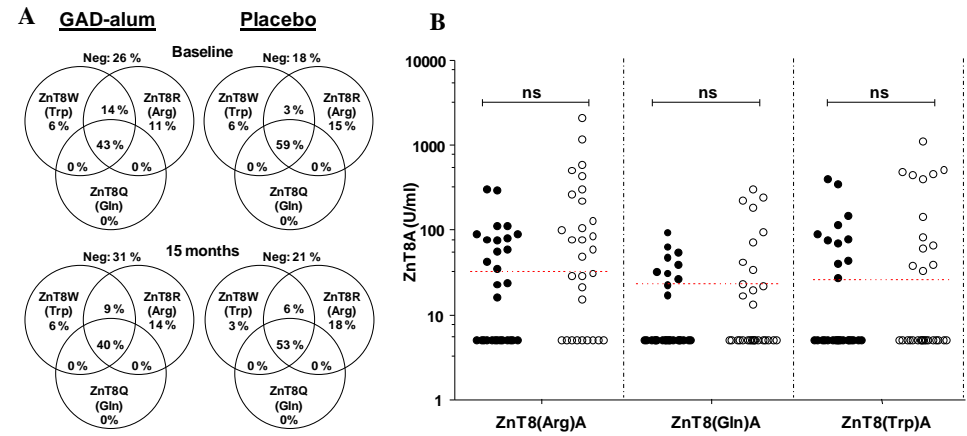


Figure 19. ZnT8A titers from patients in the phase II trial and 4-year follow-up. (A) The percentage of patients who were negative or single, double or trippel positive for one of the three ZnT8A variants during the phase II trial. (B) ZnT8A titers in GAD-alum (black circles) and placebo (empty circles) patients participating in the 4-year follow-up. Dotted line indicates the cut-off limits.

The three variants of ZnT8A, yet another T1D associated autoantibody, showed during the phase II trial no significant longitudinal differences at baseline, 3 months (not shown) and 15 months within the GAD-alum or placebo groups, nor between them (Fig. 19A). These findings were later confirmed during the 4-year follow-up (Fig. 19B), further supporting the specificity of GAD-alum treatment.

Detection of T1D unassociated Tetanus toxoid antibodies and allergy associated total IgE

To further assess the specific effect and to search for a possible allergy-associated effect in response to GAD-alum treatment, tetanus toxoid and total IgE antibodies were measured in samples from the phase II trial. Tetanus toxoid titers were analyzed in samples collected prior to treatment and after 3 and 9 months (Fig. 20A). Total IgE was analyzed in samples collected pre treatment and 15 months after the first injection (Fig. 20B).

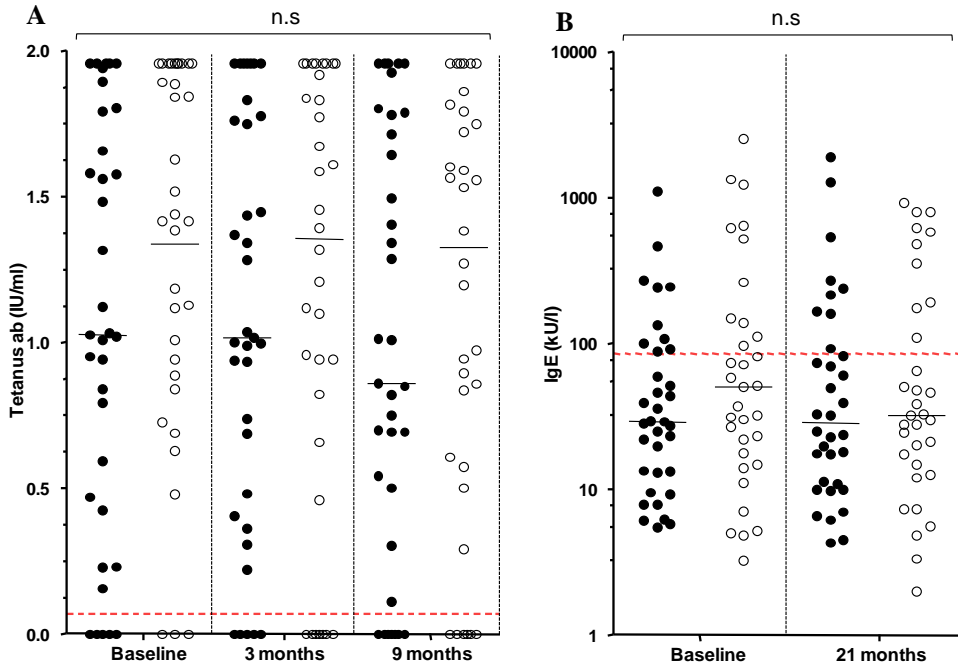


Figure 20. Tetanus toxoid and total IgE levels in patient's sera during the phase II trial. Tetanus toxoid titers (A) and total IgE (B) in GAD-alum (black circles) and placebo (empty circles) treated patients from the phase II trial. Horizontal lines indicate the median, and dotted lines the cut-off values.

