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The Perfect Storm?

Gluten and type 1 diabetes

Emma Adlercreutz



**LUND
UNIVERSITY**
Faculty of Medicine

DOCTORAL DISSERTATION

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Sweden.

To be defended at Medicinskt Forskningscentrum, Universitets-
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<p>Abstract Aim: The general objective of the present research was to study the association between type 1 diabetes and celiac disease, and the effects of dietary gluten on the risk of developing type 1 diabetes. More specific aims were as follows: to estimate the prevalence of celiac-disease-associated autoantibodies in children with type 1 diabetes; to study the connection between non-HLA genetic risk markers and autoantibodies associated with type 1 diabetes and celiac disease during the first year after diagnosis of type 1 diabetes; to investigate the effect of gluten on the risk of developing type 1 diabetes; to evaluate the impact of perinatal factors on the risk of developing both type 1 diabetes and celiac disease.</p> <p>Methods: Serum samples from children with (Studies I and II) or without (Study I) type 1 diabetes were analyzed using a combined deamidated gliadin peptide and tissue transglutaminase antibody assay, and also a tissue transglutaminase autoantibody radioligand binding assay. BALB/c mice and NOD mice were used in Study III, and FACS, qPCR, and immunohistology were performed to assess the effects of a gluten-free diet versus a standard diet on expression of the NK cell receptor NKG2D and its ligands. In Study IV, the Medical Birth Register was used to identify all singletons born in Sweden between 1987 and 1993, and the Swedish National Inpatient Register was used to identify cases of type 1 diabetes and celiac disease. Thereafter, multinomial logistic regression models were employed to estimate odds ratios with 95% confidence intervals for having type 1 diabetes, celiac disease, or both these disorders in relation to factors known to be associated with celiac disease.</p> <p>Results and conclusions: There was a discrepancy in levels of the celiac-related antibodies between children with type 1 diabetes in Denmark and those in Sweden, independent of HLA genotype, which suggests a difference in exposure to environmental factors between these two neighboring countries. Approximately 5% of children with type 1 diabetes developed celiac disease autoimmunity (CDA) during the first year of type 1 diabetes. Furthermore, a subpopulation was homozygous for IL18RAP, which may modulate the risk of developing CDA in type 1 diabetes. In animal experiments, dietary gluten seemed to affect the immune system of both immune-competent BALB/c mice and diabetes-prone NOD mice, and a gluten-free diet lowered expression of NKG2D and its ligands. Comorbidity of type 1 diabetes and celiac disease was associated with several perinatal risk factors, including Caesarean section, birth during summer, female gender, and being born to mother born in Sweden.</p>		
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Date December 6, 2014

The Perfect Storm?

Gluten and type 1 diabetes

Emma Adlercreutz



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“Success consists of going from failure to failure without loss of enthusiasm.”
-Winston Churchill

To my mother, Charlotte - this thesis would have remained a dream had it not been for you!

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- I Emma H Adlercreutz, Jannet Svensson, Dorthe Hansen, Karsten Buschard, Åke Lernmark, Henrik Mortensen, Daniel Agardh. Prevalence of celiac disease autoimmunity in children with type 1 diabetes: Regional variations across the Öresund strait between Denmark and southernmost Sweden. *Pediatric Diabetes*, 2014 Aug 18 (Epub ahead of print).
- II. Emma H Adlercreutz, Dorthe Hansen, Henrik B Mortensen, Åke Lernmark, Karsten Buschard, Daniel Agardh, Jannet Svensson. Risk of celiac disease autoimmunity is modified by non-HLA genetic markers during the first year of clinical type 1 diabetes. *J Diabetes Endocrinol*, 2014, (2) 58–64.
- III. Emma H Adlercreutz, Christian Weile, Jesper Larsen, Kåre Engkilde, Daniel Agardh, Karsten Buschard, Julie C. Antvorskov. A gluten free diet lowers NKG2D and ligand expression in BALB/c and NOD mice. *Clin Exp Immunol*, 2014 Aug; 177 (2):391–403.
- IV. Emma H Adlercreutz, Carl Johan Wingren, Raquel Perez Vincente Juan Merlo, Daniel Agardh. Perinatal risk factors and comorbidity of type 1 diabetes and celiac disease. *Acta Paediatr* 2014 (Epub ahead of print).

Abbreviations

AB	Antibody
AGA	Anti-gliadin antibodies
ALN	Auricular lymph nodes
BALB/c	Laboratory-bred strain of the House Mouse
CCR5	Chemokine receptor type 5
CD	Celiac Disease
CD8	Cluster of differentiation 8 (a transmembrane glycoprotein)
CI	Confidence Interval
DAMPs	Danger-associated molecular patterns
DQ2	Haplotype HLA-DQA1*0501-DQB1*0201
DQ8	Haplotype HLA-DQA1*0301-DQB1*0302
DX5	Antigen on mouse NK cells
ELISA	Enzyme-linked immunosorbent assay
EMA	Endomysial autoantibodies
FACS	Fluorescence-activated cell sorting in Flow cytometry
FoxP3	Forkhead box protein 3
GADA	Autoantibodies to glutamic acid decarboxylase
GF	Gluten Free
GWAS	Genome-wide association studies
HLA	Human leukocyte antigen
HRQoL	Health-related quality of life
IAA	Insulin autoantibodies
IA-2A	Insulinoma-association studies
ICA	Antibodies to islet antigen 2 autoantibodies
IEL	Intraepithelial lymphocyte
IL	Interleukin
KIRGI1	Killer cell lectin-like receptor group 1

mAB	Monoclonal antibodies
MHC	Major histocompatibility complex
MYD88	Myeloid differentiation primary response gene (88)
NCR	Natural cytotoxicity receptors
NK cell	Natural Killer cell
NKG2D	Natural Killer group 2 receptor
NKT cell	Natural Killer T cell
NOD	Non Obese Diabetic
OR	Odds ratio
PAMPs	Pathogen-associated molecular patterns
PAS	Protein A Sepharose
PCR	Polymerase chain reaction
PD1	Programmed cell death 1
PLN	Pancreatic lymph node
PRR	Pattern recognition receptor
qPCR	Quantitative real-time PCR
RBA	Radioligand binding assay
RT-qPCR	Reverse transcription-quantative polymerase chain reaction
RU	Relative units
SGA	Small for gestational age
SGF	Strictly gluten free
SNP	Single nucleotide polymorphism
SPF	Specific pathogen-free
SSTD	Strictly standard diet
STD	Standard diet
T1D	Type 1 diabetes
TCR	T cell receptor
TLR	Toll-like receptor
Th cell	T helper cell
tTG	Tissue transglutaminase
ZnT8A	Zinc transporter 8 autoantibodies

Introduction

Type 1 diabetes

Type 1 diabetes (T1D) is one of the most common chronic illnesses of childhood, and it represents a multifactorial disease that is characterized by loss of insulin production by the β cells in the pancreatic islets, which gives rise to hyperglycemia. T1D is associated with certain genotypes of human leukocyte antigen (HLA), and T1D patients show increased predisposition to other chronic diseases with autoimmune features, such as celiac disease and autoimmune thyroiditis, features that suggest common pathogenetic mechanisms of disease development. In parallel with other autoimmune diseases, the global incidence of T1D is increasing, most rapidly among children younger than 5 years of age (1, 2). After Finland, Sweden has the second highest reported incidence of T1D in the world (3).

The pathogenesis of T1D is not fully understood, although it is known to involve an autoimmune response and production of autoantibodies leading to loss of pancreatic insulin-producing β cells. At the time of diagnosis of T1D, the majority of the β cells have already been eradicated through lymphocytic infiltration of innate immune cells that generate cytokines and thereby promote increased infiltration of islet-specific T cells, predominantly CD8⁺ and CD4⁺ T cells, B cells, and macrophages (4-7). T cells, together with the cytokines they produce (e.g., INF- γ , IL-1 β , and IL-17), may cause apoptosis in β cells and hence play an important role in both the induction and the immune-mediated mechanisms that finally destroy the β cells (8-10). After a long sequence of autoimmune processes, clinically overt diabetes occurs when β cell mass is lowered below a certain threshold level that causes loss of insulin production (11).

The appearance of autoantibodies is the first detectable sign of emerging β cell destruction. Five disease-associated autoantibodies have been shown to predict T1D (12, 13): ICA (islet cell antibodies), GADA (glutamic acid decarboxylase autoantibodies), IAA (insulin autoantibodies), IA-2A (insulinoma-associated protein 2 autoantibodies), and ZnT8A (zinc transporter 8 autoantibodies). The function of these autoantibodies in the development of T1D has not been re-

vealed, although there is evidence that they can increase the immune precipitation of antigens presented to B cells. Still, these autoantibodies are the first sign of autoimmunity and may appear as early as the first year of life in children developing T1D (14, 15). Moreover, studies have demonstrated that the number of detectable autoantibodies is unequivocally related to the risk of progression to clinically manifest T1D (16-19), indicated by data showing that being positive for three or more autoantibodies increases the risk of T1D by 60–100% over a period of 5–10 years (20).

At the time of clinical diagnosis, the majority of the β cells have been eradicated by lymphocytic infiltration in pancreatic islets, a process called insulinitis (4-7). The insulinitis usually involves infiltration of innate immune cells, which produce cytokines that promote β cell apoptosis and also increase infiltration of islet-specific T cells that attack and destroy β cells. T cells, mainly CD8 and CD4 T cells, play a prominent role in the induction of T1D, and the inflammatory infiltrate in islets consists primarily of T lymphocytes, but also comprises B cells and macrophages (11).

Risk factors suggested to be associated with T1D include virus infections (especially enterovirus) (21, 22), infections with other pathogens (e.g., *Mycobacterium avium* subspecies *paratuberculosis*, the etiological agent of paratuberculosis) (23), exposure to pollutants (24), increased height velocity (25), and childhood obesity (26). Pre- and perinatal factors, or circumstances connected with those factors, may also influence the risk of T1D. Low socio-economic status and lower level of education in the mother (27, 28), birth delivery type, birth weight, infant growth, neonatal jaundice, and maternal age (29-33) have all been found to be related to the risk of T1D. Various dietary factors have also been reported to be correlated with an increased risk of T1D, and these include cow milk proteins, a short period of breast feeding, early introduction to solid foods, fruit/berries, and root vegetables, and low levels of vitamin D (34-37). Furthermore, it has been proposed that the amount, timing, and mode of introducing dietary gluten affect the risk of T1D (38), and it has been reported that introduction of gluten before the age of 3 months is a risk factor for islet autoimmunity (34, 35, 39).

Celiac disease

Celiac disease (CD) is a chronic immune-mediated disorder that is triggered by ingestion of gluten and appears in genetically predisposed patients. Approximately 1% of the population in Western countries is affected by CD, making it one of the most common chronic disorders today (40-42). Although there are variations

between, as well as within, countries, there is an overall increase in industrial countries, including northern Europe and the United States, as well as in the developing countries of Asia (43, 44). The rise in incidence rates can probably be partly explained by heightened awareness of the disease, but epidemiological studies conducted in several different countries have independently reported a twofold increase over the past 20 years thus indicating a true rise in incidence (45-48).

Unique to Sweden, the incidence of CD in children younger than 2 years of age displayed an epidemic pattern in 1984–1996 (49), and during that period reached levels higher than previously observed in any population. After the mid-1990s, the incidence of clinically reported CD in young children in Sweden decreased again, although follow-up screenings demonstrated a prevalence of 3% in this country (50). However, there is a striking difference between the Scandinavian countries, particularly between Denmark and Sweden. The incidence in 100,000 children younger than 15 years of age was 6.9 in Denmark but 12.6 in Sweden, even though the populations of these two neighboring countries are assumed to share a similar distribution of HLA genotypes (51, 52).

CD has been reported to be associated with a number of autoimmune endocrine disorders, chiefly T1D and thyroid disease (53). Similar to T1D, a prerequisite for developing CD is genetic susceptibility linked to certain HLA genotypes. In addition, CD patients must be exposed to dietary wheat gluten or related prolamins (storage proteins in wheat, barley, and rye) in order to remain immunoreactive. CD was first recognized 2,000 years ago by Areteaus, who described a chronic illness involving diarrhea and the passage of undigested or unconcocted food. It was not until 1888 that Samuel Gee observed a connection between malabsorption and diet in emaciated patients, a condition that he described as “the coeliac affection” (54). In the 1940s, the Dutch pediatrician Willem Dicke identified wheat as the causative factor of CD (55), and shortly thereafter gluten was isolated as the antigen responsible for triggering the inflammation in the small intestine (56).

Oral intestinal capsule biopsies were developed in the 1950s, which enabled visualization of the damage to the proximal jejunal mucosa caused by gluten. The characteristic histopathological features of the intestinal mucosa in a CD patient include increased numbers of intraepithelial lymphocytes (IEL), elongated crypts, and varying degrees of villous atrophy, and this picture was originally described by Marsh and subsequently modified to form the current histological criteria (57, 58).

There are several markers of CD (59), among which anti-gliadin antibodies (AGA), reticulín autoantibodies (60, 61), and endomysial autoantibodies (EMA) (62) have long been applied as diagnostic tools in the clinical setting.

Identification of tissue transglutaminase (tTG) as the main endomysial autoantigen in CD (63) enabled development of immunoassays for anti-tTG antibodies with nearly 100% diagnostic sensitivity and specificity (64). Immunoassays that exploit the known tTG-modifying effect of gliadin peptides and detect antibodies against deamidated gliadin peptides (DGP) have also proven useful as serological markers of CD (65-67).

Depending on the clinical presentation and tTG autoantibody levels, the current diagnostic criteria for CD are based on both serology and intestinal biopsy. In clearly symptomatic children, observation of anti-tTG antibody levels exceeding 10 times the upper normal limit confirmed by EMA and HLA, along with a clinical response to a gluten-free diet (GFD), is sufficient for diagnosis (68, 69). In other patients with ambiguous symptoms, the recommendation is to perform an intestinal biopsy to confirm villous atrophy (68, 69).

The clinical spectrum of CD is broad and includes classical presentation of malabsorption with diarrhea, nonclassical extraintestinal features, subclinical or asymptomatic forms, and potential disease characterized by positive serology with normal intestinal mucosa on biopsy (70-72). Due to this diversity of symptoms, there is a risk of the diagnosis being delayed or missed (73-75). Nevertheless, the increased awareness that the broad clinical spectrum of CD can include extraintestinal manifestations is probably one of the reasons why this disease is now diagnosed more often in the elderly (76-78).

Overlap of CD and T1D

In children, the prevalence of T1D with biopsy-confirmed CD varies from 2.4% in Finland to 16.4% in Algeria (79), and diagnosis of CD is frequently preceded by T1D (80, 81). The age of T1D onset is usually lower in patients that also develop CD than in those without such comorbidity (82, 83). Also, coexistence of these two diseases is even more common in patients with longer duration of T1D (84, 85). Several investigations have addressed the question of whether the T1D- or the CD-associated autoantibodies appear first, but the results have been ambiguous. The BabyDiab study (86) suggested that CD-associated autoantibodies invariably develop later than T1D-associated autoantibodies. In contrast, the DIPP study (87) demonstrated that children with HLA-conferred susceptibility to T1D developed tTG autoantibodies at a younger or the same age as they developed T1D-associated autoantibodies.

CD and T1D share a number of risk factors that can be attributed to common genes, environmental exposures, and immune dysregulation (Fig. 1). The strong-

est association between T1D and CD entails the shared risks of having HLA-DR and DQ genotypes (see the section on HLA below), but non-HLA genes have also been discovered. Recent genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) associated with either T1D or CD, and several loci were found to overlap between the two diseases, including IL18RAP, CCR5, CTL4, IL2, TAGAP, and PRKCQ, which are involved in the immune system (88).

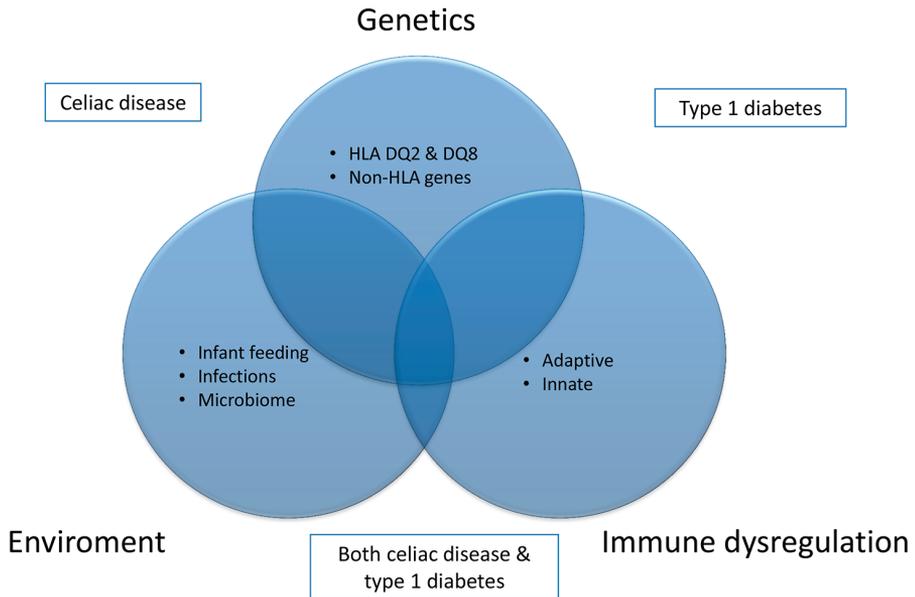


Figure 1. Risk factors associated with type 1 diabetes and celiac disease.

In the clinical setting, the majority of patients with T1D that also have CD present with mild or no symptoms (89-91), and only less than 10% of those individuals are expected to have any evident gastrointestinal symptoms (92). Instead, in many children T1D and CD, the conditions can be accompanied by growth failure and delayed puberty (84, 93, 94), and therefore screening by serological testing is performed on a regular basis at many care centers treating T1D patients (69, 95). Many T1D patients with CD are virtually asymptomatic, and thus compliance with a strict GFD can be challenging and accordingly often poor (96).

HLA in T1D and CD

The HLA genes are the human equivalent of the major histability complex (MHC) containing over 200 genes located on the short arm of chromosome 6 (6p21.3) (97). The HLA genes encode for MHC class II molecules that are found on the surface of antigen-presenting cells and are central for activation of cells in the immune system.

There is strong linkage disequilibrium between the neighboring alleles in the HLA complex, that is, specific DQ and DR alleles are non-randomly associated with each other and form haplotypes (98). In T1D, the high-risk genotype is DR3–DQ2.5 in *cis* position (DRB1*03-DQA1*05:01-DQB1*02:01)/DR4–DQ8 (DRB1*04-DQA1*03-DQB1*03:02) (*cis* is DQA1*05:01 and DQB1*02:01 on the same chromosome), which is found in 50% of the patients (99, 100). In CD, 90% of patients have one copy of the DR3–DQ2.5 *cis* haplotype, and the remaining 10% carry either DR4–DQ8 or the DQ2.5 risk alleles, although in *trans* position in the genotype DR7–DQ2.2 (DR7-DQA1*02:01-DQB1*02:02) / DR5–DQ3.5 (DR5-DQA1*05:01 DQB1*03:01) (101). Moreover, there is a gene–dose effect of DR3–DQ2 (101-104) in which DR3–DQ2 homozygosity confers the highest risk of CD (105). However, carrying one of these haplotypes is necessary, albeit not sufficient, for T1D and CD, which suggests that other factors are required for development of either of these disorders.

Wheat and gluten

The history of the human race dates back approximately 2.5 million years, but wheat was not domesticated until much more recently (i.e., about 10,000 years ago) in the Fertile Crescent (southwestern Asia) (106). Farming is the most radical event in history, no other process has had such an impact on the development of humankind (107). Before the emergence of agriculture, humans were gatherers and hunters, and did not store food or stay in one place for long, and the native diet consisted primarily of fruits, vegetables, and meat, with little exposure to grain (108). Introduction of farming led to a surplus of food, which facilitated population growth and cleared the way for new forms of social organization (109). More than 25,000 accessions of wheat have been domesticated since this plant was first cultivated (70, 110, 111). However, only during the last 500 years has there been a rise in the gluten content in food containing wheat, and breeding over past centuries has led to an increase in gluten that has enhanced crop yield (110, 112). The global consumption has increased more rapidly for wheat

compared to all other cereals, and today wheat is one of the most important food sources in the world, contributing 50% of the calories consumed in industrialized and developing countries (113).

The protein content of modern wheat varies between 7% and 22% (114). The word *gluten* actually refers to the entire protein component of wheat, although this term often also encompasses the prolamin storage proteins found in wheat (gliadin and glutenin), rye (hordeins), and barley (secalins). These prolamins differ from those detected in other cereals in two ways: first, they are present in larger amounts and have a higher molecular mass; second, they contain greater proportions of proline and glutamine, and the presence of these extremely hydrophobic repeated sequences render the prolamins insoluble in water (114, 115).

The prolamins of wheat are classically divided into two groups: the monomeric gliadins and the polymeric glutenins. The former prolamins are further classified into three groups of peptides called α/β -gliadins, γ -gliadins, and ω -gliadins (116). There are at least 50 gliadin epitopes that exert damaging effect in CD, since they are resistant to gastrointestinal enzymes (117), they have amino acid sequences that are specific to HLA:DQ2, they have preferred glutamine residues for tTg-mediated deamidation (118), and they can affect intestinal permeability and exert immunomodulatory activities (119, 120). Different types of wheat contain varying amounts of gliadin and glutenin components, and the toxicity of the individual components has not yet been determined. Indeed, it is plausible that each gluten protein has a unique toxicity profile and distinct T cell stimulatory sequences (110). It has been reported that some immunomodulatory gliadin peptides activate specific T cells, whereas others are able to induce a pro-inflammatory innate immune response (120). Gliadins have been shown to stimulate several constituents of the adaptive immune system, such as regulatory T cells, Th17 cells, and dendritic cells (121). Moreover, gliadin fragments have been shown to stimulate Toll-like receptor 4 (TLR4) and activate innate cells such as monocytes and macrophages (122), and also to regulate crosstalk between natural killer (NK) cells and dendritic cells by stabilizing HLA-E on the cell surface (123).

The potential spectrum of conditions referred to as gluten-related disorders has been expanded beyond the well-recognized and well-defined CD and dermatitis herpetiformis, and now includes other disorders with etiologies that have been associated with gluten. Non-celiac gluten sensitivity is a recently identified pathology that is defined as the onset of a variety of manifestations related to ingestion of wheat, rye, and barley in patients in whom CD and wheat allergy have been excluded (70, 124). This condition shares characteristics with both CD and irritable bowel syndrome (IBS), but it does not meet the diagnostic criteria for either of those disorders (125). The diagnosis of non-celiac gluten sensitivity is

based on the presence of a symptomatic reaction to gluten with a negative serology, negative immune-allergy test, normal duodenal biopsy, and resolution of symptoms when following a GFD (70).

Wheat allergy is an IgE-mediated reaction to wheat that involves cross-linking of IgE by repeat sequences in gluten peptides that triggers the release of chemical mediators such as histamine from basophils and mast cells. Affected individuals are usually sensitized during infancy, and most children outgrow wheat allergy, with rates of resolution of 29% by 4 years of age and 65% by 12 years of age. (126). The prevalence of wheat allergy varies between countries but has increased in recent years, similar to what is seen for all food allergies (127). The reactions typically occur within one hour of ingesting wheat and include cutaneous, gastrointestinal, and respiratory symptoms.

Baker's asthma is the main form of occupational asthma in many countries, and it is caused by another type of adverse reaction to wheat protein in which the afflicted person is sensitized to α -amylase but not gliadins (128); more precisely, this entails an IgE-mediated reaction to inhaled flour (129). Wheat-dependent exercise-induced anaphylaxis (WDEIA) also involves an IgE-mediated reaction to wheat and encompasses a wider range of clinical presentations compared to other allergic disorders, and patients with WDEIA are sensitized to Ω -5-gliadin (130). Wheat can also cause food protein-induced enteropathy (FPIES), which can emerge between the ages of 0 and 24 months, although usually within the first few months of life. FPIES presents as diarrhea in most patients, with mild to moderate steatorrhea in about 80% of cases, and failure to thrive is also common (131, 132).

Gut immunity

The disproportionate prevalence of autoimmune diseases in developed countries was the original basis for what is known as the “hygiene hypothesis”, which suggests that decreased exposure to both pathogenic and symbiotic microorganisms in childhood alters natural development of the immune system and predisposes to the loss of self-tolerance (133). There is now an accumulating body of evidence indicating that the commensal microbiota actively regulates the host immune system (134). The immune system of vertebrates is divided into two principal categories: one called innate immunity, which is evolutionally conserved and is the first line of defense against invading microorganism; and the other referred to as adaptive immunity, which responds to the infections in a time-delayed but antigen-specific fashion.

When operating optimally, the alliance between the host immune system and the resident microbiota interweaves the innate and adaptive arms of immunity in a dialogue that selects, calibrates, and terminates responses in the most appropriate manner. The gastrointestinal immune system faces unique challenges relative to other organs, because it must continuously confront an enormous microbial load. The tissues of the gastrointestinal tract are rich in myeloid and lymphoid cells, many of which reside in organized lymphoid tissues. It has long been recognized that the gut microbiota play a critical role in the development of organized lymphoid structures and in the function of cells of the immune system (135).

An important component of mature intestinal immune homeostasis is the development of oral tolerance to benign commensal bacteria and harmless antigens (136). This process is influenced by various factors during neonatal development and is achieved by complete colonization of the gut during the newborn period (137, 138). One of the major causes of onset and modulation of the newborn gut microbiota is the mode of delivery. Babies born by natural delivery develop microbial communities similar to their mothers' vaginal microbiota, whereas those born by Caesarean section develop microbial communities similar to their mothers' skin microbiota (139). The pioneer bacteria can originate from both vaginal and fecal microbiota through cross-contamination during birth, the mammary glands during breast-feeding, the mother's skin, the mouth, and the environment. Colostrum and breast milk contain live microbes, metabolites, IgA, and immune cells, as well as cytokines (140). Thus, in addition to host genotype, physiological conditions, and medical practices, it is clear that the mode of delivery, gestational age, and the mode of feeding have a profound effect on development of the microbiota (141-143).

Research using germ-free mice led to the discovery that the gut microbiota are required for normal generation and/or maturation of gut-associated lymphoid tissues (GALTs). These tissues are immune structures in which antigens can be taken up and presented by antigen-presenting cells, and hence they are essential for lymphocyte functions that lead to inflammation or tolerance. The microbiota also have an important impact on development of secondary lymphoid structure, as exemplified by the observation that germ-free mice are characterized by smaller Peyer's patches and reduced numbers of CD4+ T cells and IgA-producing plasma cells (144). In the intestine, tertiary lymphoid structures such as isolated lymphoid follicles or cryptopatches are induced after birth as a result of commensal exposure (145).

It is also well known that the resident microbiota regulate the development of specific lymphocyte subsets in the gut. There are three main types of lymphocytes: T cells, B cells, and NK cells. Furthermore, there are three major classes of T cells called cytotoxic T cells, T helper (Th) cells, and regulatory T cells

(Tregs), which play specific roles in the destruction of antigens. Th cells are also assigned to three different subsets (denoted Th1, Th2, and Th17) based on their cytokine secretion profiles.

Homeostasis in the gut mucosa is maintained by a system of checks and balances between potentially proinflammatory cells, which include the following: Th1 cells that produce interferon- γ ; Th17 cells that generate IL-17a, IL-17f, and IL-22; diverse innate lymphoid cells with cytokine effector features resembling those of Th2 and Th17 cells; and anti-inflammatory Tregs. Th17 cells are a specific lineage of CD4⁺ Th cells that are crucial for host defense and participate in the development of autoimmune disease by producing proinflammatory cytokines (146). Th17 cells preferably accumulate in the intestine, which suggests that development of these cells is regulated by gut-intrinsic mechanisms. This assumption is supported by studies in which the presence of intestinal Th17 cells was greatly reduced in antibiotic-treated or germ-free mice, thus demonstrating a pivotal function of the microbiota in development of Th17 cells (147-150).

The T cells designated Tregs comprise a subset of CD4⁺ T cells that also accumulate in the intestine, where they help to maintain gut homeostasis. Tregs are primary controllers of immune responsiveness and peripheral immunological tolerance (151), and they also regulate several organ-specific autoimmune diseases such as T1D (152). Notably, it has even been observed that establishment of tolerance and active suppression of inflammatory responses to food and other orally ingested antigens could not be induced in the absence of signals from the gut flora (153). To some extent, the development of peripherally derived Tregs also depends on the gut microbiota, as indicated by a study showing a pronounced decrease in the number of Tregs in the colonic lamina propria of germ-free mice (154). In addition to the direct influence of the microbiota on the immune machinery associated with induction of oral tolerance, commensal-specific Tregs can promote class switching to IgA in an antigen-specific manner and thereby control the host relationship with the microbiota via multiple mechanisms (155). For example, commensal gut microflora DNA has been found to limit conversion of Tregs elicited by their interaction with Toll-like receptor 9 (TRL9) (156). Therefore, an important function of the microbiota is associated with the capacity of these microorganisms to condition cells to respond to infectious challenge both systemically and locally. For instance, commensals can tune innate cells in a way that allows them to rapidly respond to pathogen encounters (138).

Another central player in the innate immune system is the intestinal epithelial cell (IEC). The IECs contain tight junctions that normally regulate passage of nutrients and inhibit translocation of pathogenic organisms. An intact intestinal epithelium is necessary to control transport of antigens to dendritic cells, and increased gut permeability caused by damage to the epithelium results in

exposure to antigens that can potentially trigger an autoimmune response in predisposed individuals. IECs also interact directly with immune cells and produce and respond to a variety of cytokine stimuli. In animal models, alterations of the intestinal flora give rise to many changes in IEC development and function. For instance, it has been observed that turnover of IECs occurred at a slower rate in germ-free mice than in mice with normal intestinal microflora (157).

Toll-like receptors (TLRs) are evolutionarily conserved innate receptors that are expressed in various immune and non-immune cells in mammals. TLRs interact with components of Gram-positive and Gram-negative bacteria to mediate both innate and adaptive immunities, as well as other functions of mucosal barrier cells (158). Together with other receptors, the TLRs constitute a class of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) expressed by microorganisms or danger-associated molecular patterns (DAMPs) from damaged tissue. TLRs play a crucial role in defense against pathogenic microbial infection, because all PRRs recognize various PAMPs in various cell compartments and trigger the release of inflammatory cytokines and type 1 interferons for host defense (159, 160).

The immune system is controlled not only by its symbiotic relationship with the microbiota, but also through its exquisite sensitivity to the nutritional status of the host. In conclusion, there is considerable evidence that multidirectional interaction occurs between the diet, the immune system, and commensal microflora (138).

Role of the gut in T1D

Much of the knowledge regarding the role of the intestinal microbiome in the development of T1D has been obtained in studies using diabetes-prone and germ-free animals. The results of that work, combined with epidemiological data from humans, have enabled us to begin to sort out the many aspects of the intestinal microbiome that directly affect the risk of T1D and development of this disease.

An immunological connection between the pancreas and the intestine has been demonstrated in animal models. For example, it has been shown that T cells activated in the gastrointestinal tract home into the pancreatic islets that express mucosal homing receptor MadCAM-1 (161). It has also been reported that orally administered ovalbumin induces proliferation of T cells not only in the mesenteric lymph nodes, but also in the pancreatic lymph nodes (PLNs) (162), suggesting that dietary and microbe-derived antigens can act as stimuli for the local immune cells in the pancreas.

In other research, incidence of spontaneous T1D in non-obese diabetic (NOD) mice was found to be ameliorated in the absence of MYD88, an adaptor protein used by multiple TLRs, although protection against diabetes in MYD88-negative NOD mice was retracted by antibiotic treatment and under germ-free conditions (163). Furthermore, in some experiments antibiotics against Gram-positive bacteria prevented autoimmune diabetes in bio-breeding (BB) T1D-prone rats (164), and feeding heat-killed *Lactobacillus casei* fed to NOD mice was also observed to prevent diabetes (165). In addition, differences in gut microbiota between T1D-prone and diabetes-resistant BB rats before the diagnosis of T1D have been reported (166). These data indicate that commensal microflora are involved in preventing T1D.

Several studies have pointed to the role of dysregulation of the intestinal barrier. Function of this barrier is compromised by changes in intestinal permeability, which in turn facilitates access of infectious agents and dietary antigens to mucosal immune elements and can eventually lead to immune reactions that damage the pancreatic β cells (167). Increased gut permeability has been demonstrated in both animal models and humans with T1D (168).

NK cells in T1D

NK cells are lymphocytes that exert strong defense mechanisms against microorganisms and they can also efficiently limit neoplastic cell growth. Further on they have also been shown to participate in autoimmunity (169). NK cells can secrete cytokines and chemokines and have the ability of killing other cells without any prior stimulation. NK cell activation results from the balance of signals produced by activating and inhibitory receptors (170). NK cells exert sophisticated biological functions that are attributes of both innate and adaptive immunity, thereby blurring the functional borders between these two arms of the immune response (171). In T1D, NK cells have been found to infiltrate the pancreatic islets at onset of the disease in humans and murines (172, 173), although the precise activities of these cells in this context are still a matter of controversy.

NKG2D and natural cytotoxicity receptors (NCRs) are the main types of activating receptors on NK cells. The NCRs (NKp30, NKp44, NKp46, and NKp80) are expressed exclusively by NK cells and belong to the superfamily of immunoglobulins (174). NKG2D is expressed on human and mouse NK cells, as well as on cytotoxic cells (e.g., CD8+ and $\gamma\delta$ T cells), and it detects stress-induced MHC class I-like molecules that are aberrantly expressed during viral infection and tumor transformation (175).

Studies have suggested that NK cell ligands recognized by NKG2D and NKp46 are expressed on the pancreatic β cells of NOD mice during development of T1D and on β cells from T1D patients, and therefore these molecules might play a key role in the destruction of pancreatic β cells achieved by NK cells (176, 177). Moreover, an investigation using NOD mice showed that immunization with NKp46-Ig and NCR1-Ig fusion proteins significantly reduced T1D without depleting the NK cell population (176). Furthermore, compared to NK cells in the PLNs or spleen, NK cells within an insulinitis lesion display an activated phenotype that expresses higher levels of KIRG1 (killer cell lectin-like receptor group 1), PD-1 (programmed cell death 1), and IL-2R (IL-2 receptor; CD25), and CD69, and this is correlated with increased β cell destruction. Activated NK cells in an insulinitis lesion express CD107a, which is a marker of granule exocytosis, and thus they may directly induce apoptosis in β cells by causing perforin- and granzyme-mediated cytotoxic damage (178).

Interestingly, impaired NK cell function has been observed in the blood of T1D patients and in lymphoid tissues of NOD mice (175, 179, 180). Also, studies have indicated that a GFD may prevent T1D by generally reducing the number of NKp46+ NK cells and cellular proliferation, and down-regulating the activating receptor NKG2D on NK cells and thereby rendering them less autoreactive (181). These findings are supported by data showing that, compared to healthy subject, children with T1D have reduced numbers of NK cells, aberrant NKG2D signaling, and lower NK cell activity (182).

Gluten and T1D in animal models

Dietary exposures affect the functional diversity of the gut microbiome by altering the proportions of various members of the microbial community. A study of fecal DNA from 18 human subjects and 33 mammalian species showed that differences in the structure and function of the intestinal microbiome were influenced by whether the host was a herbivore or a carnivore (183).

The mechanisms by which early gluten exposure affects the immune system are not fully understood, although animal models have suggested that dietary gluten can modulate the incidence of T1D by changing the gut microbiome (184). It has also been observed that NOD mice that were not exposed to gluten seemed to develop T1D later than such animals fed a standard diet (185-187), and dietary gluten had a significant negative quantitative impact on the generation of Tregs in both NOD and standard BALB/C mice, findings that are implying that gluten may be an important factor in T1D (188). Enhanced T cell reactivity to

wheat-derived polypeptides has also been demonstrated, and this response was characterized by secretion of INF-g and IL-17A (189). Furthermore, it appears that dietary gluten increases murine NK cell activity against pancreatic β cells, as indicated by a recent study showing that a gluten-containing diet increased NK cell activation *in vivo* in BALB/c and NOD mice (181).

Further investigations in humans are needed to evaluate the concept that dietary gluten also induces intestinal inflammation and T cell activation, effects that might contribute to development of islet autoimmunity by what are known as bystander mechanisms. However, some data have been published indicating that gliadin stimulation of small intestinal biopsies *in vitro* can induce an unbalanced immune response in T1D patients (190).

Aims

The overall aim for this thesis was to study the association between T1D and CD, and to elucidate the effects of dietary gluten on the risk of developing T1D. More specific aims were as follows:

- I To estimate the prevalence and levels of CD-associated autoantibodies in children with T1D (Study I).
- II To explore the association between T1D and CD-related autoantibodies and non-HLA genetic risk markers during the first year after diagnosis of T1D (Study II).
- III To investigate the effect of gluten on the risk of developing T1D (Study III).
- IV To evaluate the influence of perinatal factors on the risk of developing both T1D and CD (Study IV).

Subjects

Study I

In Study I, serum samples were collected at the time of T1D diagnosis from the following subjects: 662 children in Sweden with a median age of 10.2 years and diagnosed with T1D between 1995 and 2006 (originally included in the “Skåne study”) (13); 1,080 children in Denmark with a median age of 10.3 years and diagnosed with T1D between 1996 and 2006 (identified in the Danish Diabetic Register). As controls, we analyzed serum samples from 309 and 283 healthy school children in Sweden (median age 12.6 years) and Denmark (median age 15.6 years), respectively.

Study II

In Study II, serum samples obtained from 261 children attending 18 pediatric departments at hospitals in 15 countries in Europe and in Japan between 1999 and 2000 were provided by the Hvidoere Study Group on Childhood Diabetes. The sera were collected 1, 6, and 12 months after diagnosis of T1D. The median age of the subjects 1 month after diagnosis of T1D was 9.6 years.

Study III

Both BALB/c and NOD mice were used in Study III (Fig. 2), and they were purchased at an age of 4 weeks from Taconic Europe A/S and Taconic US, respectively. Upon arrival at our laboratory, the animals in each strain were divided equally into two groups, one of which was fed a GFD and the other a standard diet (STD). We also used additional NOD mice, which we bought as breeding pairs (Taconic) and divided into two groups that were fed either a strict GFD

(SGFD) or a strict STD (SSTD) during breeding in our laboratory to ensure that the pups were exposed to the same diet *in utero* and during and after weaning. The mice were kept in a specific pathogen-free (SPF) animal facility (temperature $22 \pm 2^\circ\text{C}$, 12-h light cycle, air changed 16 times per hour, humidity $55 \pm 10\%$) with free access to water and food.

Study IV

In study IV, 768,395 singletons were identified in the Swedish Medical Birth Registry (SMBR) (Fig. 3). Among those, 4,327 had T1D, 3,817 had CD, and 191 had both disorders according to the Swedish National Inpatient Registry.

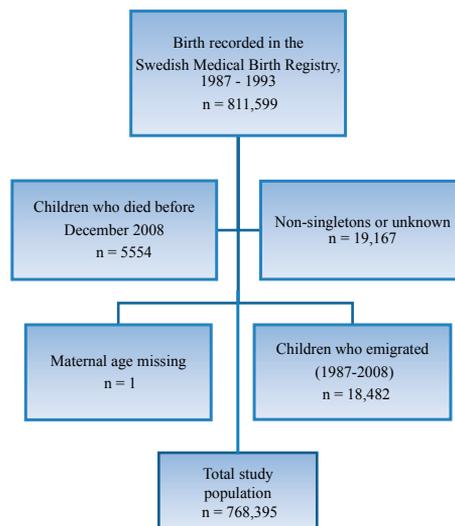


Figure 2. Flow chart presenting the children included in Study IV.

Methods

Studies I and II

Combined deamidated gliadin peptide (DGP) and tissue transglutaminase (tTG) antibody assay

In Studies I and II, we analyzed conjugated IgA and IgG (IgAG) against DGP/tTG using a QUANTA Lite™ h-tTG/DGP Screen ELISA kit (INOVA Diagnostics, San Diego, CA, USA) according to the instructions of the manufacturer. Briefly, ELISA plates coated with DGP and purified human erythrocyte tTG (htTG) were incubated with diluted patient serum samples. Antibodies bound to the ELISA wells were detected with horseradish peroxidase-labeled anti-human IgAG. Antibody levels were calculated from the optical density of each sample in relation to the reactivity of a positive control and were expressed in arbitrary units (AU). Cut-off limits were defined as negative at < 20 AU and positive at 20 AU.

Tissue transglutaminase autoantibody radioligand binding assay

In Studies I and II, an in-house radioligand binding assay (RBA) was used to detect tTG antibodies (191). In short, human tTG was synthesized by *in vitro* transcription and translation as described by Grubin *et al.* (42). Protein A Sepharose (PAS) (Zymed Laboratories, Inc., San Francisco, CA, USA) was used to separate free and antibody-bound 35S-tTG. PAS detects all subclasses of IgG except IgG3 and also some forms of IgA and IgM. The relative amount of tTG antibodies was expressed as U/mL. In Study I, a cut-off level < 7 U/mL was used as the upper normal limit representing the 92nd percentile of healthy controls. In Study II, the cut-off was set at < 4 U/mL representing the 95th percentile (192).

Genotyping

In Study II, genotyping of rs917997 (IL18rap), rs333 (CCR5), rs45450798 (PTPN2), and rs2816316 (RGS1) was done at KBioscience in the United Kingdom using an in-house KASPar system.

HLA genotyping

In Study I, HLA-DQB1 and HLA-DQA1 alleles were determined using sequence-specific oligonucleotide probes and a DELFIA hybridization assay (Perkin Elmer, Boston, MA, USA) as described elsewhere (23), or by direct sequencing of exon 2 of DRB1 according to the Immuno Histocompatibility Working Group. In Study II, genotyping of the HLA-class DRB1 locus was achieved by direct sequencing of exon 2 of DRB1 according to the Immuno Histocompatibility Working Group, and we also used previously analyzed and available data from the Hvidøre group on glycated hemoglobin (HbA1c), C-peptide, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide (GLP-1), and glucagon.

Outcome definitions

In all studies, T1D was defined as one of the following: fasting plasma glucose ≥ 7.0 mmol/L, 2-hour post-oral glucose tolerance test ≥ 11.1 mmol/L, or random plasma glucose concentrations ≥ 11.1 mmol/L plus symptoms of diabetes mellitus (193). Celiac disease autoimmunity (CDA) was defined as being positive for both IgAG-DGP/tTG and IgG-tTG in Study I, and as being positive for both IgAG-DGP/tTG and IgG-tTG detected in two consecutive blood samples in Study II. Individuals who seroconverted from antibody positive to antibody negative during the two follow-up visits were considered to be CDA transient.

Study III

The experimental mice received either a STD; (non-purified Altromin) or a GFD (modified Altromin) that had previously been found to prevent development of diabetes in NOD mice (185). Both the diets were nutritionally adequate with similar levels of protein, amino acids, minerals, vitamins, and trace elements. The diets were prepared to ensure the same content of milk and soya proteins known to be diabetogenic.

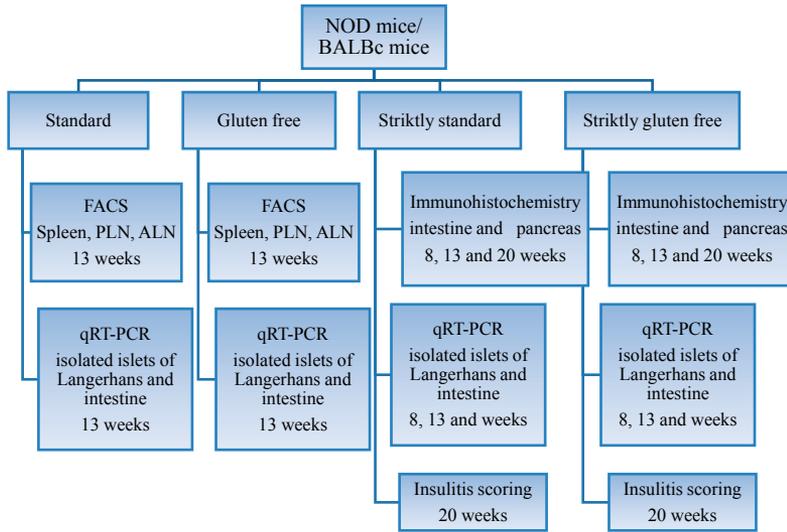


Figure 3. Flow chart describing the animals used in Study III.

Cell purification and flow cytometry (FACS)

Thirteen-week-old BALB/c and NOD mice on the GFD or STD were sacrificed, and the spleen (S), pancreatic lymph nodes (PLNs), and auricular lymph nodes (ALNs) were isolated. ALNs were chosen as control lymphoid organs to ascertain whether the effect of gluten is rigidly confined to PLNs, or if gluten also has an effect on systemic immunity, as had been reported by other investigators (194). Cells from each organ were pooled, and single-cell suspensions were prepared. Surface staining with the relevant mAb was initiated, and the cells were incubated for 30 min. Fc block (CD16/CD32) was purchased from BD Pharmingen (2-4G2; IgG2b, κ) and added to reduce Fc receptor-mediated binding. The cells were fixed and subsequently analyzed using a LSR-II flow cytometer (BD Bioscience, San Jose, CA, USA). Isotype control antibodies were used to determine the amount of non-specific binding, and an AmCyan-conjugated LIVE/DEAD fixable aqua dead cell staining kit from Invitrogen (Carlsbad, CA, USA) (catalogue no. L34957) was used to exclude dead cells.