The lack of any significant differences in tetanus toxoid titers, neither between nor within the groups at any time point, further supports the specificity of treatment. In addition, as total IgE levels did not differ between the groups and no severe clinical allergic symptoms, other than minor skin reactions at the site of injection, have been reported [160], GAD-alum treatment is not likely to induce an allergic response.

The *in vitro* GAD₆₅ enzyme activity and GADA epitope distribution

Ever since GAD-alum treatment was found to increase GADA titers, there have been concerns to whether the induced titers could result in adverse events. In particular development of Stiff Person Syndrome (SPS) which is associated to high GADA titers, impaired GAD₆₅ enzyme activity and decreased GABA synthesis, have been suggested as a

possible risk. As previous studies have reported that sera from SPS patients inhibits the *in vitro* GAD₆₅ enzyme activity [108], while sera from T1D patients do not [109], we analyzed the GAD₆₅ inhibitory capability of sera from patients participating in the phase II trial and 4-year follow-up. The analysis showed that sera from both GAD-alum and placebo patient inhibited the *in vitro* GAD₆₅ enzyme activity to a lesser extent at baseline, and at 3, 15 and 48 months compared to the SPS serum (Fig. 21 A-C). The activity did not either differ within, nor between the GAD-alum and placebo patients at any time-point.

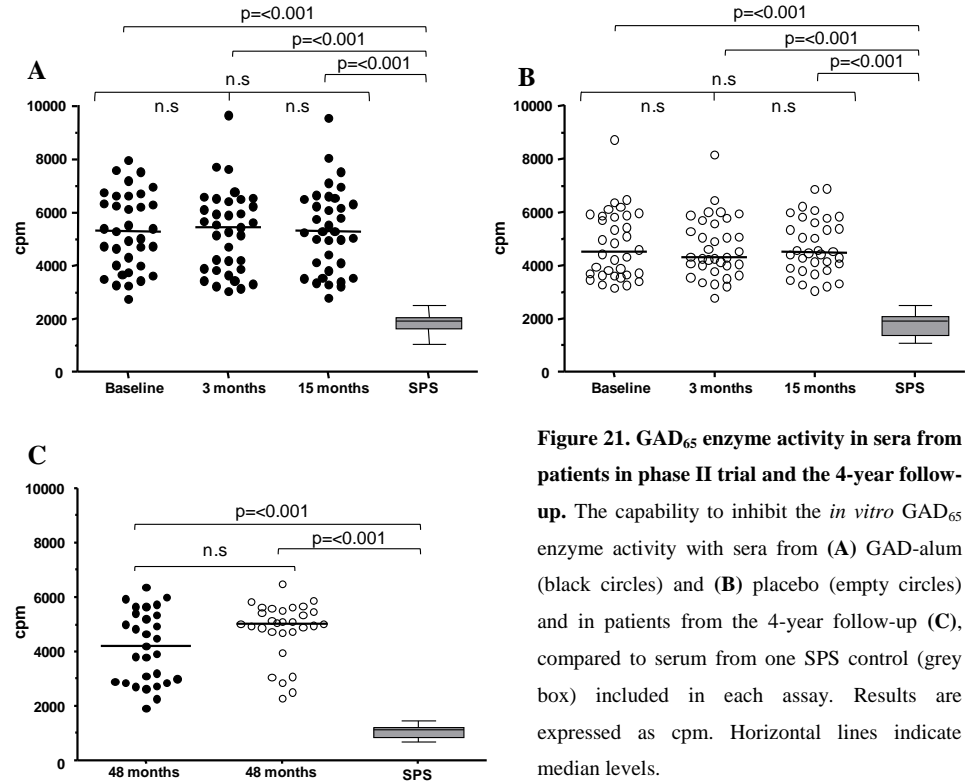


Figure 21. GAD₆₅ enzyme activity in sera from patients in phase II trial and the 4-year follow-up. The capability to inhibit the *in vitro* GAD₆₅ enzyme activity with sera from (A) GAD-alum (black circles) and (B) placebo (empty circles) and in patients from the 4-year follow-up (C), compared to serum from one SPS control (grey box) included in each assay. Results are expressed as cpm. Horizontal lines indicate median levels.