FACS antibodies

The monoclonal antibodies (mAbs) allophycocyanin (APC)-H7-conjugated rat anti-mouse CD8αAb [immunoglobulin (Ig)G2a, κ; catalogue number:560182] and fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD71 (IgG11, κ; catalogue no. 553266) were purchased from BD Pharmingen (San Jose, CA, USA). The mAbs phycoerythrin (PE)-conjugated rat anti-mouse CD49b (DX5) (IgM, κ; catalogue number: 48597182), PE-cyanin 7 (Cy7)-conjugated rat anti-mouse CD314 (NKG2D), and xxx (IgG21, κ; catalogue no. 25588282) were obtained from eBioscience (San Diego, CA, USA).

Islet isolation

Islets of Langerhans were isolated from single BALB/c or NOD mice. Briefly, collagenase (754 U/ml; Sigma-Aldrich, St. Louis, MO, USA) in RPMI-1640 was injected into the pancreatic duct of an animal, after which the pancreas was removed and subjected to further collagenase digestion [40]. Islets were manually selected to obtain 90–95% pure islet isolates, as estimated by visual inspection. Isolated islets were immediately placed in Trizol (Invitrogen) for subsequent RNA extraction.

RNA isolation

Intestinal sections were kept in RNA later until they were subjected to RNA extraction, before which they were transferred to Trizol and homogenized mechanically using a Polytron (Kinetica, Lucerne, Switzerland). Isolated pancreatic islets were not homogenized prior to RNA extraction. Total RNA was isolated from islets or intestinal sections using Trizol reagent (Invitrogen), and the yield and quality of the RNA were assessed on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

qRT-PCR

RNA was extracted from tissues for qPCR analyses: samples of duodenum and isolated islets of Langerhans from both NOD mice and BALB/C mice at different age (Fig. 3).

Approximately 1.0 μg of total RNA was reverse-transcribed into cDNA using a qScript kit (Quanta Biosciences, Gaithersburg, MD, USA) according to the recommendations of the manufacturer.

Specific mRNA levels were quantified on a Lightcycler II instrument (Roche, Penzberg, Germany) using SYBR II qPCR mixture (Takara Bio, Otsu, Japan). Primers were designed for an annealing temperature of 61 °C using Primer3 software [41] and were synthesized by TAGCopenhagen (Copenhagen, Denmark). Diluted, purified, and sequence-verified (GATC Biotech, Constance, Germany) PCR products were used to create a standard curve for each primer pair. Expression levels were calculated as absolute quantification in relation to the relevant standard curve using Lightcycler software version 4.05, and the expression values were normalized to the housekeeping gene beta actin. Cycling parameters were set to obtain similar reaction efficiencies of 1.9–2.0.

The parameters for qPCR cycling were initial denaturation at 95 °C, followed by 45 cycles of 10 s denaturation at 95 °C, 5 s annealing at primer-specific temperature (56–60 °C), and 15 s extension at 72 °C. After PCR, a melting curve analysis was conducted. Any reactions with a CP value > 40 or a nonspecific peak in that analysis were treated as negative reactions and given the value '0' for statistical analysis.

Histology and insulinitis scoring

Twenty-week-old NOD mice fed the SSTD or SGFD were used to determine diet-induced differences in lymphocyte infiltration (insulinitis scoring). Hematoxylin and eosin-stained pancreas sections were evaluated to determine the insulinitis score using the following scale: 1 = intact islets, 2 = peri-insulinitis, 3 = moderate insulinitis (50% of the islets infiltrated), and 4 = severe insulinitis (> 50% of the islets infiltrated). At least 20–25 islets from each mouse were scored blind.

Immunohistology

Immunohistochemical staining was performed on snap-frozen sections of small intestine. Tissues were fixed in Stefanini, rinsed in phosphate-buffered saline (PBS), and incubated first with 2.5% bovine serum albumin (BSA) in PBS for 1 h, and thereafter with primary anti-NKG2D (Santa Cruz NKG2D s-c5494; Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:50 and anti-CD3 (ab16669; Abcam, Cambridge, UK) 1:100 in 1% BSA for 2 h. Binding was visualized using donkey anti-goat Cy3 (705-166-147; Jackson ImmunoResearch, West Grove, PA, USA) (1:200) and donkey anti-rabbit Alexa Fluor 488 (711-546-152; Jackson ImmunoResearch) (1:200). Vectashield 4', 6-diamidino-2-phenylindole (DAPI) was used to visualize nuclei.

Study IV

In Study IV, all singletons born in Sweden between 1987 and 1993 were identified in the Swedish Medical Birth Registry established in 1973 to collect medical information on almost all (98%) of children born in Sweden (195), and is supervised by the Swedish National Board of Health and Welfare. Cases of T1D and CD were identified in the Swedish National Inpatient Registry, which compiles information about hospitalizations including discharge diagnosis.

Information on income and social allowance was obtained for each subject from the Income and Taxation Registry, which is run by Statistics Sweden. Also, data on location where the mother were born and length of residence in Sweden (when applicable) were acquired from the total population register (Statistics Sweden). The registries containing data on emigration and national mortality were used to identify subjects who emigrated or died before 14 years of age.

Linkage between the different registries was carried out by Statistics Sweden and the National Board of Health and Welfare using a unique personal identification number given to each child and parent residing in Sweden. After the linkage was performed, Statistic Sweden substituted the personal identification numbers with encrypted numbers to ensure the anonymity of the subjects before supplying us with the database.

Assessment of variables

Cases of CD and T1D were defined according to the International Classification of Diseases (ICD) 9th and 10th editions, with 579.0 (ICD 9) or K 90.0 (ICD10) for CD, and 250 (ICD 9), E14 (ICD 10), or E10 (ICD10) for T1D. The cases were then categorized into the following groups: neither CD nor T1D, only CD, only T1D, and both CD and T1D. Category neither CD nor T1D was used as the reference.

Maternal age at the time of birth was categorized as < 20, 20–24, 25–29, 30–34, 35–39, or > 39 years, and the category < 20 years was used as reference in the analyses.

Children were identified as small for gestational age (SGA) when the birth weight (grams) was two standard deviations (SD) below the expected birth weight for the gestational age (days). The expected birth weight was calculated using a gender-specific formula previously derived from intrauterine ultrasound measurements of children born at term without complications (196). Children not born SGA were used as reference in the analyses. The season of birth was categorized as fall/winter (September–February) or spring/summer (March–August), using

the former as reference category. Children delivered by Caesarean section were compared with those that were delivered vaginally.

The mothers were classified according to their smoking habits during early pregnancy, and children born to mothers who had not smoked during pregnancy were compared with those born to mothers who had smoked. Information on smoking habits was obtained from the Medical Birth Registry and had been gathered by a midwife at the first antenatal care visit (i.e., between weeks 6 and 12 of gestation).

Information about congenital malformations was retrieved from the Swedish Medical Birth Registry, in which a subset of the ICD codes for such morphological defects stipulated as ICD-9 codes 740 to 759 and ICD-10 codes Q00 to Q99 are considered to represent significant malformations. Children with a significant congenital malformation were compared with those without such a diagnosis.

Register data were used to define the mothers' education according to the highest level completed in 1990. This variable was categorized into four groups: elementary school or lower (9 years); lower secondary school (12 years total); higher education (>12 years); information not available. The category "higher education" was used as the reference group in the comparisons.

The Income and Assets Registry was used to ascertain whether the parents were receiving social welfare allowance the year before the birth of the child (yes vs. no), and also to acquire information on the income of the parents the year before and after their child was born. Social welfare allowance was defined as having received such a subsidy in any amount. This type of benefit is granted by the Swedish Social Service Agency to applicants that are deemed unable to provide for themselves. The income variables were categorized in groups by tertiles, adding a fourth category including missing information.

Statistical analysis

In all four studies, a p-value < 0.05 was considered significant.

In Study I, the chi-square test was used to evaluate differences in frequency between the cohorts. The Kruskal-Wallis and Dunn's multiple comparison tests were applied to assess differences in antibody levels. Correlations were evaluated using Spearman rank correlation (r). Data analysis was carried out using the Statistical Package for Social Sciences version 21.0 (SPSS INC, Chicago, IL, USA).

In Study II, we used a compound symmetric repeated measurement model to estimate the impact of different variables (visit, age, sex, seasonality, HLA, autoimmunity, and gender) on IgAG-DGP/tTG levels and the effect of IgAG-DGP/tTG levels on the variables C-peptide, HbA1c, GLP-1, GIP, and glucagon. Where appropriate, the outcome variable was log-transformed to meet the assumption of a normal distribution. All models were adjusted for visit, sex, age (linear), and HLA genotype. We used linear regression and the chi-square test to assess differences at the 12-month visit. Data analysis was carried out using the Statistical Analysis Software (SAS 9.2, Cary, NC, USA).

In Study III, pairwise comparison of groups was done using Student's *t*-test, and the X^2 method was employed for evaluation of the insulinitis score data. Unless specified otherwise, the groups were only compared pairwise: GFD *versus* STD and SGFD *versus* SSTD. Data analysis was carried out using the Statistical Analysis Software (SAS 9.2, Cary, NC, USA). Flow cytometry data were analyzed using FACS diva software (BD Bioscience).

In Study IV, multinomial logistic regression models were applied to estimate odds ratios (OR) with 95% confidence intervals (CIs) for having T1D, CD, or both diseases in relation to factors known to be associated with CD (i.e., maternal age, SGA, Cesarean delivery, maternal smoking, preterm birth, birth cohort, sex, season of birth, mother's country of birth, and congenital malformations). Data analysis was carried out using the Statistical Package for Social Sciences version 20.0 (SPSS Inc., Chicago, IL, USA).

Ethical aspects

The serum samples used in Study I originated from the Skåne Study, which was approved by the Human Research Ethics Committee of the Faculty of Medicine, Lund University, Lund, Sweden, and from the Danish Diabetes Registry with authorization from the Ethics Board in Copenhagen, Denmark. For Study II, the local ethics board in each of the 15 countries approved the use of samples from the Hvidøre study group. The animal experiments (Study III) were approved by the Animal Experiments Inspectorate in Denmark and were performed in accordance with international guidelines for the care and use of laboratory animals. Use of database information in Study IV was authorized by the Regional Ethics Review Board in southern Sweden.

Results and discussion

Prevalence of celiac disease autoimmunity in children with type 1 diabetes: regional variations across the Øresund strait between Denmark and southernmost Sweden (Study I)

In Study I, 17.2% and 11.7% of the children with T1D investigated in Sweden (group 1) and Denmark (Group 2), respectively, were positive for IgAG-DGP/tTG ($p = 0.001$), and the corresponding rate was 9.4% for control subjects in Sweden (Group 3; $p = 0.001$) and 5.7% for controls in Denmark (Group 4; $p = 0.003$). The frequency of IgG-tTG in Group 1 (6.3%) did not differ from that in Group 2 (4.6%) ($p = 0.149$), and the frequencies in those two groups were higher compared to Group 3 but not significantly higher compared to Group 4. In Group 3, 9.4% were IgAG-DGP/tTG positive compared with 5.7% in Group 4 ($p = 0.110$), whereas the opposite was true for IgG-tTG, that is, 0.3% compared with 2.8% ($p = 0.002$). In total, 6.0% of the children in Sweden with T1D were defined as having celiac disease autoimmunity (CDA) as compared to 4.5% of the children with T1D in Denmark ($p = 0.2$) (Table 1).

Among the children with T1D, those in Sweden had higher levels of both IgAG-DGP/tTG (median 12.0 [range 0–238] AU) and IgG-tTG (median 2.4 [range 1–8746] U/mL) compared to those in Denmark (medians 10 [range 0–208] AU [$p < 0.001$] and 1.5 [range 1–924] U/mL [$p < 0.0001$], respectively). Thus occurrence of CDA did not differ significantly between the Swedish and the Danish children with T1D, although both the frequency and levels of IgAG-DGP/tTG were higher in the former group. The higher antibody levels in the Swedish T1D subjects was not explained by differences in frequency of the HLA risk haplotypes DQ2 and DQ8, which suggests dissimilarities in exposure to environmental factors between the study populations.

IgAG-DGP/tTG has a lower diagnostic specificity for CD compared to conventional tTG autoantibodies (197), but it may nonetheless indicate non-celiac gluten sensitivity (NCGS) (198, 199). NCGS is a gluten-associated condition that is characterized by gastrointestinal or extraintestinal symptoms related to anti-gliadin antibody positivity (200) and also occurs in children with T1D (201).

The higher levels of IgAG-DGP/tTG may also reflect higher gluten consumption in Sweden compared to Denmark, because several national dietary surveys have established that food habits differ between these two neighboring countries. In Denmark, there is for example no tradition of feeding infants gruel (a follow-on formula containing flour) as there is in Sweden. This is reflected by results reported by Weile *et al.* (202) showing that in 1987 the estimated content of gliadin in the officially recommended diets for infants in the two countries differed substantially, with the diet in Sweden containing more than 40 times more gliadin than that in Denmark (4,400 mg vs. 100 mg) at the age of 8 months, and four times more (3,600 mg vs. 900 mg) at the age of 12 months. In turn, the infant diet in Denmark contained a larger amount of the lower-gluten-containing rye flour compared to the infant diet in Sweden.

The mentioned levels were noted during the high-incidence period of CD, in the years of 1984–1996 in Sweden, when gluten consumption in children younger than 12 months of age was extremely high. The children in our study were diagnosed with T1D between 1995 and 2006 at a median age of 10 years; hence most of them were born during this critical period. Since that time, the gluten content in the follow-on formulas has decreased in Sweden, but it is still traditional to wean babies onto gruel containing large amounts of gluten (203). In contrast, a recent study of children in Denmark showed that gluten is introduced slowly and in small amounts according to the national guidelines (204). Furthermore, there is also a long tradition of using rye as the main grain in bread in Denmark, whereas there is a high consumption of wheat in Sweden (205). Of these two grains, wheat contains the highest concentrations of gluten.

Recent surveys have also demonstrated that Sweden differs markedly from Denmark in regard to consumption of bread, seen as much greater intake of white bread (28.1 g/d in Sweden and 22.8 g/d in Denmark) and crisp bread (45.5 g/d in Sweden and 5.0 g/d in Denmark) than non-white bread (28.3 g/d in Sweden and 119.2 g/d in Denmark) (206). Several studies have suggested that rye bread and whole-grain bread have a number of health benefits over white bread (207). For example, hormonally active compounds in grains called lignans may protect against hormone-mediated diseases, and concentrated sources of lignans include rye meal, whole grain oats, and whole grain wheat (208). It is only during the past century that humans have consumed refined grain products. Before that time, grain was ground in grist mills, which did not completely separate the bran and germ from the white endosperm, and produced only limited amounts of purified flour (207). Studies have also shown that consumption of high-fiber rye bread, as compared with refined wheat bread, significantly increased the first phase of insulin secretion, which seemed to improve β cell function and reduce fasting insulin levels and 24-hour urinary C-peptide excretion (209, 210).

A most surprising and notable finding in Study I was also the high prevalence of CDA (nearly 3%) in the control group in Denmark, which consisted of presumably healthy schoolchildren. CD was previously considered to be uncommon in Denmark, and earlier studies had demonstrated very low prevalence of the condition in this country (211). However, more recent data suggest that the prevalence may be higher than previously noted in clinical investigations due to a supposedly large number of undiagnosed children and evidence suggesting a rise in prevalence (52). Our results also indicate that the prevalence of CD might be higher in Denmark than has been assumed. A survey based on questionnaires concerning CD-related symptoms revealed a prevalence of CD of 1.2% in Denmark (212), and an earlier study of blood donors suggested that the prevalence is much higher than is clinically detected (213). As some cases of CD present with no evident symptoms or only extraintestinal manifestations (214-217), screening of the general population is needed to explore the true prevalence of this condition in Denmark. This is further underlined by a recent investigation in Sweden in which symptoms were found to be equally common among screening-detected CD cases and non-CD children, and also that the frequency of screening-detected CD was similar in groups with and without any CD-related symptoms (218).

Table 1. Outcome of analysis of autoantibodies associated with celiac disease in children with type 1 diabetes in Sweden (Group 1) and Denmark (Group 2), and in healthy controls in Sweden (Group 3) and Denmark (Group 4).

	Group 1	Group 2	Group 3	Group 4	P-value
N	662	1080	309	283	
IgAG-DGP/tTG, n (%)	114 (17.2)	126 (11.7)	29 (9.4)	16 (5.7)	P# = 0.001 P'' = 0.4
IgG-tTG, n (%)	42 (6.3)	50 (4.6)	1 (0.3)	8 (2.8)	P# = 0.15 P'' = 0.02*
CDA ^a , n (%)	40 (6.0)	49 (4.5)	1 (0.3)	8 (2.8)	P# = 0.20 P'' = 0.02

Celiac disease autoimmunity = CDA.

Symbols: # indicates for comparisons between Group 1 and Group 2;'' denotes comparisons between Group 3 and Group 4. *Fischer's exact test.

Table 2. Distribution of HLA genotypes in children with type 1 diabetes in Sweden (Group 1) and Denmark (Group 2) found to be positive for IgAG-DGP/tTG or IgG-tTG, or for both antibodies (defined as celiac disease autoimmunity, CDA).

HLA haplotype	Swedish children with T1D				Danish children with T1D			
	All n (%)	IgAG-DGP/tTG positive n (%)	IgG-tTG positive n (%)	CDA n (%)	All n (%)	IgAG-DGP/tTG positive n (%)	IgG-tTG positive n (%)	CDA n (%)
DQ2/2	46 (7.0)	19 (16.7)	9 (21.4)	9 (22.5)	33 (3.6)	8 (7.6)	4 (9.8)	4 (10)
DQ2/X	64 (9.7)	11 (9.6)	7 (16.7)	7 (17.5)	129 (14.2)	23 (21.9)	9 (22.0)	9 (22.5)
DQ2/DQ8	216 (32.7)	42 (3.7)	14 (33.3)	14 (35)	349 (38.3)	44 (41.9)	18 (43.9)	17 (42.5)
DQ8/DQ8	44 (6.7)	5 (4.4)	3 (7.1)	2 (5)	49 (5.4)	5 (4.8)	2 (4.9)	2 (5)
DQ8/DQX	209 (31.6)	27 (23.7)	9 (21.4)	8 (20)	261 (28.7)	19 (18.1)	5 (12.2)	5 (12.5)
DQX/DQX	82 (12.4)	10 (8.8)	0 (0)	0 (0)	89 (9.8)	6 (5.7)	3 (7.3)	3 (7.5)

DQ2 denotes (DQA1*0501-DQB1*0201); DQ8 (DQA1*0301-DQB1*0302); DQX is other haplotype than DQ2 and DQ8.

Risk of celiac disease autoimmunity is modified by non-HLA genetic markers during the first year of clinical type 1 diabetes (Study II)

Analysis of serum samples collected from children 1, 6, and 12 months after diagnosis of T1D showed that 8.0% of the subjects were positive for IgAG-DGP/tTG in two or more consecutive samples, and 4.6% were confirmed positive for IgG-tTG and consequently defined as having CDA. Of these children, 10.1% were positive for IgAG-DGP/tTG at the first visit 1 month after T1D diagnosis, 8.0% at the 6-month visit, and 11.5% at the 12-month visit. Also, in the radioligand binding assay, 6.3% were positive for IgG-tTG at 1 month, 5.8% at 6 months, and 7.4% at 12 months. The 5% prevalence of CDA in this international cohort agrees well with the findings of other investigations showing that CD affected 2.4% to 16.4% of children with T1D (79).

It is well known that the prevalence of CD differs between countries, and the prevalence in Europe based on population screenings ranges from 1:500 in Croatia to 1:33 in Sweden (50, 109). The fluctuation in CD antibody levels over time has been elucidated in a longitudinal prospective study of children at increased risk of T1D (219), in which it was found that subjects with transient CDA were likely to have low CD antibody levels and were also positive for multiple islet autoantibodies 1 month after diagnosis. Speculatively, these observations might be explained by a general inflammatory state of the gastrointestinal tract and a leaky gut, leading to increased passage of multiple antigens and in turn activation of the immune defense system.

The results of studies concerning the effect of glycemic control in patients with T1D have been conflicting, with a GFD being associated with improvement in some cases and with deterioration in others (220, 221). We did not find a correlation between levels of IgAG-DGP/tTG and HbA1c. This observation might be explained by small variations in HbA1c during the first year after diagnosis of T1D, but it also concurs with a recent study of potential CD in T1D patients that did not reveal any significant differences in HbA1c between groups with or without confirmed CD (222). Furthermore, we found no correlation between levels of IgAG-DGP/tTG and GAD, IA2, ICA, GLP-1, GIP, or glucagon, possibly due to the small number of patients defined as having CDA in our study. However, our findings regarding GIP and GLP-1 agree with another study in which untreated patients with CD with HLA susceptibility for T1D were studied (223).

HLA and non-HLA loci

Analysis of HLA distribution showed that 58.7% of the children in the cohort with T1D carried at least one DQ2 allele and 62.5% at least one DQ8 allele, and 8.5% had HLA haplotypes other than DQ2 and DQ8 (Table 2). Nearly one third of the children who had T1D but not CDA also carried DQ2/DQ8, which suggests that efforts should be made to find additional genes outside the HLA complex that are associated with the risk of CD.

In work conducted by other investigators (88), three CD non-HLA genes RGS1, IL18RAP and TAGAP were found to be associated with T1D, and two other T1D loci (CCR5 and PTPN2) were also found to be linked to CD. Therefore, we extended our study to include these non-HLA loci (224), which revealed that levels of IgAG-DGP/tTG were higher in children carrying the AA genotype of IL18RAP. Furthermore, a similar trend, albeit not statistically significant, was found for children with high levels of IgAG-DGP/tTG and carrying CCR5^{+/+}, whereas no association was found with either RGS1 or PTPN2.

The CCR5 gene encodes the protein CCR5, which belongs to the beta chemokine receptor family of integral membrane proteins. CCR5 is predominantly expressed on T cells, macrophages, dendritic cells and microglia (225). This protein is probably involved in inflammatory responses to infection, although its exact role in normal immune function is unclear. Altered levels of CCR5 ligands have been observed in patients with recent-onset T1D (226). In the NOD mouse model of T1D, CCR5 and its ligand CCL4 have been reported to play several essential roles in development of the disease (227).

IL18RAP encodes the IL18 receptor, and binding of this receptor mediates activation of IL18R1, which, in co-expression with IL18, is required for activation of NK cells. This is particularly interesting when considering our cohort of children with T1D, because our experiments using NOD mice demonstrated that a GFD reduces both the level of NKG2D and expression of NKG2D ligands (Study III). We speculate that children with T1D carrying both AA IL18RAP and HLA-DQ2 and/or DQ8 haplotypes are at higher risk of activation of NK cells that can lead to chronic inflammation of the gut, and CDA after exposure to dietary gluten.

A gluten-free diet lowers NKG2D and ligand expression in BALB/c and non-obese diabetic (NOD) mice (Study III)

We studied the effect of a GFD in fully immune-competent BALB/C mice and in NOD mice. BALB/C is a laboratory-bred strain of the house mouse, and it is one of the inbred strains that are most widely used in animal experimentation. NOD mice represent the preferred model for studies of the etiopathogenesis of T1D, along with the BioBreeding rat (228). Using both these strains allowed us to study the effect of gluten in both healthy animals and those predisposed to disease.

In both BALB/c and NOD mice, FACS showed that NKG2D expression on DX5⁺ cells was decreased in the spleen and ALNs of 13-week-old animals that were fed a GFD. This diet also reduced expression of the proliferation marker CD71 on DX5⁺ and CD8⁺ cells in spleen, PLNs, and ALNs in both BALB/C and NOD mice, although the decrease in CD71 on DX5⁺ cells in PLNs of the BALB/C mice was not statistically significant. Furthermore, the GFD lowered the number of CD8⁺ cells in spleen, PLNs, and ALNs in both mouse strains. The main diet-induced differences in NKG2D and DX 5 occurred more frequently in the spleen compared to the PLNs. This observation suggests a systemic effect of gluten intake on immune activation, which is supported by earlier studies demonstrating an impact of gluten on both intestinal and systemic immunity (194, 229).

Analysis by qRT-PCR revealed that expression levels of NKG2D and DX5 in intestinal tissue were markedly altered by diet. A GFD significantly reduced the levels of these two receptors in both BALB/C and NOD mice. In isolated pancreatic islets, substantial changes in NKG2D and DX5 expression levels were detected only in animals fed the SGFD, in which NKG2D expression was significantly decreased and DX5 expression was doubled compared to animals fed the SSTD. That the effect on the expression levels was most prominent in intestinal tissue is not surprising, given that such tissue is in direct contact with gluten digestion products.

It is noteworthy that we also found that a SGFD was correlated with lower NKG2D expression in islets, especially considering that DX5 expression was increased in the same tissue. It is not clear whether this rise in DX5 reflects an increase in NK cells, or if it implies an increased number of islet-infiltrating natural killer T (NKT) cells (expressing DX5) (230), which has been reported to prevent the development of T1D (231). Similar to humans, NOD mice exhibit defects in NKT cell development (232), and overexpression of NKT cells prevents transgenic NOD mice from developing diabetes. An increased level of NKT cells within the islets per se might be a mechanism behind the protective role of a GFD against T1D in NOD mice. Gluten-induced expression of NKG2D is of particular interest, because research has indicated that NKG2D plays a key role in immune-mediated diseases, including CD and T1D (233). Perhaps NKG2D can serve as an immune activator that can tip the balance in favor of autoimmunity and chronic inflammation. It has been observed that NKG2D can mediate cytotoxicity independently of TCR ligation in recently activated T cells (234), which is especially relevant in this context, because it suggests that gluten intake alone can establish a self-perpetuating loop of immune activation.

We also found that the GFD we used affected the expression of NKG2D ligands, and thus we performed qPCR analyses of a number of NKG2D ligands known to be expressed in both intestinal tissue and isolated islets: Mill1, Mill2, Mult1, Rae-1, and H60. In the BALB/C mice, the GFD did not markedly alter the intestinal expression pattern, because only levels of the ligand H60 were significantly reduced compared to what was noted in animals fed the STD. In NOD mice, the expression of all NKG2D ligands was reduced in the animals fed the SGFD compared to those given the SSTD. This agrees well with the reduced intestinal expression of NKG2D and DX5 and resembles the transition from low levels of NKG2D ligand expression in healthy gut epithelium that is increased in patients with CD (235), a disorder in which distressed intestinal epithelial cells upregulate the stress-inducible non-classical MHC class I MIC (MICA and MICB) upon exposure to gluten peptides (236). Lowered levels of NKG2D along with decreased levels of Mult 1 and Rae-1 might be involved in the beneficial effects of the SGFD compared to the GFD in our experiments.

The expression in isolated islets displayed a different pattern. None of the investigated ligands showed a significant change in expression levels in BALB/C or NOD mice fed the GFD. In NOD mice, expression of Mill1, Mill2, and Mult1 increased significantly in the animals fed the SGFD compared to those given the SSTD, and a similar trend (albeit not statistically significant) was seen for the remaining ligands. Thus it seems that a SGFD can lower NKG2D-mediated immunity in the intestines through direct downregulation of the NKG2D receptor, as well as through ligand expression in target tissues, which clearly suggests an effect of the timing of gluten exposure.

The observed pattern of ligand expression indicates that NKG2D ligands may have different functions, but the details of such NKG2D ligand interplay remain to be established. Perhaps NKG2D ligand upregulation can protect the islets from NKG2D-mediated cytotoxicity, because it has been proposed that NK cells can be desensitized by continuous exposure to NKG2D ligands (180, 237). However, other studies have found that the expression of NKG2D ligands decreases expression of MHC class I molecules (238), which leads to increased NK cell killing and thus implies a complex relationship between NKG2D ligand expression and MHC I. In agreement with results published by Maier *et al.* (239), but in contrast to the findings of Ogasawara *et al.* (177), our data show no dramatic difference in Rae-1 expression levels between NOD and BALB/c mice in the intestinal tissue, in isolated pancreatic islets, or with age. The explanation for the discrepancy between these results and those obtained by Ogasawara *et al.* is not apparent, but may be related to an effect of differences in rearing conditions or dietary components. Immunohistochemical staining was performed to visualize the presence of NKG2D-positive cells in intestinal tissue from NOD mice (Fig. 5).

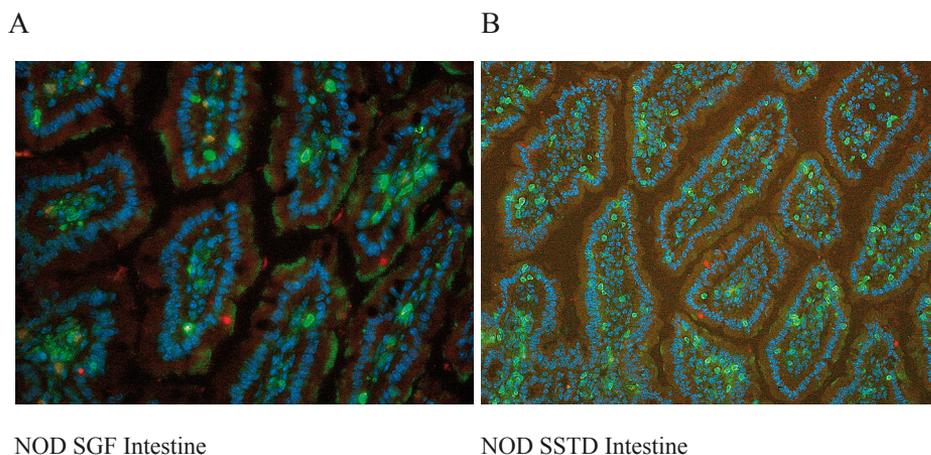
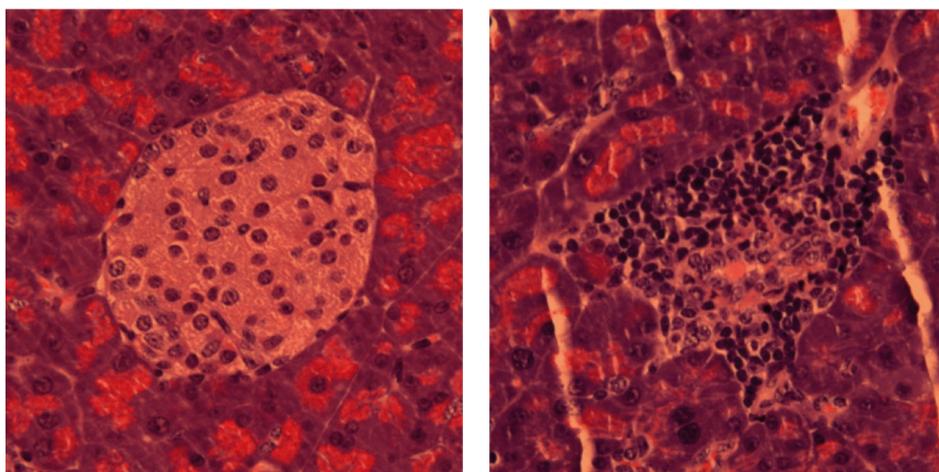


Figure 5. Sections of intestinal tissue from NOD mice fed the strictly gluten-free diet (A) and the strictly standard diet (B). Cells positive for NKG2D are red, cells positive for CD3 are green, and cell nuclei are blue.

The islet infiltration was further studied using hematoxylin–eosin-stained (H&E) pancreatic sections. The insulinitis score was significantly reduced in NOD mice fed the SGFD compared to those fed the SSTD (Fig. 6). Our results help clarify how the immune status is affected by dietary gluten, which has been proposed to be an environmental factor influencing the development of T1D, as well as the status of healthy individuals and those assumed to have non celiac gluten sensitivity (70). Our finding that gluten affects immunity in both BALB/c mice and NOD mice supports the idea that gluten can induce cellular changes in animals predisposed to disease as well as in healthy animals. The effect of gluten on expression of NKG2D and NKG2D ligands, as observed in CD patients, is not disease-specific but also occurs in NOD and BALB/c mice.

A

B



SGFD

SSTD

Figure 6. Scoring of histological grade of insulinitis performed by hematoxylin and eosin (H&E) staining of sections of pancreas from 20-week-old NOD mice fed a strictly gluten-free diet (SGFD) ($n=11$) (A) and a strictly standard diet (SSTD) ($n = 9$) (B). Islets were scored as showing no insulinitis, peri-insulinitis, moderate insulinitis, or severe insulinitis.

Perinatal risk factors and comorbidity of type 1 diabetes and celiac disease: perspectives from a Swedish childhood population (Study IV)

In a cohort comprising 768,395 singleton children, we identified 4,327 (0.6%) with T1D, 3,817 (0.5%) with CD, and 191 (0.02%) with both T1D and CD. The children with comorbidity were younger at diagnosis of T1D (median age 2.6

years) compared to those with T1D only (median age 4.7 years). Other investigators have also found age at onset of T1D to be inversely associated with risk for CD (82, 83), although there are also some conflicting results (240).

The clinical presentation of CD varies with different combinations of HLA genotypes, and individuals carrying two copies of HLA-DR3-DQ2 are at the highest risk of developing CD at an early age (29). Interestingly, among the children diagnosed with T1D in Sweden that were included in Study I, those that were DR3–DQ2 homozygous developed CDA more frequently than those with other HLA genotypes (241). Therefore, we speculated that the children with both T1D and CD that presented with T1D at an early age may have had a different HLA genotype compared to those who had T1D only and were diagnosed at an older age.

The absolute risk of CD in the study population with T1D was 4.2% (191/4,518), a level that is eight times higher than the level in the general population in Sweden. In our cohort, 0.5% (3817/763,877) had only CD ($p < 0.0001$), and 0.6% (4518/768,395) had only T1D ($p < 0.0001$). However, the prevalence of CD was probably underestimated, because it has been reported that a substantially larger number of CD cases is identified by population-based screening than by using only clinical case findings (50).

A larger proportion of the children with CD were females, regardless of T1D status, which concurs with previous studies (242, 243) and reflects the importance of gender modulation of the risk of autoimmune disorders. The reason for the female predominance among individuals at increased risk is still unresolved, although it might be attributed to either genetic and conditional effects, or a combination of both, and needs to be further explored.

Caesarean delivery was associated with a dual diagnosis of T1D and CD in the children we studied. It is well known that such delivery is a risk factor for each of these two diseases separately (244–247), and hence it is not surprising that it is also a risk factor for a dual diagnosis. Nonetheless, the finding that risk of T1D-CD comorbidity was associated with Caesarean section should be interpreted with caution, because such exposure is strongly confounded by indication and determined by factors related to the pregnancy itself (e.g., fetal distress and dystocia), and is also affected by maternal factors (e.g., lifestyle and previous Cesarean sections) and physician attitude (e.g., defensive medicine), which in turn may be conditioned by factors associated with risk of CD (248).

Our finding that children with T1D-CD comorbidity were more likely to be born during summer agrees with previous reports showing that there is a seasonal effect on the risk of the two diseases. This effect has been suggested to be due to seasonal variation in exposure to viruses or possibly also to differences in expo-

sure to sunlight during pregnancy, which can affect the maternal and thus also the infant vitamin D status and thereby modulate the future risk of developing T1D (249-251).

Children born to women of Swedish descent were at elevated risk of contracting CD, T1D, or both diseases, compared to children born by mothers with other national background. The increased risk is likely to be a result of the high proportion of DQ2 and/or DQ8 carriers in the general population in Sweden as compared to other countries. However, it has previously been reported that acculturation appears to condition the risk of CD in offspring born in Sweden (252).

In our study, mothers that gave birth to children with both CD and T1D were older than mothers whose children were not affected by either of these diseases, and a higher maternal age was also associated with a slightly increased risk of T1D only. In recent decades, the age at which women give birth has been increasing in many Western countries, and studies have identified correlations between maternal age and pregnancy complications, including preterm delivery and low-birth-weight babies, as well as various diseases in childhood such as asthma, leukemia, and tumors in the central nervous system. Pooled analyses have also provided evidence of a weak but significant linear increase in the risk of childhood T1D across the range of maternal age (253).

According to our results, socioeconomic factors were only weakly associated with an increased risk of CD and T1D, and other investigators have reported that a lower socioeconomic status is associated with a lower incidence of T1D (254) and CD (255).

Conclusions

- 6 % of the Swedish children with T1D had CDA compared to 4.5 % of the Danish. There was a significant difference in antibody levels between the two countries. The observed HLA-independent discrepancy in CD-related antibody levels suggests a difference in exposure to environmental factors between the two neighboring countries.
- Approximately 5% of T1D children are expected to be affected by CDA during the first year of T1D, and it is plausible that there is a subpopulation homozygous for IL18RAP, which can modulate the risk of developing CDA in T1D.
- Dietary gluten seems to affect the immune system of both immune-competent BALB/c mice and diabetes-prone NOD mice, and a gluten-free diet lowers expression of NKG2D and its ligands in these two strains.
- Having both T1D and CD is associated with Caesarean section, birth during summer, female gender, and being born to an ethnic Swedish mother, suggesting that perinatal factors can modify the risk of developing both these diseases.

Summary and future perspectives

The research underlying this thesis examined the association between T1D and CD and the effects of modification of the innate immunity by dietary gluten. The immune system balances on a fine line to distinguish self from non-self in order to maintain the integrity of the host (256), and disturbance of this fine line can result in over activity to self-antigens that can lead to autoimmunity. A significant increase in the incidence of autoimmune diseases has been observed worldwide over the last two decades (257), and this includes T1D and CD (45-48). During the same period, there have been numerous changes in our environment with respect to aspects such as sanitation, healthcare, and diet (258-261).

The known remarkable difference in incidence of CD between Denmark and Sweden made me curious as to whether this finding also applies to CD in children with T1D. Such a possibility has never before been tested by the same laboratory using the same diagnostic methods.

The observed disparity in levels of CD-related antibodies that could not be explained by differences in HLA alleles might instead be the result of differences in exposure to environmental factors. It is well-known that dietary habits, and especially weaning practices, differ between Denmark and Sweden, but there are still several aspects that remain ambiguous. For example, the influence of diet on immunity and autoimmunity remains to be investigated. Moreover, population-based screenings and dietary surveys combined with studies of the gut microbiota would cast more light over why T1D prevalence differs between neighboring countries like Sweden and Denmark.

Can the answer be differences in infant feeding practice? It is now assumed that there is a certain age interval that provides a “window of opportunity” for gluten introduction. Researchers recently proposed that both early (infant age < 4 months) and late (age \geq 7 months) introduction of gluten should be avoided, and that gluten should be introduced while an infant is still being breastfed. (262).

Is it the total amount of gluten intake that differs during weaning? It is well known that infant feeding patterns vary substantially between countries and that Swedish babies have a higher gluten intake at a younger age compared to infants in other countries (263). The finding that gliadin may actually activate a zonulin-dependent enterocyte intracellular signaling pathway and thus increase intestinal permeability suggests a cycle in which dietary intake of gliadin would lead to increasing intestinal permeability via the activation of zonulin by gliadin and in turn to increasing exposures to the body upon continued gliadin intake (264).

Do gut microbiota differ between people living in Denmark and Sweden, and can that explain the difference in T1D prevalence between the two countries? Diet is one of several factors that can determine the composition of the gut microbiota (183). During toddlerhood, the intake of solid food and the maturation of the immune system profoundly modify the gut microbiota profiles toward an adult microbiota setting. What we eat during the weaning period probably has a profound impact on shaping of the gut microbiota, and this microbial community plays a crucial role not only in immune function, but also in energy storage and metabolism, barrier integrity, development of the autonomic nervous system, epithelial cell proliferation, and intestinal motility (206).

The present finding that CDA was also common in the healthy children that served as controls strengthens the argument for performing a population-based screening CD study in Denmark. Results from a population-based screening in Sweden suggested that as many as two thirds of CD cases can be missed when applying a case-finding strategy (50).

We also investigated the distribution of CDA in an international cohort of children with T1D and found a subgroup of children with homozygosity for IL18RAP, indicating that non-HLA genetic markers may modulate the risk of CDA in children with T1D. Genome-wide association studies (GWAS) have identified 39 CD-associated loci (265), and in many cases suggested that T cell development and the innate immune system are causally related to CD. An important finding of the mentioned studies was the strong overlap seen in the association signals with other autoimmune diseases (266). T1D and CD share > 50% of their associated loci. SNPs analysis of IL18RAP, a non-HLA region known to be associated with CD, has also provided strong evidence of a link with T1D. Interestingly, IL18RAP was found to be inversely associated with T1D but positively associated with CD (88). The exact arrangement of the shared genetics between CD and T1D remains to be determined. One possibility is that there is a common genetic background with respect to autoimmunity and inflammation, and that the final clinical outcomes are determined by further combinations of more disease-specific variation in HLA and non-HLA genes, in interaction with epigenetic and environmental factors.

The interrelationship between immune system, genetics, and gut microbiome has gained increasing attention in recent years. It appears that the host genome affects the composition of the microbiome, and that variants of a particular gene can influence the composition and diversity of the microbiota (267).

We used an animal model to further study the effects of dietary glutes on the immune system, and our results demonstrated that a GFD had an impact on both immune-competent BALB/c mice and NOD mice, the latter of which develop T1D spontaneously. However, even though the NOD mice model can be highly useful for elucidating mechanisms and creating new ideas, it is necessary to bear in mind the huge difference between autoimmune diabetes in mice and humans (268). For example, cereals are a natural source of food for mice, whereas humans have evolved over millions of years without any gluten in the diet. This calls for further research to compare the effects of a GFD and a gluten-containing diet on the human immune system.

We also need to further identify key elements that define the crosstalk between the gut microbiota, immunity, and metabolism. Observational investigations with sufficiently large cohorts of HLA susceptible individuals at risk of T1D, such as The Environmental Determinants of Diabetes in the Young (TEDDY) study (269), will be needed to pinpoint possible relationships between infections and/or dietary exposures and the appearance of islet autoantibodies. Identifying the environmental triggers of islet autoimmunity would offer enormous potential for improving primary prevention therapeutics.

In Study IV, we explored perinatal risk factors for comorbidity of CD and T1D. Our large cohort implied that children who suffer from both these disorders from an early age might have a different HLA genotype compared to those with T1D diagnosed at an older age. This corresponds well with the findings of Study II showing that CD autoimmunity in children with T1D was developed by a larger proportion of those who were DR3–DQ2 homozygous compared to those with other HLA genotypes.

Some attempts have been made to investigate the effects of a GFD on autoimmunity in human preclinical T1D (270) and in children at high risk of developing T1D (271), but the studies have been very small and have arrived at conflicting results. Maybe it is too late to start a GFD when preclinical diabetes has already occurred? Might it be better to apply the intervention much earlier? Or should these genetically susceptible individuals have a diet that includes a very moderate amount of gluten? Should the mother already at conception be on a GFD during pregnancy to completely prevent exposure to gluten for a child at high risk of T1D? Previous studies have underlined the importance of the timing introduction of gluten, but can autoimmunity be prevented by never introducing gluten at all?

And how is quality of life affected by a GFD. Previous studies have shown that health-related quality of life (HRQoL) in children and adolescents treated for CD is influenced by age at diagnosis, disease severity at onset, and number of years on a GFD (271). Studies have shown, that in Sweden, most children with CD do not have an impaired HRQoL, whether treated or not (272), and it has also been shown that children with T1D have a good HRQoL compared to both healthy children and children with T1D in other countries. But how does a dual diagnosis of CD and T1D affect HRQoL? There are arguments against GFD in assumable asymptomatic children related to aspects such as difficulty in handling the changes in diet, lifestyle, and medication management that are inherent in the care of children with both T1D and CD, and a lack of clear benefits in symptom-free children (273). In children with T1D, there is evidence that a GFD can be beneficial (274, 275), although in symptom-free cases the benefits are limited to weight gain and changes in bone mineral density (276-279). Earlier studies have also shown that adherence to a gluten-free diet is poor among children with T1D (280-282).

Only two very small studies have evaluated HRQoL in patients with both T1D and CD (283, 284), and the results are conflicting. We recently conducted a larger investigation in which all children with T1D in Skåne County (Sweden) were invited to participate and the well-validated DISABKIDS questionnaire was applied. Preliminary results show that quality of life is compromised in children with both T1D and CD compared to those with only T1D. The differences between these two groups were found to be particularly pronounced in the “mental health domain” of the questionnaire, which contains items about independence (e.g., autonomy and ability to live without restrictions due to the disease) and emotions (e.g., anxiety, anger, and worries).

Populärvetenskaplig sammenfattning

Vårt immunförsvar måste ständigt vara redo att försvara oss mot angrepp utifrån. Det måste kunna agera selektivt för att skydda oss mot främmande mikroorganismer som virus och bakterier. Om immunförsvaret istället missbedömer kroppens egna celler för att vara inkräktare och attackerar dessa, inträffar det vi kallar autoimmunitet. De autoimmuna sjukdomarna har ökat överallt i världen under de senaste två decennierna. En högst angelägen fråga att försöka besvara är varför det är så. Ökningen är för snabb för att kunna orsakas av genetiska förändringar, vilket gör att svaret måste sökas i livsstilsfaktorer och miljön runt omkring oss. Celiaki (glutenintolerans) och typ 1 diabetes (T1D) är de vanligast förekommande autoimmuna sjukdomarna hos barn och föremål för studier i detta avhandlingsarbete.