In addition to differences in GADA titers and inhibited GAD₆₅ enzyme activity, GAD₆₅-specific monoclonal antibodies and their recombinant Fab (rFab) have previously been used to map GADA epitopes associated with SPS- or T1D patients. The GADA epitope defined by monoclonal antibody b96.11 is located in the middle region of GAD₆₅, and appears to be associated with progression to T1D [58, 106-107]. In contrast, SPS patients recognize a GADA epitope defined by monoclonal antibody b78 which is located in the C-terminal

region [59], and has been associated with the inhibition of GAD₆₅ enzyme activity [108]. We have previously reported minor changes in these and other epitope frequencies in patients participating in the phase II trial [184]. Analysis of both epitope distribution and GAD₆₅ enzyme activity was performed using the whole phase II study population, and serum from only one SPS patients was used as a reference control.

To further study possible changes in GADA phenotypes in those patients responding with the highest GADA titers, we selected a subgroup of patients that displayed the most elevated GADA titers at 3 months during the phase II trial. In addition, we included a number of SPS patients, a group of high GADA titer T1D patients as well as a group of high GADA positive healthy children at risk for T1D. Even though the SPS patients were the only group not selected based on high GADA titers they still displayed higher levels than the other groups (Paper II).

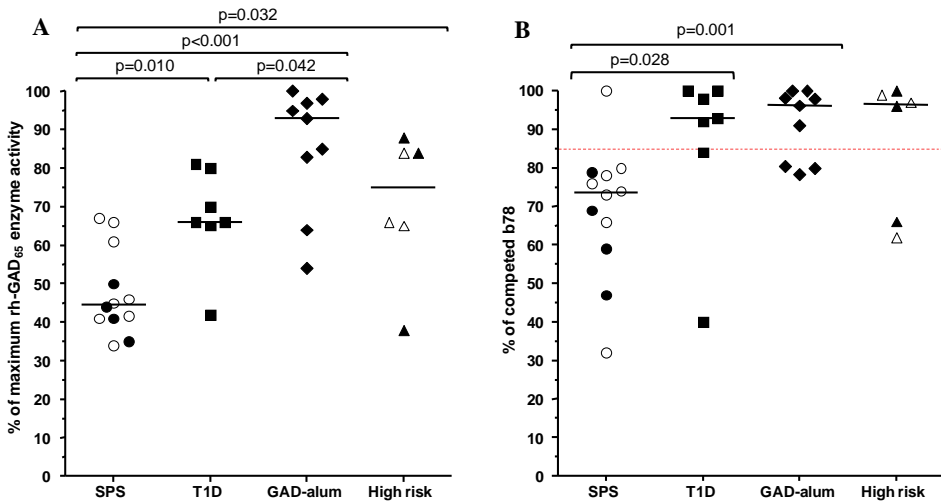


Figure 22. GAD₆₅ *in vitro* enzyme activity and frequency of GADA with the b78 defined epitope. Recombinant human GAD₆₅ *in vitro* enzyme activity (A) and b78 epitope defined GADA frequency (B) in SPS (circles, n=12), T1D (squares, n=7), GAD-alum (rhombuses, n=9) and High-risk (triangles, n=6) groups. Empty circles in the SPS group (n=8) represent individuals with coexistent T1D whereas empty triangles in the High-risk group (n=3) represent individuals that developed T1D after sampling.

The GAD₆₅ enzyme activity is expressed as a percentage of the maximum enzymatic activity. Binding to GAD₆₅ in the presence of rFab b78 presented as a ratio of competed / non-competed samples. A higher binding to GAD₆₅ in the presence of rFab indicates a lower proportion of GADA binding to the respective epitope. Samples with a calculated value below the 85% cut-off limit, represented as a dotted line, were regarded as positive for binding to the respective epitope. Black horizontal lines represent the median.

The SPS group also showed a significantly lower GAD₆₅ *in vitro* enzyme activity compared to the other groups (Fig. 22A), as well as a higher frequency of GADA towards the SPS defined b78 epitope (Fig. 22B) as compared to GAD-alum- and T1D patients. Analysis of GADA directed to the b96.11 defined epitope described to be T1D-associated, revealed no significant differences between the groups (data not shown). These findings further support the notion that even patients responding with very high GADA titers to GAD-alum treatment, do not develop GADA with an SPS associated phenotype. Furthermore, as no clinical neurological adverse events have been reported, our findings support the safety of GAD-alum immunomodulation.