Celiaki utvecklas hos genetiskt predisponerade individer som ett resultat av miljöpåverkan. En nödvändig faktor är naturligtvis intag av gluten, men flera andra faktorer tros bidra till sjukdomsutvecklingen. Man tror att hur vi avvänjer våra spädbarn spelar in, det vill säga i vilken form vi introducerar gluten för det späda barnet, vid vilken tidpunkt detta sker samt om det sker under samtidig amning eller ej. Infektioner som barnet utsätts för i tidig ålder, kanske redan i mammas mage, samt bakteriefloran i barnets magtarmkanal tros också spela roll i utvecklingen av celiaki. Intoleransen mot gluten orsakas av en immunologisk reaktion mot tunntarmens slemhinna, som efter en tid stöts bort. Sjukdomen kan ha mycket varierande symtombild, allt ifrån närmast symtomfrihet till uttalade besvär i form av viktnedgång, diarréer, buksmärtor, benskörhet och infertilitet. Många patienter går på grund av den varierande bilden och de ofta ospecifika symtomen odiagnostiserade under lång tid. Vid celiaki bildar kroppen antikroppar som tycks vara mycket sjukdomsspecifika och som sannolikt är involverade i sjukdomsutvecklingen. Sådana antikroppar kan nu analyseras i blodprov och är värdefulla sjukdomsmarkörer som idag används vid utredningen av celiaki. Traditionellt har diagnosen ställts genom att man tagit ett vävnadsprov från tunntarmen, en så kallad biopsi, med hjälp av ett gastroskop eller en kapsel som förts

ner i tunntarmen. Numer kan diagnosen hos barn med symtom fastställas även utan biopsi, om antikropps nivåerna i upprepade prover är tillräckligt höga.

Man vet ännu inte vad som är den utlösande faktorn för T1D. Man måste ha en viss ärftlig känslighet för att man skall kunna utveckla sjukdomen. Olika riskfaktorer har föreslagits, bland annat virusinfektioner, tidig exponering för komjölk, snabb tillväxt under barnaåren, låga halter av D-vitamin samt intag av gluten. Kroppen använder normalt insulin för att reglera sockernivåerna i blodet, så vi kan utnyttja socker som energisubstrat. Vid T1D bildar kroppen antikroppar mot bukspottskörtelns β celler som bildar insulin, varför insulinproduktionen gradvis avtar. När endast ca 10-20% återstår insjuknar man med symtom såsom ökad törst, ökade urinmängder, trötthet, dimsyn och i värsta fall syraförgiftning (ketoacidosis). Detta är ett livshotande tillstånd som kännetecknas av svåra buksmärter, kräkningar och ansträngd andning, orsakad av ett för lågt pH i kroppen. Patienter med T1D löper risk att utveckla andra autoimmuna sjukdomar, framför allt thyroideasjukdomar och celiaki. Ca 10 % av alla patienter med T1D utvecklar för eller senare också celiaki.

Förekomsten av celiaki och T1D varierar mellan olika etniska grupper och mellan olika länder. Även mycket närliggande länder kan ha stor skillnad i sjukdomsfrekvens. Detta kan inte förklaras av enbart genetiska skillnader, utan tyder på att miljöfaktorer spelar in. Syftet med denna avhandling var att undersöka samband mellan celiaki och T1D samt att utforska hur gluten påverkar immunförsvaret.

I studie I ville vi undersöka förekomst av glutenrelaterade antikroppar hos barn med T1D. Eftersom de två grannländerna Sverige och Danmark har mycket olika frekvens av både T1D och celiaki, ville vi i dessa länder kartlägga om den stora skillnaden i celiakifrekvens också kunde påvisas i den högriskgrupp som barn med T1D utgör. Vi ville också undersöka hur de celiakirelaterade antikropparna förhöll sig hos friska barn på båda sidor om Öresund.

Vi fann att antikropps nivåerna skilde sig mellan de svenska och danska barnen med T1D. Denna skillnad var oberoende av de genetiska markörer (HLA) vi analyserade, vilket tyder på att variationen är beroende av miljöfaktorer. Vi fann också att många fler friska danska barn än förväntat hade celiakirelaterade antikroppar. Detta fynd antyder att celiaki är underdiagnostiserat bland barn i Danmark.

I studie II undersökte vi hur celiakirelaterade antikroppar fördelade sig i en internationell kohort med barn med T1D under första året efter diagnos. Prover togs vid tre tillfällen; tre, sex och tolv månader efter T1D diagnos. Vi fann att risken för att ha celiakirelaterade antikroppar vid upprepade tillfällen modifierades av vissa genetiska markörer (IL 18RAP). Av detta drar vi slutsatsen att risken att

utveckla celiaki när man har T1D bland annat påverkas av genetiska markörer och inte endast av miljöpåverkan.

I Studie III undersökte vi hur glutenfri respektive gluteninnehållande diet påverkade specifika delar av immunförsvaret hos möss. Vi undersökte både laboriemöss (BALB/C) med normalt fungerande immunförsvaret och möss framavlade för att utveckla T1D, så kallade NOD-möss. Vi fann att gluten i mössens diet påverkade en speciell receptor på en speciell sorts vit blodkropp, NKG2D receptorn som finns på NK- (Natural Killer) celler. NK-celler deltar normalt i immunförsvaret genom att döda främmande mikroorganismer och kan också förhindra cancercellers tillväxt. De har också visat sig vara involverade i utveckling av autoimmunitet. Våra fynd tyder på att gluten i kosten alltså kan påverka delar av immunförsvaret som är inblandade i utvecklingen av T1D.

I Studie IV undersökte vi perinatale (kring födelsen) riskfaktorer för samtidig celiaki och T1D. För att kunna studera dessa faktorer använde vi flera svenska nationella register, bland annat det medicinska födelseregistret och slutenvårdsregistret. Denna studie omfattade samtliga barn (768,395 personer) som fötts i Sverige under åren 1987 till 1993. Vi fann att risken att utveckla samtidig celiaki när man har T1D ökade om man var född med kejsarsnitt, om man var född på sommaren, var av kvinnligt kön eller hade en svenskfödd mamma.

Sammanfattningsvis visar resultat från denna avhandling att både omgivande miljöfaktorer, olika speciella genetiska markörer, samt perinatale riskfaktorer tycks påverka samsjuklighet mellan T1D och glutenintolerans, samt att gluteninnehåll i kosten kan påverka immunförsvarets funktion.

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Paper I

Original Article

Prevalence of celiac disease autoimmunity in children with type 1 diabetes: regional variations across the Øresund strait between Denmark and southernmost Sweden

Adlercreutz EH, Svensson J, Hansen D, Buschard K, Lernmark Å, Mortensen HB, Agardh D. Prevalence of celiac disease autoimmunity in children with type 1 diabetes: regional variations across the Øresund strait between Denmark and southernmost Sweden. *Pediatric Diabetes* 2014.

Objectives: The aim was to determine the prevalence of celiac disease autoimmunity in children with type 1 diabetes (T1D) diagnosed in Denmark and Sweden.

Methods: A total of 662 Swedish children with T1D were matched with 1080 Danish children with T1D and 309 healthy children from Sweden and 283 from Denmark served as controls. Sera were analyzed for the presence of IgA and IgG (IgAG) autoantibodies against deamidated gliadin peptide (DGP) and tissue transglutaminase (tTG) with enzyme-linked immunosorbent assay (ELISA) and IgG-tTG separately in a radioligand binding assay (RBA). Human leukocyte antigen (HLA)-DQB1 and DQA1 genotyping were determined in the T1D cohorts.

Results: In the Swedish T1D cohort, 17.2% (114/662) were IgAG-DGP/tTG positive compared with 11.7% (126/1080) in the Danish T1D cohort ($p = 0.001$) and with 9.4% (29/309) Swedish ($p = 0.001$) and 5.7% (16/283) Danish ($p = 0.003$) controls. In the Swedish T1D cohort, both levels of IgAG-DGP/tTG and IgG-tTG were higher compared with the levels in the Danish T1D ($p < 0.001$). In the control group, 2.8% of the Danish children were positive for both IgAG-DGP/tTG and IgG-tTG, compared to 0.3% of the Swedish. Presence of HLA-DQ2 was equally distributed among 89 children with T1D positive for both IgAG-DGP/tTG and IgG-tTG.

Conclusion: The discrepancy in levels of IgAG-DGP/tTG and IgG-tTG between Swedish and Danish T1D cohorts was independent of HLA and suggests that regional variations in comorbidity of celiac disease in T1D is caused by difference in exposure to environmental factors.

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The possible interaction between type 1 diabetes (T1D) and celiac disease (CD) is unclear but has been attributed to shared links to the human leukocyte antigen (HLA) DR3-DQA1*05:01-DQB1*02

(i.e., DR3-DQ2) and DR4-DQA1*03:01-DQB1*03:02 (i.e., DR4-DQ8) haplotypes (1). Although genome-wide association studies suggest that genes outside the HLA complex may also contribute to both T1D

and CD (2), it is likely that the two autoimmune disorders share a multi-factorial etiology initiated by environmental factors (3–7).

Sweden and Denmark are two neighbor Scandinavian countries distanced only 3.5 km at the narrowest point. Although the general population shares similar distribution of HLA genotypes (in fact southernmost Sweden was part of Denmark until the 17th century), the incidence of childhood-onset T1D differs greatly, from 22/100 000 in Denmark to 40/100 000 in children less than 15 yr of age in Sweden (8–10). Even more pronounced differences have been reported for CD, where the incidence varies between 6.9 and 12.6 in 100 000 children younger than 15 yr of age in Denmark and Sweden, respectively (11, 12).

The reason for the discrepancy in CD incidence in the general population between the two countries is not known, but differences in national infant feeding recommendations of gluten-containing foods have been suggested as one possible explanation (13). In populations at risk for CD, such as patients with T1D, previous studies have reported a similar prevalence of CD between the countries (10.4–12.3%), in Denmark (14, 15) as compared to Sweden (9.1–10%) (16, 17). However, no direct comparative study between two nearby countries has been performed in order to establish if the comorbidity of CD in T1D is country-specific or dependent on other factors.

In this study, we aimed to determine the occurrence of autoantibodies associated with CD in two HLA genotyped T1D cohorts diagnosed across the Øresund strait between Denmark and southernmost Sweden. We hypothesized that children with T1D from Sweden are at higher risk to develop celiac disease autoimmunity than Danish children with T1D, independent of HLA.

Materials and methods

Included were 1742 children and adolescents diagnosed with T1D between the years 1995 and 2006 of whom 662 Swedish T1D children (Group 1) were selected from the Skåne study (situated in the southern region of Sweden) and 1080 Danish T1D children (Group 2) retrieved from the Danish diabetic register (Table 1). The samples were collected at the time of diagnosis of T1D. In addition, 309 Swedish (Group 3) and 283 Danish (Group 4) healthy school children from each country were included and served as controls (Table 1). Serum samples from each of the participants were analyzed after approval from the Regional Ethics Board in Lund and from the ethical board of Copenhagen.

Combined deamidated gliadin peptide and tissue transglutaminase antibody assay

Conjugated IgA and IgG (IgAG) against deamidated gliadin peptide (DGP)/tissue transglutaminase (tTG) were analyzed using the commercial the QUANTA Lite™ h-tTG/DGP Screen ELISA kit (INOVA Diagnostics, San Diego, CA, USA) and run according to the instructions of the manufacturer (18). This assay has a diagnostic sensitivity and specificity of 100 and 89%, respectively (18). Briefly, DGP and purified human erythrocyte tTG (htTG)-coated enzyme-linked immunosorbent assay (ELISA) plates were incubated with diluted patient serum samples. Antibodies bound to the ELISA wells were detected with horseradish peroxidase labeled anti-human IgAG. Antibody levels were calculated from the optical density of the sample in relation to the reactivity of a positive control and expressed as arbitrary units (AU). Cut-off limits 20 AU or greater was defined as positive.

Tissue transglutaminase autoantibody radioligand binding assay

Tissue TG autoantibodies were analyzed in a radioligand binding assay (RBA) as previously described (19, 20). This in-house assay has a diagnostic sensitivity and specificity of 94% and 94%, respectively (21).

Briefly, IgG-tTG antigen/antibody complexes were bound to 30% protein A sepharose (PAS) (Zymed Laboratories, Inc., San Francisco, CA, USA). The PAS detects all subclasses of IgG except for IgG3 and also some of IgA and IgM. (22) The relative amount of tTG antibodies was expressed as U/mL. A cut-off level ≥ 7 U/mL was used for the upper normal limit representing the 95th percentile of healthy controls (23).

Celiac disease autoimmunity

For the purposes of this study, celiac disease autoimmunity (CDA) was defined as being positive for both IgAG-DGP/tTG and IgG-tTG.

HLA genotyping

HLA-DQB1 and -DQA1 alleles were determined by sequence-specific oligonucleotide probes using a DELFIA hybridization assay (Perkin Elmer, Boston, MA, USA) as previously described (24) or by direct sequencing of exon 2 of DRB1 according to the Immuno Histocompatibility Working Group.

Statistical analysis

Differences in frequency between the cohorts were tested using the Chi-squared test and the

Table 1. Patient characteristics from the Swedish cohort of children with type 1 diabetes (T1D) (Group 1) and the Danish cohort with T1D (Group 2) and from healthy children from Sweden (Group 3) and Denmark (Group 4)

	Group 1	Group 2	Group 3	Group 4
N	662	1080	309	283
Female:male ratio (F/M) (%)	305/357 (46)	518/542 (49)	141/158 (46)	130/153 (46)
Median age (range) at sample (yr)	10.2 (1.0–17.9)	10.3 (0.6–17.8)	12.6 (11.9–14.0)	15.6 (14.1–17.0)

Table 2. Outcome of celiac-associated autoantibodies in children with type 1 diabetes (T1D) from the Swedish cohort (Group 1) and the Danish cohort (Group 2) and from healthy controls – Swedish (Group 3) and Danish (Group 4)

	Group 1	Group 2	Group 3	Group 4	p value
N	662	1080	309	283	
IgAG-DGP/tTG, n (%)	114 (17.2)	126 (11.7)	29 (9.4)	16 (5.7)	p# = 0.001 p* = 0.4
IgG-tTG, n (%)	42 (6.3)	50 (4.6)	1 (0.3)	8 (2.8)	p# = 0.15 p* = 0.02*
CDA, n (%)	40 (6.0)	49 (4.5)	1 (0.3)	8 (2.8)	p# = 0.20 p* = 0.017

CDA, celiac disease autoimmunity; DGP, deamidated gliadin peptide.

p values <0.05 for comparisons between Group 1 and Group 2 are marked with #, comparisons between Group 3 and Group 4 are marked with *.

*Fischer's exact test.

Kruskal–Wallis and Dunn's multiple comparison tested differences in antibody levels. Correlations were evaluated by Spearman rank correlation (r) using the *SPSS* software version 21. P values <0.05 were considered significant.

Results

In the Swedish T1D cohort (Group 1), 17.2% (114/662) were IgAG-DGP/tTG positive compared with 11.7% (126/1080) in the Danish T1D cohort (Group 2) (p = 0.001) and with 9.4% (29/309) Swedish controls (Group 3) (p = 0.001) and 5.7% (16/283) Danish controls (Group 4) (p = 0.003), respectively. The frequency of IgG-tTG in Group 1 6.3% (42/662) and Group 2 4.6% (50/1080) did not differ (p = 0.149), but were

higher compared with the controls in Group 3, but not compared with Group 4. In Group 3, 9.4% (29/309) were IgAG-DGP/tTG positive compared with 5.7% (16/283) in Group 4 (p = 0.1089), whereas the opposite was true for IgG-tTG in 0.3% (1/309) compared with 2.8% (8/283) (p = 0.002) (Table 2).

In Group 1, both levels of IgAG-DGP/tTG [median 12.0 (0–238) AU] and IgG-tTG [median 2.4 (range 1–8746) U/mL] were higher compared with that of Group 2 [median 10 (range 0–208) AU; p < 0.001 respective median 1.5 (range 1–924) U/mL; p < 0.0001] (Figs 1 and 2).

HLA was available in 661/662 (99.8%) in Group 1 and in 910/1080 (84.3%) in Group 2 (Table 3). The DQ2 haplotype was found in 326/661 (49.3%) in Group 1 compared with 511/910 (56.2%) in Group 2 (p =

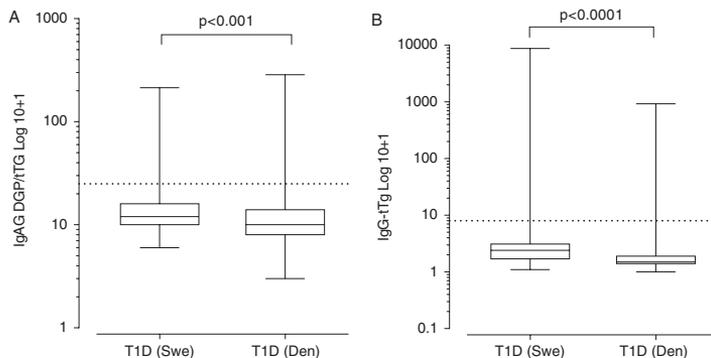


Fig. 1. Outcome of screening Swedish and Danish children with type 1 diabetes (T1D) for IgAG-DGP/tTG [enzyme-linked immunosorbent assay (ELISA)] (A) and for IgG-tTG antibodies [radioligand binding assay (RBA)] (B). Solid horizontal lines mark median levels. Dotted lines mark cut-off level of positivity.

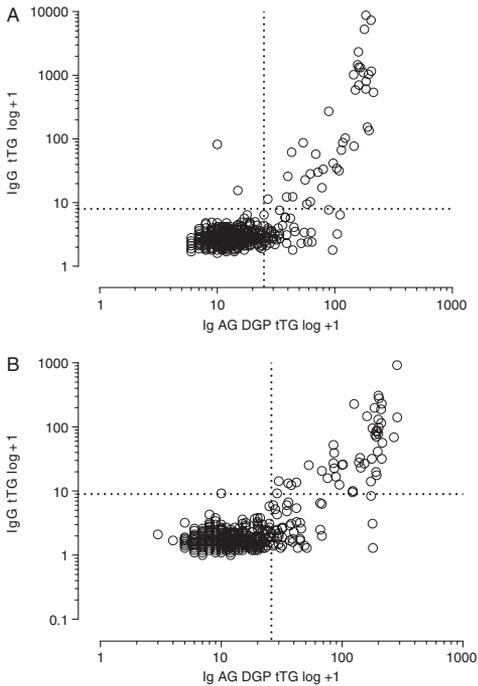


Fig. 2. Correlation between levels of IgAG-DGP/tTG [enzyme-linked immunosorbent assay (ELISA)] and IgG-tTG [radioligand binding assay (RBA)] in Swedish children with T1D, $r=0.317$ and $p < 0.010$ (A) and in Danish children with T1D, $r=0.073$ and $p < 0.0001$ (B). Children positive for both antibodies were defined as having celiac disease autoimmunity (upper right quadrant). Dotted lines mark cut-off level of positivity.

0.007). The DQ8 haplotype was equally distributed in Group 1 (469/661 or 70.1%) and Group 2 (659/910 or 72.4%) ($p=0.5120$), respectively. Among the 89 children with CDA, the DQ2 haplotype appeared 30/40 (75.0%) in both groups (Table 3).

Table 3. Distribution of human leukocyte antigen (HLA) genotypes in Swedish (Group 1) and Danish (Group 2) type 1 diabetes children positive of IgAG-DGP/tTG or IgG-tTG, or both antibodies defined as celiac disease autoimmunity (CDA)

HLA haplotype	Group 1				Group 2			
	IgAG-DGP/tTG		IgG-tTG		IgAG-DGP/tTG		IgG-tTG	
	All	Positive	Positive	CDA	All	Positive	Positive	CDA
DQ2/2	46 (7.0)	19 (16.7)	9 (21.4)	9 (22.5)	33 (3.6)	8 (7.6)	4 (9.8)	4 (10)
DQ2/X	64 (9.7)	11 (9.6)	7 (16.7)	7 (17.5)	129 (14.2)	23 (21.9)	9 (22.0)	9 (22.5)
DQ2/DQ8	216 (32.7)	42 (3.7)	14 (33.3)	14 (35)	349 (38.3)	44 (41.9)	18 (43.9)	17 (42.5)
DQ8/DQ8	44 (6.7)	5 (4.4)	3 (7.1)	2 (5)	49 (5.4)	5 (4.8)	2 (4.9)	2 (5)
DQ8/DQX	209 (31.6)	27 (23.7)	9 (21.4)	8 (20)	261 (28.7)	19 (18.1)	5 (12.2)	5 (12.5)
DQX/DQX	82 (12.4)	10 (8.8)	0 (0)	0 (0)	89 (9.8)	6 (5.7)	3 (7.3)	3 (7.5)

DQ2 denotes (DQA1*0501-DQB1*0201); DQ8 (DQA1*0301-DQB1*0302); DQX is other haplotype than DQ2 and DQ8.

Discussion

In this study, the occurrence of CDA between Swedish and Danish T1D children was the same. Yet, both the frequency and levels of IgAG-DGP/tTG were higher among Swedish as compared to Danish T1D children. The higher levels of antibodies among Swedish as compared to Danish T1D children were furthermore not explained by differences in frequency of the HLA risk haplotypes DQ2 or DQ8, pointing to the direction of difference in exposure of environmental factors between the study cohorts.

Although IgAG-DGP/tTG has a lower diagnostic specificity for CD as compared to conventional tTG autoantibodies (25), it may still indicate gluten sensitivity (26, 27), also called 'non-celiac gluten sensitivity' (NCGS), a gluten-associated condition clinically characterized by gastrointestinal or extra-intestinal symptoms associated with anti-gliadin antibody positivity (28) and frequently found in children with T1D (29). The higher levels of IgAG-DGP/tTG may also reflect a higher gluten consumption in the Swedish as compared to the Danish participants. It is well established from several national dietary surveys that the food habits differ between the two nearby countries. There is a long tradition in Denmark to use rye as the main grain in bread, whilst in Sweden there is a high consumption of wheat (30) which contains much higher concentrations of gluten.

A surprising and notable finding in our study was that the healthy children who served as the Danish control group had a prevalence of CDA near 3%. Indeed, this is higher than previously reported at 1.2%, albeit the earlier screening was based on questionnaires for CD-related symptoms (31). Our results indicate the prevalence of CD could be as high as in Sweden (32). This is also supported by a recent study based on the Danish national health register which shows that the incidence is increasing in Denmark (12). As the clinical presentation of CD sometimes appears with no evident

symptoms or only with extra-intestinal manifestations (33) (34–36) screenings performed among the that general populations are needed to explore the true prevalence of CD also in Denmark.

Some weaknesses of this study should be mentioned. Owing to its retrospective design, there was no information on diagnosis of CD or recordings on gluten-free diet. The lack of testing consecutive samples for persistence of autoantibody positivity is also a limitation. The strength on the other hand is the comparison of two T1D cohorts where all samples were collected at the diagnosis of T1D, during the same study period, using the same methods assessed in one laboratory and matched for HLA.

In conclusion, this study found a discrepancy in IgAG-DGP/tTG and IgG-tTG between Swedish and Danish T1D that was independent of HLA. We suggest that the previously reported regional variations in comorbidity of CD and T1D might be caused by difference in exposure to environmental factors between the two countries and speculate that the differences in levels of autoantibodies between the study populations might mirror a higher consumption of gluten-containing cereals among the Swedish T1D children that need to be further explored.

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Paper II

Risk of Celiac Disease Autoimmunity is Modified by Non-HLA Genetic Markers During the First Year of Clinical Type 1 Diabetes

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Abstract: *Aims:* This study plotted the prevalence of celiac disease associated antibodies in relation to demographic patterns, genetic and metabolic markers during the first year after diagnosis in a multinational cohort of children with T1D.

Material and Methods: Sera from a total of 261 children (128 males, 133 females) (0.5-16.3 years) with T1D recruited at 18 European centers were screened at 1, 6 and 12 months after diagnosis, using a combined ELISA measuring both IgA and IgG antibodies against deamidated gliadin peptide and tissue transglutaminase (IgAG-DGP/ITG) and a radioligand binding assays measuring IgG-tTG. Children positive in both assays in two consecutive samples were defined as having celiac disease autoimmunity (CDA). Associations between CDA and genotypes of HLA, IL18 rap, CCR 5, PTPN2 and correlations with islet autoantibodies (ICA, GADA, IA2 and IA) and HbA1C and C-peptide were performed.

Results: At the one, six and twelve month visit after T1D diagnosis, the prevalence of IgAG-DGP/ITG across all sites were 24/238 (10.1%), 18/224 (8.0%) and 25/218 (11.5%), respectively, and 11/238 (4.6%) were confirmed positive for IgG-tTG and defined as having CDA. A subpopulation of T1D children with high levels of IgAG-DGP/ITG were defined and associated with the genotype AA of IL18RAP.

Conclusions: In our multinational cohort of children with T1D, approximately 5% were affected by CDA during first year after diagnosis of T1D. Independent of country of residence, a subpopulation being homozygote for IL18RAP were found with high levels of IgAG-DGP/ITG, indicating that non-HLA-DQ2/DQ8 genetic markers may modulate the risk for developing CDA in T1D.

Keywords: Autoimmunity, celiac disease, children, HLA, genes, type 1 diabetes.

INTRODUCTION

Type 1 Diabetes (T1D) and celiac disease (CD) are two autoimmune disorders that often co-exist in individuals carrying HLA-DR3-DQ2 and/or DR4-DQ8 haplotypes [1]. The highest risk genotype in T1D is DR3-DQ2/DR4-DQ8 genotype accounting for up to 30-50% of the genetic risk [2]. In CD, more than 90% express the HLA-DR3-DQ2 haplotype and 5-10% HLA-DR4-DQ8 [1]. Moreover, HLA-DR3-DQ2 homozygotes seem to be at even higher risk for CD and at an earlier age than heterozygotes, suggesting a possible gene dose effect depending on the HLA haplotype [3]. Carrying either DR3-DQ2 or DR4-DQ8 is prerequisite to develop CD, but both these HLA risk haplotypes are also common among the general population suggesting that other genes might be involved in the pathogenesis

[4]. In gene wide association studies (GWAS) at least 39 non-HLA genes that confer a predisposition to CD have been identified, most of which are involved in inflammatory and immune responses [5].

Prospective longitudinal studies have demonstrated that the natural history of autoantibodies in CD may fluctuate over time and that spontaneous normalization is common and among T1D patients in particular [6, 7]. This phenomenon of so called transient celiac disease autoimmunity (CDA) has not yet been clarified, but seems to appear near diagnosis of T1D. It is not clear whether the natural history of CD associated antibodies are a parallel phenomenon that occur spontaneously over time in HLA-risk individuals or if other non-HLA risk loci may explain why subgroups of patients with T1D develop CD and others revert to transient CDA.

In this study, we examined a multinational cohort of children with T1D for their CDA status plotted at three different time-points during the first year after diagnosis. Specifically, we aimed to characterize if

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presence of CDA found among subgroups of T1D children could be attributed to demographic patterns, genetic or metabolic markers.

MATERIALS AND METHODS

The Hvidoere Study Group on Childhood Diabetes is a multinational collaboration of pediatric diabetes centers for the study and follow-up of children and adolescents with T1D as described elsewhere [8]. A total of 261 children (128 males, 133 females) with new onset T1D was included from 18 different clinical centres representing 15 European countries and Japan during 1999 to 2000 [9]. Samples were collected one month after diagnosis at a median age 9.6 (range 0.5–16.3) years and subsequently at six and twelve months after diabetes onset, respectively (Table 1). Sera were missing in 53 children: among 11 children at the one month visit, 27 at the six month visit and 30 at the twelve month visit.

For the purposes of this study, we used CDA as outcome defined as being antibody positive in two separate assays detected in two consecutive blood samples. Individuals who seroconvert from antibody positive to antibody negative during the six and twelve month follow-up visits were defined as being CDA transient. Furthermore, previous analyzed and available data on glycosylated hemoglobin (HbA1c) (Table 1) C-peptide, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide (GLP-1) and glucagon were used to define the metabolic control (data not shown). HLA and non-HLA genotyping had previously been performed as described [9].

Conjugated IgA and IgG (IgAG) against Deamidated Gliadin Peptide (DGP) and Tissue Transglutaminase (tTG) Immunoassay – IgAG-DGP/tTG (ELISA)

Sera were screened for IgAG-DGP/tTG using a commercial ELISA kit (the QUANTA Lite™ h-tTG/DGP Screen, INOVA Diagnostics, San Diego, CA) and run according to the instructions of the manufacturer [10]. Briefly, DGP and purified human erythrocyte tTG (htTG) coated ELISA plates were incubated with diluted patient serum samples. Antibodies bound to the ELISA wells were detected with horseradish peroxidase labelled conjugated anti-human IgAG. Antibody levels were calculated from the optical density of the sample in relation to the reactivity of a positive control and expressed as arbitrary units (AU). In this study, ≥ 20 AU was used to define cut-off level of positivity. This assay

previously provided a diagnostic sensitivity of 100% and specificity 89% in our CD material in children [10].

Tissue Transglutaminase Autoantibody Radioligand Binding Assay

Anti-tTG antibodies were also measured in radio ligand binding assays (RBA) as previously described [11, 12] with a few modifications. In short, tTG was produced using the *in vitro* coupled transcription and translation kit, TNT® SP6 Coupled Reticulocyte Lysate System (Promega Corp.) and [³⁵S] methionine (PerkinElmer™, Boston, MA, USA) with 1 µg plasmid. For the assessment of anti-tTG autoantibodies, a protein A Sepharose (PAS), which binds IgG of all subtypes except for IgG3 and some of IgA and IgM [13], was used to separate the free from antibody bound ³⁵S-tTG. Cut-off levels for a positive value was determined using quantile-quantile (QQ) plots to identify deviation from normality in the distribution of 398 healthy blood donors and set at ≥ 4 U/mL, representing the 95th percentile. This assay has previously demonstrated a 94% diagnostic sensitivity and 99% specificity in our CD material in children [14, 15].

Islet antibodies IA-2, Insulin antibodies IA, Glutamic acid decarboxylase antibodies GADA, Islet cell antibodies ICA, C-peptide, HbA1c, GLP-1, GIP and glucagon was measured as previously described [9, 16].

Genotyping

In both cohorts typing of the HLA-class II DRB 1 locus was performed by direct sequencing of exon 2 of DRB1 according to the Immuno Histocompatibility Working Group. DR 03 (DQ2) and DR 04/04 (DQ8) were the HLA groups we focused on in this study, since their connection to CD as previously described [1]. Genotyping of rs917997 (IL18rap), rs333 (CCR5), rs45450798 (PTPN2), rs2816316 (RGS1) was done using an in-house KASPar system at KBioscience, UK.

Statistics

Data analysis was carried out using the Statistical Analysis Software (SAS 9.2, Cary, NC USA). We used a compound symmetric repeated measurement model to estimate the impact of different variables (visit, age, sex, seasonality, HLA, autoimmunity and gender) on IgAG-DGP/tTG levels and the impact of IgAG-DGP/tTG levels on the variables C-peptide, HbA1c, GLP-1, GIP as well as glucagon. The outcome variable was log-

transformed to meet the assumption of a normal distribution where appropriate. All models were adjusted for visit, sex and age (linear) as well as HLA genotype. To test for differences at visit 12 month in Table 3, we used linear regression and chi-square. A p -value <0.05 was considered significant.

RESULTS

Prevalence of CDA

At the one, six and twelve month visit after T1D diagnosis, 24/238 (10.1%), 18/224 (8.0%) and 25/218 (11.5%) of the children with T1D were positive for IgAG-DGP/tTG as compared to 15/237 (6.3%), 13/222 (5.8%) and 16/216 (7.4%) for the IgG-tTG with the RBA, respectively. In total, 19/238 (8.0%) were positive for IgAG-DGP/tTG in two or more consecutive samples and 11/238 (4.6%) of the children were confirmed positive for IgG-tTG and consequently defined as having CDA. Two children were persistently IgG-tTG positive, but IgAG-DGP/tTG negative; in one of them the levels of IgG-tTG decreased from 929 to 13 U/mL whereas the level of the IgAG-DGP/tTG was just below the cut-off level of positivity (19.1 AU). The other child with discrepant results had high levels of IgG-tTG in all three samples (167, 154 and 160 U/mL), but were negative for IgAG-DGP/tTG. Antibody titers for both assays are shown in Figure 1A and 1B.

In children with initial positive levels, 13 children were IgAG-DGP/tTG transient as compared to 4

children who were IgG-tTG positive. Transient IgAG-DGP/tTG levels were lower in males (7.4% (SEM 2.9%)) as compared to females ($p=0.02$). IgAG-DGP/tTG levels in contrast to IgG-tTG decreased with age (8.0% (SEM 3.9%)) ($p=0.04$) when adjusted for HLA, gender and visit.

There was no correlation between levels of IgAG-DGP/tTG and GAD, IA2, ICA, GLP-1, GIP or glucagon nor HbA1c. T1D patients identified as being transient for any CD antibodies compared to those without any CD antibody, also had several other islet autoantibodies one month after onset (Figure 2).

HLA and other Non-HLA Loci

The HLA genotypes from the participants specified by country are shown in Table 1. The HLA distribution showed that 58.7. % in the cohort of children with T1D carried at least on allele DQ2, 62.5% at least one DQ8 and 8.5% had other HLA haplotypes than that of DQ2 and DQ8 (Table 2).

When stratifying for HLA, levels of IgAG-DGP/tTG were higher in those having either DQ2 and/or DQ8 compared to those without these HLA risk haplotypes (12.4 % \pm 5.7 %) ($p=0.04$). In addition, levels of IgAG-DGP/tTG were also higher in children with T1D carrying the genotype AA of IL18RAP (19.5 \pm 6.8 %) ($p=0.01$). A similar trend, albeit not significant, was found for children with high levels of IgAG-DGP/tTG

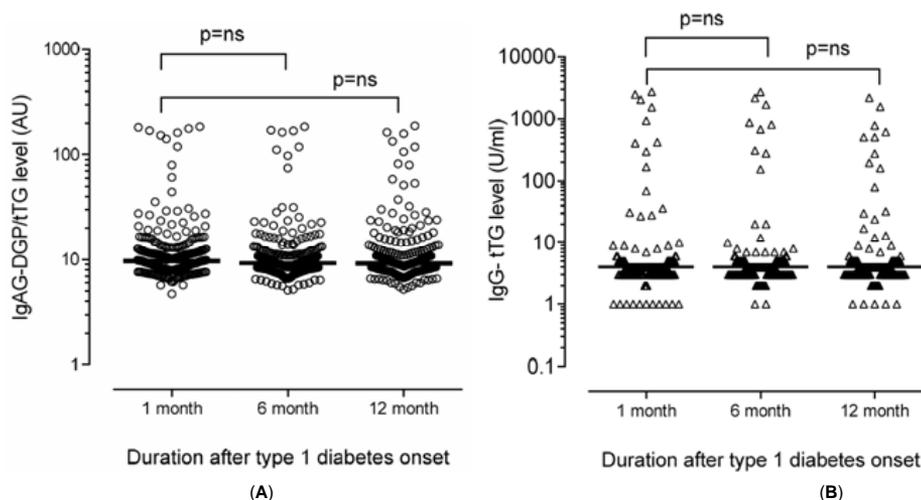


Figure 1: Antibody titers of IgAG-DGP/tTG (A) and IgG-tTG (B) at 1, 6 and 12 month after diabetes onset.

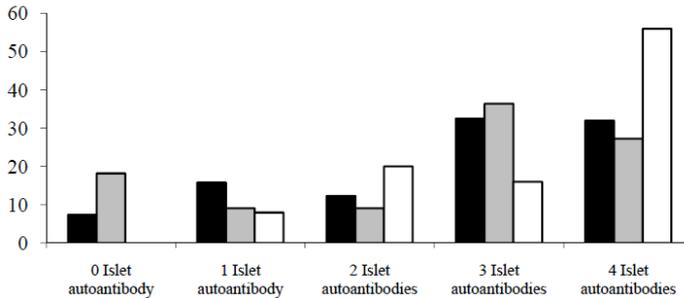


Figure 2: Number of children positive for one or more islet autoantibodies at one month after diagnosis of type 1 diabetes having celiac disease autoimmunity (CDA) (grey bars), being CDA transient (white bars) or free from CDA (black bars).

Table 1: Demographics of the Study Population by Country of Residence

Country	N	Mean Age (Years)	Mean HbA1c (%)*	Sex (F/M)	HLA-DQ2 and/or DQ8 (%)	T1D with Transient CDA (N)	T1D with CDA (N)
Belgium	11	6.8	7.5	9/2	100	0	1
Denmark	31	8.9	8.0	13/18	94	4	0
France	7	8.7	8.0	1/6	86	2	0
Germany(Ulm)	2	5.5	8.0	2/0	100	0	0
Germany (Berlin)	17	7.9	8.0	6/11	94	2	2
Holland	1	10.2	5.6	2/1	100	1	0
Ireland	16	9.4	8.6	8/8	100	1	0
Italy Parma	14	9.3	7.2	9/5	64	1	1
Italy Chieti	10	6.9	7.0	7/2	100	4	0
Japan	3	9.9	7.7	2/1	33	0	0
Macedonia	21	9.4	9.0	13/9	82	2	0
Norway	9	8.8	7.9	4/6	90	2	0
Portugal	27	8.8	7.7	13/15	93	0	2
Spain	6	9.6	6.7	4/2	100	1	0
Sweden	13	11.0	7.3	3/10	92	1	1
Switzerland	15	9.0	7.4	7/8	80	1	0
UK Leicester	14	10.0	8.1	9/5	100	2	3
UK Glasgow	11	9.8	7.3	7/4	91	1	1

*At 12 month visit. HbA1c was centrally analyzed at Steno Diabetes center.

and carrying CCR5 +/+ (7.8 +/- 4.0 %) (p=0.053) whereas no association was found for neither RGS1 nor PTPN2.

DISCUSSION

The fluctuation in CD antibody levels over time is well described in previous longitudinal prospective studies on T1D [17]. In line with previous studies [16, 18], we found a 5% prevalence of the children with T1D

being persistently positive for CD associated autoantibodies; a term which we coin celiac disease autoimmunity (CDA). We also found that children with T1D with transient CDA were likely to have low CD antibody levels and also being positive for multiple islet autoantibodies one month after diagnosis. Some had seroconverted to CD antibody negative despite of having initially high levels at diagnosis. The reason for this phenomenon of transient autoantibodies is unclear, but could be attributed to a general inflammatory state

Table 2: Distribution of HLA Genotypes among Children with T1D with and without Celiac Disease Autoimmunity (CDA)

HLA Genotype	T1D with CDA (n=11)	T1D without CDA (n=211)	T1D with Transient CDA (n=25)	All T1D (n=247)
DQ2/DQ2, n (%)	1 (9.1)	23 (10.9)	4 (16)	28 (11.3)
DQ2/DQ8, n (%)	3 (27.3)	64 (30.3)	12 (48)	79 (32.0)
DQ2/X, n (%)	3 (27.3)	32 (15.2)	3 (12)	38 (15.4)
DQ8/DQ8, n (%)	2 (18.2)	21 (10.0)	3 (12)	26 (10.5)
DQ8/X, n (%)	2 (18.2)	47 (22.3)	2 (8)	51 (20.)
X/X, n (%)	0 (0)	20 (9.5)	1 (4)	21(8.5)

X denotes other HLA genotypes than DQ2 or DQ8.

Table 3: Characteristics of 230 Children with T1D, with and without Celiac Disease Autoimmunity (CDA) 12 Month after Onset

Feature	T1D with CDA (n=11)	T1D with Transient CDA (n=24)	T1D without CDA (n=195)	P-Value
Age at onset (Median, range)	9.4 (3.0-14.2)	7.4 (2.7-13.5)	9.8 (0.2-16.3)	P=0.046
Gender (Boys/girls)	4/7	7/17	100/95	P=0.06
HbA1c* (Median, range)	7.6 (5.7-9.3)	7.7 (5.6-10.9)	7.7 (4.9-16.4)	P= 0.90
DQ2 or DQ8 /nonDQ2/nonDQ8/ Missing	11/0/0	23/1/0	172/19/4	P=0.80

*Visit 12 month.

of the gastrointestinal tract and a leaky gut that leads to an increased passage of multiple antigens. Still, it remains unresolved why only some of the patients with T1D develop CDA and others keep or develop tolerance to gluten antigens.

Earlier studies on the effect of glycemic control in patients with T1D have been conflicting, with some studies showing improvement and some deterioration on gluten-free diet [19, 20]. We did not find a correlation between levels of IgAG-DGP/TG and HbA1c. This could be due to small variations in HbA1c during the first year after diagnosis of T1D, but are also in line with a recent study on potential CD in patients with T1D where no significant difference in HbA1C between the groups with or without confirmed CD or potential CD were found [21].

GLP-1 is a gastrointestinal peptide hormone that regulates glucose homeostasis through stimulation of insulin secretion from pancreatic beta cells and promotes insulin synthesis [22, 23]. Along with GLP-1, GIP acts to stimulate insulin release. GIP has direct

anabolic effects on adipose tissue, by stimulating glucose import, fatty acid synthesis, lipogenesis and inhibiting lipolysis [24].

Our finding that there were no correlation between the levels of IgAG-DGP/TG and the gut hormones GIP and GLP-1 is in line with previous findings in untreated patients with CD, HLA susceptible for T1D [25].

As expected, we found a majority of our T1D patients with CDA to carry HLA-DQ2 (63.6%) and the remainder DQ8. However, near one third of the children with T1D without CDA also carried DQ2/DQ8 which suggests that additional genes associated with CD risk outside the HLA complex should be searched for. In a previous study, three CD non-HLA genes RGS1, IL 18RAP and TAGAP were found to be associated with T1D and two other T1D loci, CCR5 and PTPN2, were also found to be linked to CD [26]. Therefore, we extended our investigation to also include these non-HLA loci in our analysis [27]. Interestingly, we could only confirm a correlation between elevated levels of IgAG-DGP/TG and IL

18RAP. This gene encodes for the IL18 receptor and the binding of this receptor mediates the activation of IL18R1 which in co-expression with IL18 is required for the activation of NK-cells. Our finding in our cohort of children with T1D is particularly interesting since recent studies in our NOD mouse model demonstrate that a gluten-free diet reduces the level of NKG2D and the expression of NKG2D ligands (Clin Exp Immunol. 2014 Mar 28). We speculate that children with T1D carrying both AA IL 18RAP and HLA-DQ2 and/or DQ8 haplotypes, are at a higher risk of activating NK cells that leads chronic inflammation in the gut in CDA after exposure to dietary gluten.

Some limitations of the present study should be mentioned. Due to its retrospective design, a small-bowel biopsy was not performed to confirm the diagnosis of CD. Neither do we have any report of symptoms, diet or other coexisting diseases. The studied cohort is, however, non-selected and comprised of consecutively enrolled children of both genders and all ages as well as from different geographic regions and ethnicity. Furthermore, we used a three-step algorithm in order define those T1D children in our cohort to have highest risk for CD using a combination of both highly out-put sensitive and specific serological tests for CD to discriminate those who were most likely to have CDA in our T1D population. As we first screened all children with the IgAG-DGP/tTG, that previously yield a 100% sensitivity for CD [10], and then retested all samples with the highly specific IgG-tTG measured with a RBA method [28]. We did this in three consecutive samples to test for persistence, thus we feel confident that the children with persistent antibody positivity in both assays had ongoing CDA.

In conclusion, we demonstrate that CDA occur among 5% of children with T1D. We also confirm that HLA-DQ2 or DQ8 is necessary, but not sufficient to develop CDA, but that other non-HLA genes such as the IL18RAP may contribute to additional increased risk for CDA in a subpopulation of children with T1D.

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ABBREVIATIONS

CDA	=	celiac disease autoimmunity
CD	=	celiac disease
DGP	=	deamidated gliadin peptides
GIP	=	glucose-dependent insulinotropic polypeptide
GLP	=	glucagon-like peptide
HbA1c	=	glycated hemoglobin
HLA	=	human leukocyte antigen
IAA	=	insulin autoantibodies
IA-2	=	islet antibodies 2
ICA	=	islet cell autoantibodies
GAD	=	Glutamic acid decarboxylase antibodies
PAS	=	protein A sepharose
RBA	=	radioligand binding assays
T1D	=	Type 1 diabetes
tTG	=	tissue transglutaminase

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Paper III

A gluten-free diet lowers NKG2D and ligand expression in BALB/c and non-obese diabetic (NOD) mice

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Summary

The interplay between diet and immune parameters which could affect type 1 diabetes (T1D) pathogenesis is not sufficiently clarified. Intestinal up-regulation of the activating receptor natural killer group 2D (NKG2D) (CD314) and its ligands is a hallmark of coeliac disease. However, the direct effect of gluten on NKG2D expression is not known. We studied, by fluorescence activated cell sorter (lymphoid tissues) and reverse transcription-quantitative polymerase chain reaction (intestine and pancreatic islets), if a gluten-free diet (GF diet) from 4 weeks of age or a gluten-free diet introduced in breeding pairs (SGF diet), induced changes in NKG2D expression on DX5⁺(CD49b) natural killer (NK) cells, CD8⁺ T cells and in intestinal and islet levels of NKG2D and ligands in BALB/c and non-obese diabetic (NOD) mice. Gluten-free NOD mice had lower insulinitis ($P < 0.0001$); reduced expression of NKG2D on DX5⁺ NK cells in spleen and auricular lymph nodes ($P < 0.05$); and on CD8⁺ T cells in pancreas-associated lymph nodes ($P = 0.04$). Moreover, the level of CD71 on DX5⁺ NK cells and CD8⁺ T cells ($P < 0.005$) was markedly reduced. GF and SGF mice had reduced expression of NKG2D and DX5 mRNA in intestine ($P < 0.05$). Differences in intestinal mRNA expression were found in mice at 8, 13 and 20 weeks. Intestinal expression of NKG2D ligands was reduced in SGF mice with lower expression of all ligands. In isolated islets, a SGF diet induced a higher expression of specific NKG2D ligands. Our data show that a gluten-free diet reduces the level of NKG2D and the expression of NKG2D ligands. These immunological changes may contribute to the lower T1D incidence associated with a gluten-free diet.