C-peptide preservation in patients completing the phase III trial

Since patients in Sweden entered the phase III trial relatively early, compared to the rest of the European countries, a majority of the Swedish patients completed their 21 months visit and approximately 1/3 also completed the 30 months follow-up. When comparing fasting- and stimulated C-peptide secretion at 21 months, no differences between the three treatment arms were found (Fig. 23A-B). However, in the subgroup of 45 Swedish patients completing the 30 months study period, a significantly reduced loss both in fasting and in stimulated C-peptide was observed in the 2D regimen compared to placebo, suggesting an effect of GAD-alum on preservation of residual insulin secretion (Fig. 23C-D).

In addition, a larger proportion of patients in the 2D group tended to be able to achieve a stimulated peak C-peptide value above 0.2 nmol/l at the 30 months follow-up, compared to the placebo group (Fig. 23E). As even a modest residual insulin secretion, with stimulated C-peptide levels > 0.2 nmol/l, has been reported to provide clinically meaningful benefits in terms of reducing long-term complications [113], this further supports the concept of GAD-alum as treatment for T1D. To ensure that the small subgroup of patients followed for 30 months represents the entire study cohort, fasting and stimulated C-peptide in the 2D regimen was compared to the Swedish cohort and the entire European study cohort. The small subgroup did not significantly differ from the other cohorts at any time point; furthermore there were no differences in patient's baseline characteristics (data not shown).

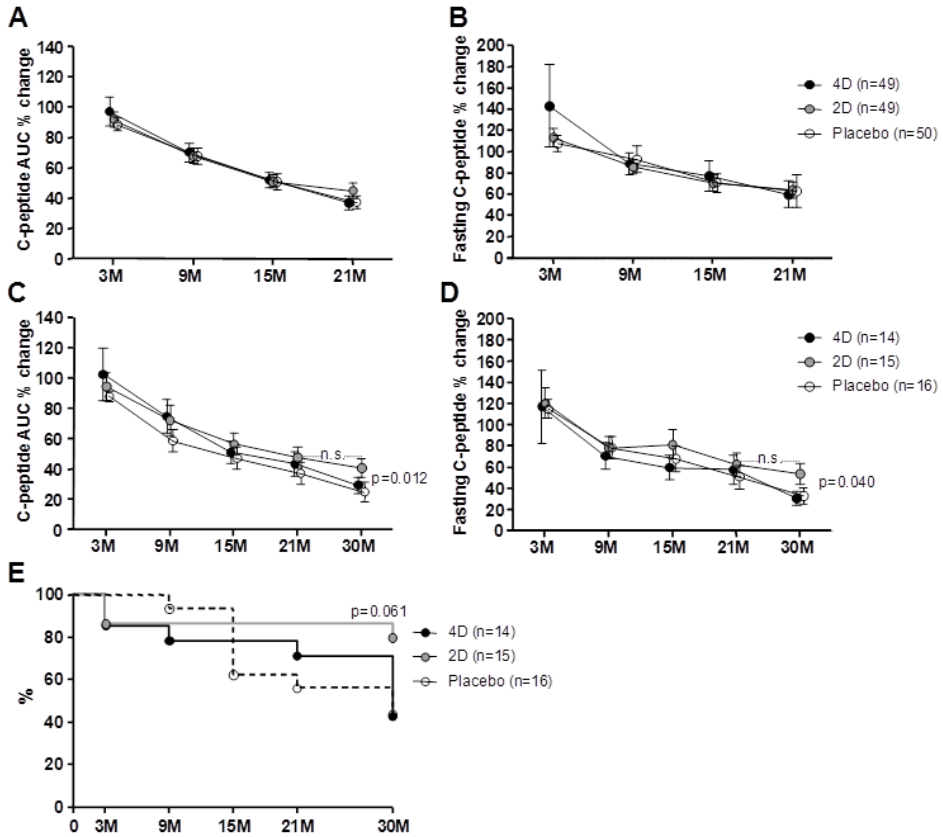


Figure 23. Changes in stimulated and fasting C-peptide, according to study group. Changes in stimulated and fasting C-peptide (mean \pm SEM), reported as a percentual change from baseline, in patients receiving 4 doses of GAD-alum (4D, black circles), 2 doses of GAD-alum (2D, gray circles) or placebo (empty circles), in the entire Swedish cohort up to 21 months (**A-B**), and for the Swedish subgroup of patients who completed the 30 month visit (**C-D**). The proportion of patients achieving a peak stimulated C-peptide level >0.2 nmol/l (**E**) from baseline to 30 months after treatment with 4 doses of GAD-alum (4D, black circles), 2 doses of GAD-alum (2D, grey circles), or placebo (empty circles). Significant differences are indicated as p-values.

The reason why clinical effect of GAD-alum was not detected until 30 months after treatment might be due to the rather small loss of C-peptide in diabetic teenagers during the first 12-15 months after diagnosis, as it has been seen in a Swedish cohort of approximately 4000 newly-diagnosed children and adolescents with T1D (Ludvigsson *et al* to be published), and also observed in a large European cohort of patients with T1D [185].

CONCLUDING REMARKS

During the last three decades, several attempts aimed to prevent or intervene the β -cell destruction in T1D have been tried with various success, and knowledge about the effects on humoral immune responses is limited. The positive clinical findings, in parallel to induced GADA levels, observed in our phase II trial using GAD₆₅ as an immunomodulator, prompted us to further analyze humoral responses following GAD-alum intervention in T1D. In this thesis I report results concerning humoral responses and safety aspects during a clinical phase II trial and a subsequent 4-year follow-up, as well as a recent European phase III trial.

In the phase II trial, we reported a transient increase of IgG3 and IgG4, and a decrease in IgG1 as part of the GAD-alum induced GADA levels, a result interpreted to be Th2-associated. This Th2 associated immune response was also observed in the following GAD-alum phase III trial including a larger group of patients, and GADA levels were induced both in patients receiving 2 and 4 GAD-alum doses. The GADA fold-change was associated with *in vitro* GAD₆₅-stimulated cytokine secretion only in patients receiving 2 doses. However, enhanced Th2-like IgG subclass distribution, reflected as increased IgG4 frequencies, was in contrast only observed in the group treated with 4 doses of GAD-alum. The inconsistencies regarding the subclass distribution between the phase II-and III trials, might be due to changed inclusion criteria (i.e. T1D duration of <18 months and <3 months, respectively) or the use of different GADA-screening assays at inclusion.

The safety aspect of GAD-alum treatment was also analyzed, with regard to the concern that induced GADA titers might be of a Stiff person syndrome (SPS)-phenotype, a disease affecting the nervous system. We assessed this through analysis of the GAD₆₅ enzyme activity after incubation with sera from patients participating in the phase II trial and with sera from SPS patients. Even though the SPS serum inhibited the GAD₆₅ enzyme activity significantly more compared to the GAD-alum- and the placebo group, and no longitudinal changes were observed during the trial, we further analyzed the GADA-phenotype in a subgroup of GAD-alum patients inducing the highest GADA titers. We investigated not only safety aspects of GAD-alum treatment in selected patients responding with the highest GADA titers during the phase II trial, but also GADA phenotypes in different high GADA titer groups. We showed that *in vitro* phenotypes of GADA from SPS patients differed from high GADA titer positive T1D patients and T1D High-risk individuals, and that GAD₆₅

injections did not induce SPS associated phenotypes in T1D patients responding with very high GADA titers to GAD-alum treatment.

Even though the primary end-point of the European phase III trial was not reached, and the study was closed after 15 months, a majority of the Swedish patients completed the 21 months follow-up and approximately 1/3 the last 30 month visit. In the small Swedish subgroup of patients that completed the trial, a significant preservation of β -cell function was observed in the 2D group compared to placebo at 30 months. However, despite the immunomodulatory effects, in parallel to a preserved fasting and stimulated C-peptide in 2D patients at 30 months, no specific immune marker associated to clinical efficacy could be identified.

The possible future of GAD₆₅ as an immunomodulator in the intervention of T1D withholds a number of issues that needs clarification. Firstly, the clinical effect observed in the small subgroup of patients completing the phase III trial needs to be confirmed, therefore we have initiated a clinical 4-year follow-up of the trial which is currently ongoing. Secondly, the different clinical outcome in the phase II trial as compared to the phase III trial needs to be elucidated. In the phase II trial it was found that C-peptide preservation was most effective in children with shorter disease duration, and as a consequence the inclusion criteria was changed for the phase III trial. As it has been shown that T1D teenagers have a small reduction in C-peptide close after diagnosis, the clinical effect of GAD-alum might be observed first at a later stage. In addition, short after initiation of the phase III trial, the outbreak of the “Swine flu” (Influenza A H1N1) resulted in a nationwide vaccination campaign in Sweden, which also might have influenced the outcome of the trial. Lastly, the efficacy of GAD₆₅ may be improved by other administration routes, adjuvants, dosages or patients groups, or in combination with other T1D associated autoantigens and/or therapies. Also, the use of GAD₆₅ for the prevention of T1D in high-risk individuals may show clinical efficacy in the future.

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