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Keywords: coeliac disease, gluten, gluten-free diet, NKG2D, type 1 diabetes

Introduction

Type 1 diabetes (T1D) and coeliac disease (CD) are both autoimmune diseases, and there is a high prevalence of patients with both diseases. Studies have revealed an average prevalence of CD among children with T1D on 2–12% and patients with CD have earlier diabetes onset [1,2]. Intake of gluten is known to affect the disease process leading to T1D. Thus, the highest incidence of disease in animal models of T1D [biobreeding (BB) rats and non-obese diabetic (NOD) mice] is found in those animals which are on a diet based on cereals [3–5], and a gluten-free (GF) diet prevents diabetes development in NOD mice [6]. The T1D incidence of

NOD mice is further reduced in animals that have never been in contact with gluten, even during fetal development [7]. Human studies have further supported the effect of a gluten-free diet on T1D development [8,9], and T1D patients without CD are found to have an abnormal immune reactivity to gluten [10,11] independent of CD-associated haplotype, which could indicate that the response to wheat proteins is diabetes-specific [12]. The potential of gluten to affect the diabetogenic process seems to be dependent upon the time of gluten introduction, both in animals [13,14] and humans [15,16].

In addition to diet, the importance of intestinal microflora on the development of type 1 diabetes has

become evident, and it has been found that gluten intake directly affects the composition of the intestinal microflora: NOD mice fed a gluten-free diet have reduced numbers of caecal bacteria and Gram-positive bacteria compared to mice fed a wheat-containing standard diet [17], and a gluten-containing diet specifically increased intestinal species of *Bifidobacterium*, *Tannerella* and *Barnesiella*, whereas *Akkermansia* was increased in microflora of NOD-mice fed a gluten-free diet [18]. Therefore, it cannot be excluded that diet-induced changes in intestinal immunity could be influenced by corresponding microbiota changes. However, Patrick *et al.* showed that a diet based on cereals was a stronger promoter of type 1 diabetes than gut microbes and more significant protection from disease was observed by a hydrolyzed casein-based diet than could be obtained by altering the microbiota environment (germ-free conditions) [19].

The intestinal immune system plays an important role in the pathogenesis of T1D, as diabetogenic T cells are activated in the intestinal compartment before infiltrating the pancreas [20,21]. However, the effect of gluten on different cell populations of the immune system has not been completely clarified. We have shown recently that gluten intake influences the proportion of multiple regulatory T cell subsets as well as T helper type 17 (Th17) cells in lymphoid tissues [22] and modify the cytokine pattern of both forkhead box protein 3 (FoxP3)⁻ T cells and FoxP3⁺ regulatory T cells towards a more inflammatory cytokine profile [23].

Natural killer group 2D (NKG2D) (CD314) is an activating receptor on NK cells, CD8⁺ T cells, NKT cells and $\gamma\delta$ ⁺ T cells [24,25]. Interleukin (IL)-15-induced up-regulation of NKG2D on intraepithelial cytotoxic T cells (IELs) and NKG2D ligands on enterocytes is a hallmark in CD and is responsible for epithelial cell destruction and subsequent villous atrophy [25]. NKG2D is also known to be involved in the development of T1D and seems to be essential for disease progression, as blockade of NKG2D prevents diabetes in NOD mice [26,27]. Furthermore, patients with T1D have aberrant signalling through the NKG2D receptor [28,29]. In NK cells, NKG2D acts as an activating receptor to mediate target cell killing, and in CD8⁺ T cells the ligation of NKG2D delivers a co-stimulatory signal to the T cell. Normally, T cell receptor (TCR) ligation triggers the armed CD8⁺ T cells to mediate target cell killing. Simultaneous ligation of TCR and NKG2D increases the cytolytic response [30] and the cytotoxic IELs become lymphokine-activated killer (LAK) cells with NK-like cytolytic function, i.e. the ability to kill target cells independent of TCR specificity [31].

The NKG2D receptor can bind to a number of ligands that are all distantly related to major histocompatibility complex (MHC) class I. In humans, MICA and MICB (MHC class I chain-related) are well-described ligands [32,33]. MICA and MICB have no mouse homologues, but

are related closely to the mouse proteins Mill1 and Mill2 [34]. Other ligands include the ULBP-family (UL16 binding protein) with the mouse homologue Mult1 [35], H-60 (minor histocompatibility antigen) [36] and the Rae-1 family [37]. All these ligands are selectively up-regulated in target cells by stress or infection, and act to induce NKG2D-mediated killing of the target cells [38].

Evidence is pointing towards a role for gluten in the development of T1D. In the multi-factorial pathogenesis of T1D, gluten could play a role in the complex interplay between predisposing genes, defective immune-regulation and inflammatory priming of the mucosal immune system. Thus, we wished to study the effect of a GF diet on specific immune populations, known to be involved in CD and T1D. The aim of the present study was to clarify the effect of a gluten-free (GF) diet on NKG2D levels on DX5⁺ (CD49b) NK cells and CD8⁺ T cells isolated from lymphoid tissues: spleen (S), pancreatic lymph nodes (PLN) and auricular lymph nodes (ALN) as control peripheral lymphoid tissue. These were compared to mice receiving the diabetes-promoting gluten-containing standard diet (STD). The experiments were performed in the NOD mice to study potential diabetes-related mechanisms as well as in fully immunocompetent BALB/c mice, to clarify the effect of gluten intake in normal healthy animals. Moreover, we studied diet-induced differences in NKG2D expression in intestine and isolated pancreatic islet and NKG2D ligand expression.

Materials and methods

Animals

Fluorescence activated cell sorter (FACS) studies were performed on 13-week-old BALB/c and NOD mice on a GF *versus* gluten-containing standard diet (STD) from 4 weeks of age: BALB/cJ BomTac were purchased from Taconic Europe A/S (Ry, Denmark) and NOD mice were delivered from Taconic US, purchased from Taconic Europe A/S. Both BALB/c mice and NOD mice arrived at 4 weeks of age and were divided equally in groups receiving the GF *versus* the STD diets upon arrival. Nine mice from each group were used for FACS studies and 11 mice from each group used for reversed transcription-quantitative polymerase chain reaction (RT-qPCR). These animals were also used in Larsen *et al.* ('Effects of dietary gluten on murine natural killer cell activity' 2014, unpublished data). To study if the effect of a GF diet on selected immune parameters could be influenced by the timing of gluten exposure, we bought breeding pairs of NOD mice (Taconic US, Taconic Europe A/S) and divided them into two groups receiving either GF or STD diet during breeding. Therefore, the pups were only exposed to one diet (either GF or STD) both *in-utero*, during weaning and after weaning. These groups of animals were named strictly gluten-free (SGF) *versus* strictly

standard diet (SSTD) and were used to study NKG2D and NKG2D-ligand expression in intestinal tissue and isolated islets by RT-qPCR and immunohistochemistry. Moreover, the animals were used to determine diet-induced differences in lymphocyte infiltration (insulinitis scoring) in 20-week-old mice.

First-generation female offspring (12 in each group) were used in the study when 8, 13, 20 weeks old to study if the effect of diet was in the prediabetic phase or later in the disease development.

The mice were kept in a specific pathogen-free (SPF) animal facility (temperature $22 \pm 2^\circ\text{C}$, 12-h light cycle, air changed 16 times per hour, humidity $55 \pm 10\%$) with free access to water and food. The animal experiments were carried out with approval from The National Animal Experimentation Board (2012-15-2934-00086), and experiments were performed in accordance with international guidelines for the care and use of laboratory animals.

Diets

The animals received either the STD, non-purified Altromin diet or a GF, modified Altromin diet (Altromin, Lage, Germany), shown previously to prevent diabetes development in NOD mice [6,7]. Both experimental diets were nutritionally adequate with a similar level of protein, amino acids, minerals, vitamins and trace elements. These two diets have been used previously at The Bartholin Institute to study the effect of a GF diet on diabetes incidence in NOD mice [6,7]. The exact composition of the STD and the GF diet is given in [6,7]. The diets were prepared to ensure the same content of milk and soya proteins, found previously to be diabetogenic. The overall protein contents of the STD and the GF diets were similar (22.9 *versus* 22.9%). The only component that differs between the two diets is that the gluten-containing proteins in the STD diet (6.9%) are replaced by more animal proteins in the GF diet. This results in a slightly increased level of animal proteins in the GF diet (15.3% *versus* the STD diet (8.4%). With regard to this slight increase in animal proteins, it cannot be excluded that this could have an effect. However, it has been shown previously that meat meal as the protein source promotes development of T1D in NOD mice [39], suggesting an even stronger diabetes-protective effect of the gluten-free diet due to its higher level of animal proteins. The two diets had the same content of amino acids, minerals, vitamins and trace elements. The weight of the mice was monitored and both groups of animals displayed similar weight distribution.

FACS antibodies

The following monoclonal antibodies (mAb) were purchased from BD Pharmingen (San Jose, CA, USA): allophycocyanin (APC)-H7-conjugated rat anti-mouse CD8a

mAb [immunoglobulin (Ig)G2a, κ ; catalogue number: 560182]; fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD71 mAb (IgG11, κ ; catalogue number: 553266). Phycoerythrin (PE)-conjugated rat anti-mouse CD49b (DX5), mAb (IgM, κ ; catalogue number: 48597182), PE-cyanin 7 (Cy7)-conjugated rat anti-mouse CD314 (NKG2D) and mAb (IgG21, κ ; catalogue number: 25588282) were purchased from eBioscience (San Diego, CA, USA).

Cell purification and flow cytometry

Mice were killed and spleen (S), pancreas-draining lymph nodes (PLN) and auricular lymph nodes (ALN) were isolated from 13-week-old BALB/c and NOD mice on GF and STD diets. The ALN were chosen as control lymphoid organs to show whether or not the effect of gluten was strictly confined to PLN, or if gluten also has an effect on systemic immunity, as shown previously [22,23]. Cells from each organ were pooled and single-cell suspensions were prepared. Surface staining was initiated with use of the relevant mAb, and cells were incubated for 1/2 h. Fc block (CD16/CD32) was purchased from BD Pharmingen (2-4G2; IgG2b, κ) and added to reduce Fc receptor-mediated binding. The cells were fixed and subsequently analysed by flow cytometry using a LSR-II (BD Bioscience, San Jose, CA, USA), and data were analysed with use of FACS diva software (BD Bioscience). Isotype control antibodies were used to determine the amount of non-specific binding and the AmCyan-conjugated LIVE/DEAD fixable aqua dead cell staining kit were purchased from Invitrogen (Carlsbad, CA, USA) (catalogue number: L34957) to exclude dead cells.

Islet isolation

Islets of Langerhans were isolated from single BALB/c or NOD mice using collagenase digestion. Following injection of collagenase (754 U/ml; Sigma-Aldrich, St Louis, MO, USA) in RPMI-1640 into the pancreatic duct, the pancreas was removed and subjected to further collagenase digestion [40]. Islets were hand-sorted to obtain 90–95% pure islet isolates, estimated by visual inspection. Isolated islets were immediately put into Trizol (Invitrogen) for RNA extraction.

RNA isolation

Intestinal sections were kept in RNA later until RNA extraction, where they were transferred to Trizol and homogenized mechanically using a Polytron (Kinetica, Lucerne, Switzerland). Isolated islets were not homogenized prior to RNA extraction. Total RNA was isolated from islets or intestinal sections using Trizol reagent (Invitrogen).

RNA yield and quality were assessed on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

qRT-PCR

RNA was extracted from a number of tissues for qPCR analyses. Duodenum ($n = 11$) and isolated islets ($n = 4$) samples from the BALB/C and NOD mice introduced to gluten at 4 weeks were used for qPCR. Duodenum sections from mice of 8, 13 and 20 weeks of age ($n = 12$, $n = 8$, and $n = 11$) from NOD mice that were kept strictly gluten-free were used for qPCR along with isolated islets ($n = 11$) from 13-week-old mice.

Approximately 1.0 μg of total RNA was reverse-transcribed into cDNA using the qScript kit (Quanta Biosciences, Gaithersburg, MD, USA), as recommended by the manufacturer.

Specific mRNA levels were quantified on a Lightcycler II (Roche, Penzberg, Germany) using SYBR II qPCR mixture (Takara Bio, Otsu, Japan). Primers (see Supporting information) were designed for an anneal temperature of 61°C using Primer3 software [41] and synthesized by TAGCopenhagen (Copenhagen, Denmark). Diluted, purified and sequence-verified (GATC Biotech, Constance, Germany) PCR products were used to create a standard curve for each primer pair. Expression levels were calculated as absolute quantification in relation to the relevant standard curve, using Lightcycler Software version 4.05. Expression values were then normalized to the housekeeping gene beta actin. Cycling parameters were set up to obtain similar reaction efficiencies of between 1.9 and 2.0.

The parameters for qPCR cycling were: initial denaturation at 95°C followed by 45 cycles of 10 s denaturation at 95°C, 5 s anneal at primer-specific temperature (56–60°C) and 15 s extension at 72°C.

Following PCR, a melting curve analysis was conducted. Any reactions with a CP value above 40 or with a non-specific peak in the melting curve analysis were treated as negative reactions and given the value '0' for statistical analysis.

Histology and immunohistology

NOD SSTD and SGF mice at 20 weeks of age were used to determine diet-induced differences in lymphocyte infiltration (insulinitis scoring). Haematoxylin and eosin-stained pancreas sections were evaluated for the insulinitis score using the following scale: (i) intact islets, (ii) peri-insulinitis, (iii) moderate insulinitis (50% of the islets infiltrated) and (iv) severe insulinitis (> 50% of the islets infiltrated) [7]. At least 20–25 islets from each mouse were scored blind.

Immunohistochemical staining was performed on snap-frozen sections of small intestine. Tissues were fixated in Stefanini, rinsed in phosphate-buffered saline (PBS) and incubated with 2.5% bovine serum albumin (BSA) in PBS for 1 h and then incubated with primary anti-NKG2D (Santa Cruz NKG2D s-c5494; Santa Cruz Biotechnology,

Santa Cruz, CA, USA) 1:50 and anti-CD3 (ab16669; Abcam, Cambridge, UK) 1:100 in 1% BSA for 2 h. Binding was visualized using donkey anti-goat Cy3 (705-166-147; Jackson ImmunoResearch, West Grove, PA, USA) (1 : 200) and donkey anti-rabbit Alexa Fluor 488 (711-546-152; Jackson ImmunoResearch) (1 : 200). Vectashield 4',6-diamidino-2-phenylindole (DAPI) was used to visualize nuclei.

Statistical analysis

Groups were compared pairwise by Student's *t*-test. The χ^2 method was used on the insulinitis score data. $P < 0.05$ was considered significant. All data are shown as mean \pm standard error of the mean (s.e.m.). Statistical significance is indicated with one, two or three asterisks in the figures, signifying levels of 0.05, 0.01 and 0.001, respectively. The groups were only compared pairwise: GF *versus* STD and SGF *versus* SSTD, unless specified otherwise.

Results

Decreased NKG2D expression in mice receiving GF diet on DX5⁺ cells in S and ALN

Cell suspensions were prepared from isolated lymphoid organs (S, PLN and ALN) from 13-week-old mice on a GF *versus* STD diet and stained for DX5, CD8, NKG2D and CD71. Because BALB/c mice do not express the NK1.1 marker we used the anti-CD49b (clone DX5) pan-NK mAb, which has been shown previously to overlap with NK1.1 staining in C57Bl/6 mice as a marker for NK cells [42]. However, DX5 is also expressed on NKT cells [43,44]. By gating on DX5⁺ cells (gating shown in Fig. 1a), we analysed the expression of NKG2D on DX5⁺ cells. The mean fluorescence intensity (MFI) on NKG2D was decreased by 21.6% in the S of NOD mice receiving the GF diet (Fig. 1b, $P = 0.0059$, $n = 3$). No significant changes were found in PLN from BALB/c or NOD mice (Fig. 1c). In the ALN (Fig. 1d), NKG2D was decreased by 35.5% ($P = 0.0371$, $n = 3$) and in BALB/c by 28.7% ($P = 0.0249$, $n = 3$) in NOD receiving the GF diet.

We also analysed NKG2D expression on CD8⁺ as a marker for cytotoxic T cells, and found a 29% ($P = 0.0351$, $n = 3$) increase in S (Fig. 2b) of BALB/c mice receiving the GF diet and an 8.8% ($P = 0.0394$) decrease in the PLN (Fig. 2c) of NOD mice receiving the GF diet. No changes were seen in ALN.

The GF diet decreases proliferation marker, CD71, on DX5⁺ and CD8⁺ cells in S and PLN and ALN

To determine gluten-induced changes in the proliferation of DX5⁺ NK cells and CD8⁺ T cells, we gated on the two cell

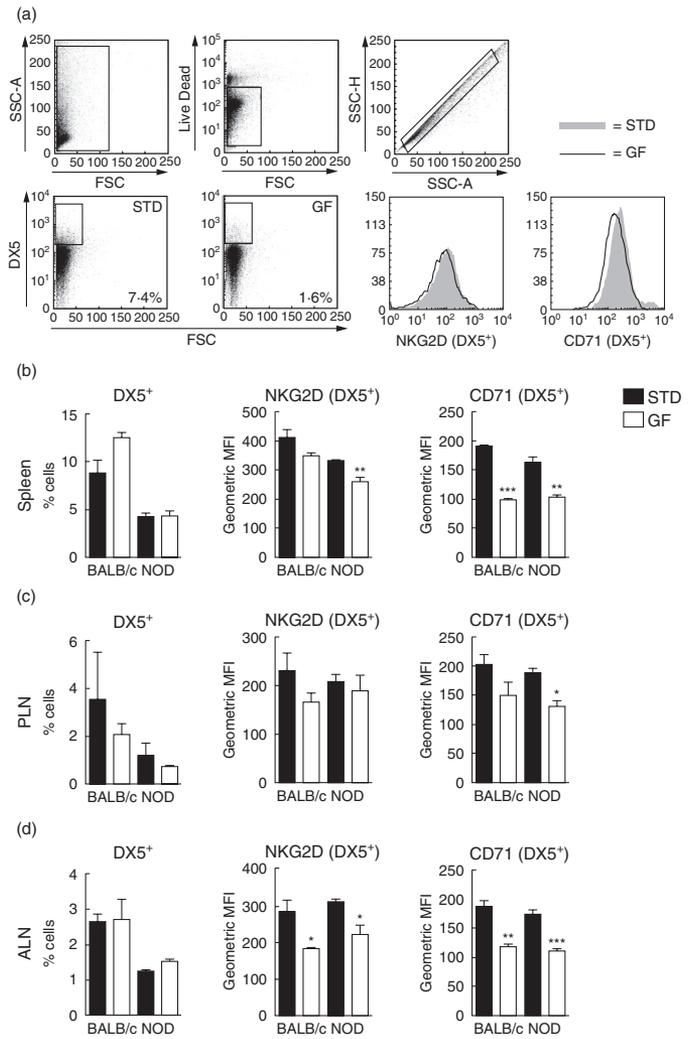


Fig. 1. Reduced level of CD71 and NKG2D expression on NK cells in mice on a gluten-free (GF) diet. (a) Representative plot of lymphocyte, singlet and live gate (upper panel). Examples of dot-plots showing the gating and percentages of DX5⁺ cells in BALB/c standard diet (STD) and BALB/c GF mice and histograms showing expression of CD71 and NKG2D on DX5⁺ cells in BALB/c and NOD spleen (S). Data are represented as mean values \pm standard deviation of three independent experiments with three mice in each group. Black bars: STD diet; white bars: GF diet. (c) The same representation as in (b) in pancreatic lymph nodes (PLN); (d) the same representation as in (b) in aricular lymph nodes (ALN). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

subsets and analysed the MFI of the transferrin receptor CD71. Transferrin receptor CD71 is a transmembrane homodimer glycoprotein, involved in uptake of iron and cell growth. The receptor expression is correlated with cellular proliferation and is thus expressed at greater levels on cells with a high proliferation rate [45–48]. We found a significant lower expression of CD71 on DX5⁺ cells in S of BALB/c (48%, $P < 0.0001$) and NOD (37%, $P = 0.0036$) mice receiving the GF diet (Fig. 1b). We found a comparable decrease in the PLN of NOD mice (30%, $P = 0.018$) receiving the GF diet (Fig. 1c). In ALN from both BALB/c and NOD mice we also found significant decreases (37%,

$P = 0.0028$ and 36%, $P = 0.0009$) in mice receiving the GF diet (Fig. 1d).

When gating on CD8⁺ cells we found a significant decrease of CD71 expression with the GF diet in both BALB/c and NOD mice in all organs analysed. In S (Fig. 2b) the decrease of CD71 was 32% ($P = 0.0002$) for BALB/c mice and 29% ($P = 0.0087$) for NOD mice. In PLN (Fig. 2c) the decrease of CD71 was 60% ($P = 0.0023$) for BALB/c mice and 50% ($P = 0.0073$) in NOD mice. Comparable effects were seen in ALN, where the decrease of CD71 was 52% ($P = 0.0001$) for BALB/c mice and 45% ($P = 0.0001$) in NOD mice.

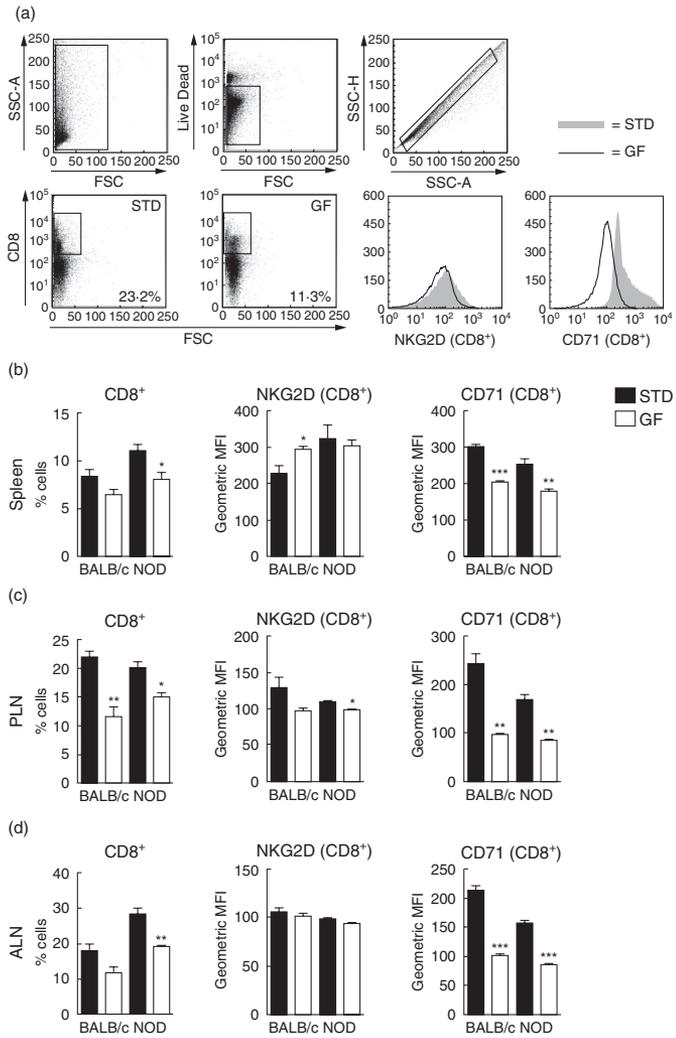


Fig. 2. Reduced level of CD71 expression and CD8⁺ cells in mice on a gluten-free (GF) diet. (a) Representative plot of lymphocyte, singlet and live gate (upper panel). Examples of dot-plots showing the gating and percentages of CD8⁺ cells in BALB/c standard diet (STD) and BALB/c GF mice and histograms showing expression of CD71 and NKG2D on CD8⁺ cells. (b) Bars represent percentages of CD8⁺ cells, and geometric mean fluorescence intensity (MFI) of CD71 and NKG2D gated on CD8⁺ cells in BALB/c and non-obese diabetic (NOD) spleen (S). Data are represented as mean values \pm standard deviation of three independent experiments with three mice in each group. Black bars: STD diet; white bars: GF diet. (c) The same representation as in (b) in pancreatic lymph nodes (PLN); (d) the same representation as in (b) in auricular lymph nodes (ALN). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

GF diet decreases CD8⁺ cells in S, PLN and ALN of BALB/C and NOD mice

We found a reduced proportion of CD8⁺ cells in the S of NOD mice receiving the GF diet (33%, *P* = 0.0338) (Fig. 2b). In PLN we found reduced CD8⁺ cells in BALB/c (47%, *P* = 0.0338) and NOD (25%, *P* = 0.0294) mice receiving the GF diet (Fig. 2c). Comparable effects were seen in ALN, where we found a reduction of 32% (*P* = 0.0046) in NOD mice receiving the GF diet (Fig. 2c).

GF diet affects NKG2D and DX5 mRNA expression in intestine and islets

We performed qRT-PCR on NKG2D and DX5 mRNA in intestines and isolated islets from 13-week-old mice, comparing GF or SGF diets with matched controls (STD/SSTD). Expression levels in intestinal tissue were markedly altered by diet (Fig. 3a). NKG2D and DX5 expression levels were reduced significantly in intestinal tissue in GF and SGF mice compared to controls. NKG2D levels were reduced to

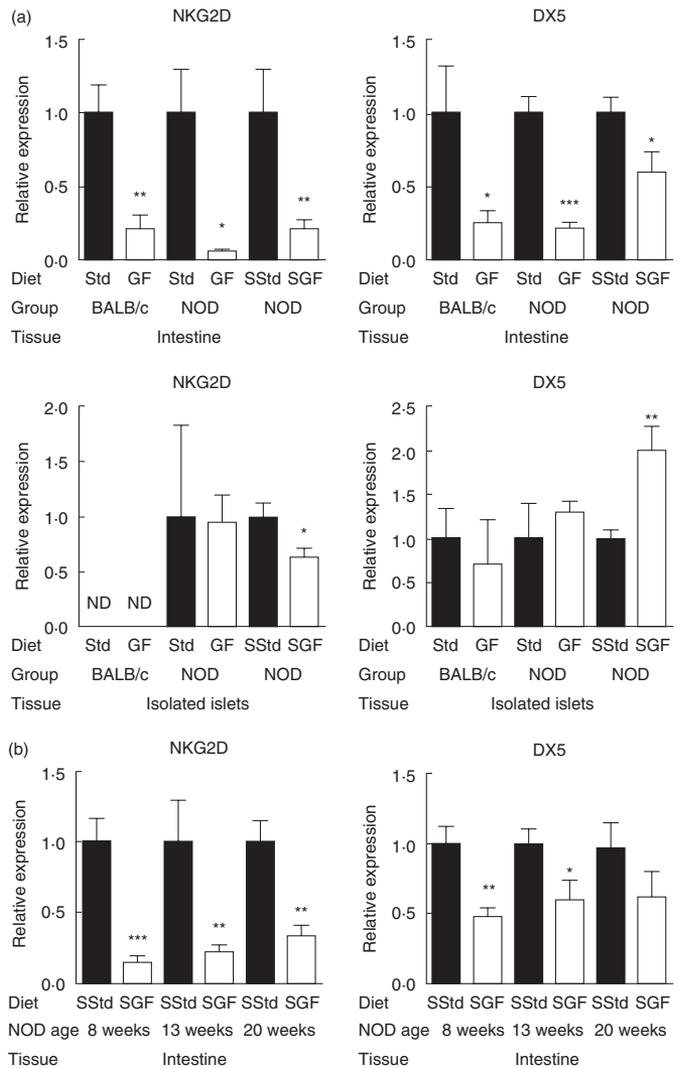


Fig. 3. Reduced mRNA levels of NKG2D and DX5 in gluten-free (GF) mice. (a) Relative messenger RNA expression levels of NKG2D and DX5 in tissues from 13-week-old mice kept on a gluten free (GF), strictly gluten-free (SGF) or matched controls, standard diet (STD) and strictly standard diet (SSTD). Expression levels of NKG2D and DX5 in intestinal tissue (upper graphs). NKG2D and DX5 expression levels in islets (lower graphs). (b) Relative messenger RNA levels of NKG2D and DX5 in intestines from SGF-fed non-obese diabetic (NOD) mice at 8, 13 and 20 weeks of age. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

21, 6 and 22% in BALB/c, NOD and NOD SGF mice, respectively ($P = 0.002$, $P = 0.01$, $P = 0.003$; $n = 11$). DX5 levels were also reduced in GF and SGF mice of both BALB/c and NOD genetic backgrounds to 26, 22 and 60% of controls, respectively ($P = 0.03$, $P < 0.0001$, $P = 0.002$; $n = 11$).

In isolated islets (Fig. 3a), NKG2D and DX5 expression levels in islets only changed significantly in SGF animals where NKG2D expression was reduced to 64% ($P = 0.04$; $n = 8$), and DX5 expression was doubled compared to animals fed the SSTD diet ($P = 0.003$; $n = 8$). No changes were observed in GF animals.

Prediabetic mRNA expression of NKG2D and DX5 in mouse tissues

To investigate the effect of a GF diet on NKG2D and DX5 down-regulation at different time-points, qRT-PCR was performed on intestines from SGF NOD mice of different ages.

NKG2D and DX5 expression levels were consistently lower in intestinal tissue of SGF mice compared to SSTD-fed controls (Fig. 3b). This effect was seen for animals of 8, 13 and 20 weeks of age. The SGF NOD mice had significantly lower expression of NKG2D at all measured time-points,

amounting to 15, 22 and 34% of SSTD at 8, 13 and 20 weeks, respectively ($P < 0.0001$, $P = 0.003$ and $P = 0.005$). DX5 expression was only significantly lower at 8 and 13 weeks, amounting to 48, 60 and 61% of SSTD at 8, 13 and 20 weeks, respectively ($P = 0.002$, $P = 0.03$ and $P = 0.20$). NKG2D expression was also compared between different age groups (Supporting information, Fig. S1). The NKG2D expression level of the SSTD groups was lower at 20 weeks than at both 8 and 13 weeks (38% of 8 weeks, $P = 0.004$). In the SGF groups, NKG2D expression was higher at 13 weeks compared to 8 and 20 weeks (260% of 8 weeks, $P = 0.01$).

DX5 expression levels decreased with mouse age in both SSTD and SGF groups (Supporting information, Fig. S1). The decrease was significant for all SSTD groups, with expression levels at 13 and 20 weeks that amounted to 43 ($P = 0.0009$) and 25% ($P < 0.0001$), respectively. The decrease was less prominent in SGF groups, where only the expression at 20 weeks was significantly lower than at 8 weeks (33%, $P = 0.004$).

GF diet affects the expression of NKG2D ligands

To study a direct effect of gluten on NKG2D ligand expression, we performed qPCR analyses of a number of known NKG2D ligands in mouse intestines and isolated islets.

The intestinal expression pattern of NKG2D ligands (Fig. 4a) did not change greatly in BALB/c mice fed a GF diet compared to a STD diet. Only the ligand H60a was reduced significantly to 42% of STD controls ($P = 0.01$; $n = 11$).

In NOD mice fed a GF diet, the expression level of Mill1 was increased 4.9-fold ($P = 0.0001$; $n = 11$), the level of Mill2 decreased to 20% ($P = 0.02$; $n = 11$) and the level of Mult1 increased threefold ($P = 0.03$; $n = 11$) compared to STD mice.

The expression levels of all NKG2D ligands were reduced in SGF NOD mice compared to SSTD controls. Thus, Mill1, Mill2, Mult1, Rae-1 and H60a was expressed at levels corresponding to 11 ($P = 0.0004$), 35 ($P = 0.01$), 45 ($P = 0.003$), 27 ($P = 0.001$) and 52% ($P = 0.26$) of controls, respectively (all $n = 12$).

A different pattern of NKG2D ligand expression was observed in isolated islets (Fig. 4b). None of the investigated ligands showed a significant change in expression level in GF BALB/c mice (upper graph) or NOD mice (middle graph) ($n = 4$). In the SGF NOD mice (lower graph), the expression of Mill1, Mill2 and Mult1 was increased significantly compared to SSTD controls, with non-significant increases observed in the expression levels of the remaining ligands. The increased expression amounted to 23-fold ($P = 0.01$), fivefold ($P = 0.05$) and 1.7-fold ($P = 0.02$) for Mill1, Mill2 and Mult1, respectively (all $n = 11$).

The NKG2D ligands Mult1 (top) and Rae-1 (bottom) showed interesting expression patterns in intestines (Fig. 5c). Thus, while Mult1 expression was increased three-

fold ($P = 0.03$; $n = 11$) in NOD GF mice, it was consistently lower in SGF mice at 8, 13 and 20 weeks, corresponding to a reduction to 54 ($P = 0.001$; $n = 12$), 45 ($P = 0.003$; $n = 12$) and 70% ($P = 0.15$; $n = 9$), respectively.

Rae-1 expression was also reduced for all GF and SGF groups, with the only significant effects for SGF mice. The GF diet reduced Rae-1 expression to 81% in BALB/c mice ($P = 0.83$, $n = 11$), 54% in NOD GF mice ($P = 0.25$, $n = 11$), 31% in 8-week SGF mice ($P = 0.01$, $n = 12$), 27% in 13-week SGF mice ($P = 0.001$, $n = 12$) and to 31% of controls in 20-week SGF mice ($P = 0.006$, $n = 8$).

Decreased islet infiltration in animals receiving SGF diet

Haematoxylin and eosin-stained (H&E) pancreatic sections were evaluated for islet infiltration. The insulinitis score was 2.1 ± 0.05 for the SGF NOD mice and 2.5 ± 0.05 for the SSTD NOD mice ($P < 0.0001$). Severe insulinitis (>50% of the islet infiltrated) was rarely observed in the SGF NOD mice, and only few intact islets were found in the SSTD NOD mice. Using immunohistochemical staining we showed the presence of NKG2D-positive cells in intestinal tissue of NOD mice (Fig. 5).

Discussion

This study provides new evidence concerning the effect of dietary gluten on immune parameters associated with disease development, and may help to understand the effect of a GF diet observed both in animal models [6,7] of disease as well as in humans [9,15,16]. We studied the effect of a GF diet in both fully immune-competent BALB/c mice and in NOD mice. This allowed us to study the effect of gluten both in healthy animals and in those predisposed to disease.

We have shown that the GF diet reduces the expression of NKG2D on DX5⁺ NK cells in S and ALN, and on CD8⁺ T cells in PLN of NOD mice. Furthermore, a GF and SGF diet diminishes the intestinal expression of NKG2D in both BALB/c and NOD mice, and a SGF diet reduces NKG2D expression in isolated islets from NOD mice. The SGF diet induced a significantly lower expression of NKG2D ligands in intestine from NOD mice. Interestingly, a GF diet markedly reduced the expression of the proliferation marker (CD71) in both DX5⁺ NK cells and CD8⁺ T cells. The reduced proliferation was found in all lymphoid organs tested and was profound both in BALB/c mice and in NOD mice.

Our study shows that a diet without gluten reduces the level of NKG2D on DX5⁺ NK cells and CD8⁺ T cells and we have consistently found lower NKG2D mRNA expression in tissues from mice on GF or SGF diets. That this effect is most prominent in intestinal tissue is not surprising, given that this is the tissue in direct contact with gluten digestion

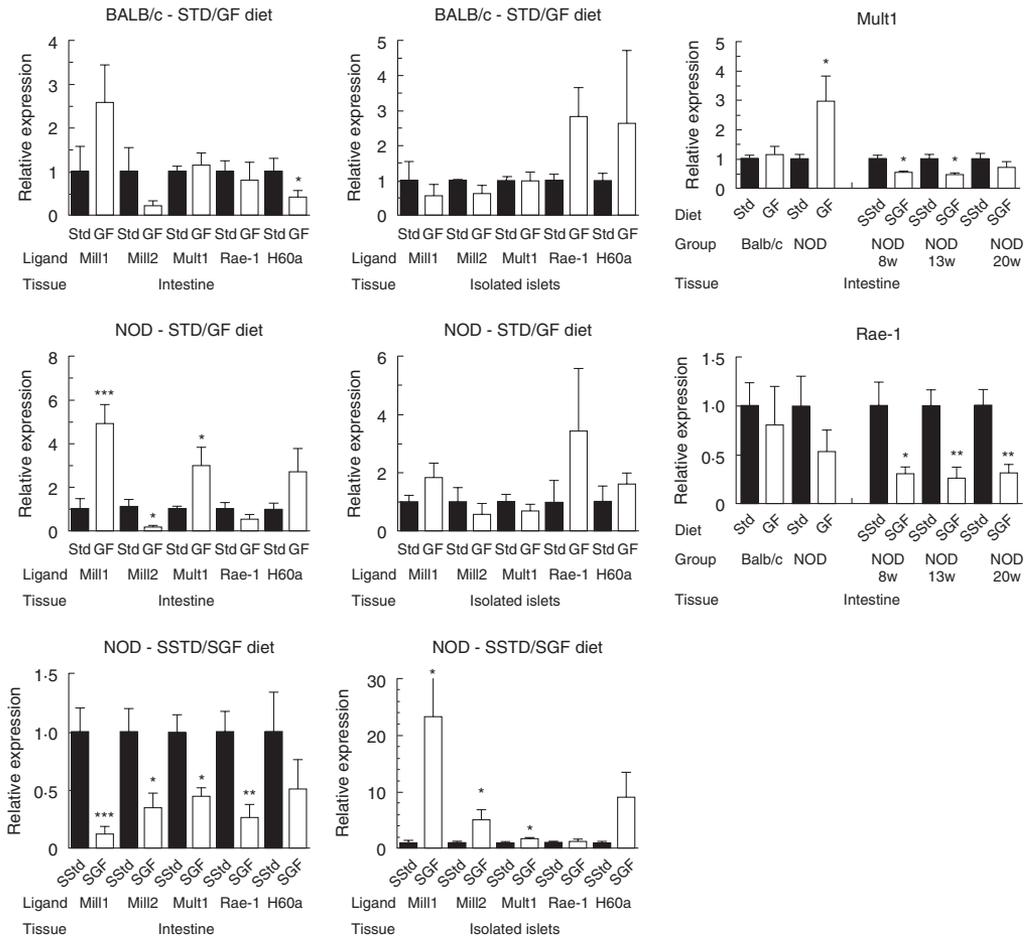
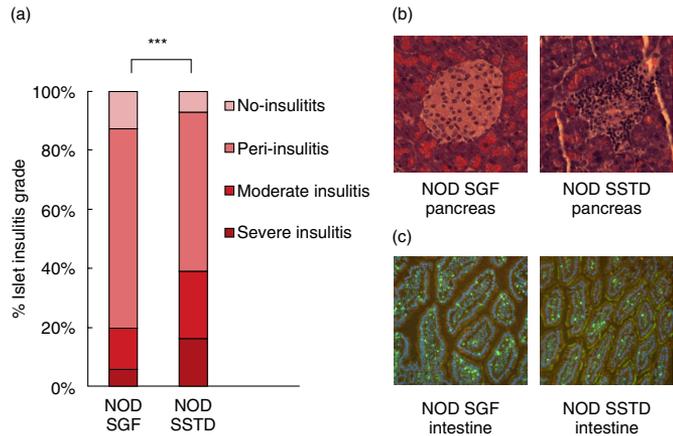


Fig. 4. Expression patterns of NKG2D ligand mRNAs in intestine and islets from BALB/c and non-obese diabetic (NOD) mice. Relative messenger RNA levels of known NKG2D ligands were quantified in intestine and islets. (a) The expression pattern of NKG2D ligands in intestinal tissue from 13-week-old BALB/c, NOD and NOD strictly gluten-free (SGF) mice. (b) Expression levels of NKG2D ligand in isolated islets of Langerhans from the same mice. (c) The expression levels of NKG2D ligands Mult1 (top) and Raet1 (bottom) in intestine, from BALB/c and NOD 13-week-old mice and from NOD SGF mice at 8, 13 and 20 weeks of age. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

products. However, we studied only changes in duodenum, and therefore we did not investigate possible important immunological changes in other parts of both the small and large intestines. Such differences could be interesting, because of the existence of distinctive functional differences in the lymphocyte populations residing at different intestinal compartments [49]. In the lymphoid tissues, we observed the main diet-induced differences in NKG2D and DX5 in S compared to PLN. This supports a systemic effect of gluten intake on immune activation, which is supported by earlier studies of gluten-induced effects on both intesti-

nal and systemic immunity [22,23]. It is surprising that a SGF diet also correlates with lower NKG2D expression in islets, especially as the same tissue shows increased DX5 expression. It is not clear whether the increase in DX5 expression reflects an increase in NK cells or if this reflects an increased level of islet-infiltrating NKT cells (expressing DX5) [44], which was found previously to prevent the development of T1D [50]. An increased level of NKT cells directly in the islets could be one mechanism behind the protective role of a GF diet in the prevention of T1D in NOD mice. Gluten-induced expression of NKG2D is of

Fig. 5. Islet insulinitis score, NKG2D and CD3 staining in non-obese diabetic (NOD) mice. (a) Scoring of histological grade of insulinitis was performed on pancreatic section stained with haematoxylin and eosin (H&E) from 20-week-old NOD mice receiving strictly gluten-free (SGF) ($n = 11$) and strictly standard diet (SSTD) ($n = 9$) diet. Islets were scored for no-insulinitis, peri-insulinitis, moderate insulinitis and severe insulinitis. * $P < 0.0001$. (b) Histological pancreatic sections stained with H&E from NOD SGF and NOD (SSTD). (c) Section of intestine from NOD SGF and NOD SSTD. The red indicates cells positive for NKG2D; the green cells are positive for CD3; and blue shows cell nuclei.



particular interest because studies indicate that NKG2D plays a key role in immune-mediated diseases, including CD and T1D [25,26]. NKG2D could serve as an immune activator that can tip the balance in favour of autoimmunity and chronic inflammation. The observation that NKG2D can mediate cytotoxicity independently of TCR ligation [31] in recently activated T cells is particularly relevant in this context, because it suggests that gluten intake alone can establish a self-perpetuating loop of immune activation.

Untreated CD patients have a decreased intestinal level of NK cells that remain low, even when the patients are treated with a GF diet [51], implying that a GF diet does not influence the level of NK cells in CD patients. We did not find any diet-induced effect on the level of DX5⁺ NK cells in isolated lymphoid tissue (S, PLN or ALN) in either BALB/c or NOD mice, which was described previously [22]. Interestingly, we found that a GF and a SGF diet reduced the intestinal expression of DX5 mRNA, both in BALB/c and in NOD mice, and that this diet-induced difference was sustained at all measured time-points and was already present in NOD mice at the age of 8 weeks. Thus, a GF diet was found to have an effect on the intestinal level of NK cells, both in normal healthy intestinal tissue (BALB/c mice) as well as in NOD mice. Moreover, we found that a SGF diet induced a higher expression of NK cells in isolated islets. Therefore, it seems that the effect of gluten on the expression of DX5 is mainly directly in the tissues and not in lymphoid organs, suggesting an innate activation by gluten directly in the tissue.

Both T1D and CD [52] are found to be CD4⁺ T cell-mediated diseases, but the activation of CD8⁺ T cells is also central in controlling the immune response that causes immunopathology in both diseases [53–55]. We found diet-induced differences in the level of CD8⁺ T cells in all tested lymphoid organs. A GF diet diminished the level of CD8⁺ T

cells in NOD mice, both in S and ALN and in PLN. In BALB/c mice a GF diet was also found to reduce the level of CD8⁺ T cells in PLN.

Interestingly, we found a consistent diet-induced effect on the level of CD71 on DX5⁺ and CD8⁺ cells in both BALB/c and NOD mice, where a GF diet reduced the expression on both NK cells and CD8⁺ T cells in all tested lymphoid organs. Increased CD71 receptor levels are found on immature proliferating cells and upon antigen stimulation of T cells, as well as cells with a high proliferation rate [45–48]. This implies that a GF diet diminishes the proliferation or activation of both NK cells and CD8⁺ T cells.

Interestingly, we found that the intestinal expression of NKG2D ligands was changed according to diet in NOD mice. The NOD mice on a GF diet from 4 weeks of age had an increased intestinal expression of Mill1 and Mult1. However, in SGF animals that had never been exposed to gluten we found a significantly reduced intestinal expression of almost all NKG2D ligands. This correlates well with the reduced intestinal expression of NKG2D and DX5. This resembles the transition from low levels of NKG2D ligand expression in healthy gut epithelium [25] that increases in patients with CD, where distressed intestinal epithelial cells up-regulate the stress-inducible non-classical MHC class I MIC (MICA and MICB) [56] upon exposure to gluten peptides. Lowered levels of NKG2D along with lowered levels of Mult1 and Rae-1 could be involved in the beneficial effects of a SGF diet compared to GF diet. Thus, it seems that a SGF diet can lower NKG2D-mediated immunity in intestines both through direct down-regulation of the NKG2D receptor as well as through target tissue ligand expression, which shows clearly that there is an effect of the timing of gluten exposure.

Isolated islets from SGF NOD mice had a higher expression of Mult1, Mill1 and Mill2 than matched SSTD

controls. This was found neither in BALB/c mice nor the GF NOD mice. The observed pattern of ligand expression could indicate that NKG2D ligands may perform different roles, but the details of such NKG2D ligand interplay remain to be established. Perhaps NKG2D ligand up-regulation could protect the islets from NKG2D-mediated cytotoxicity, because it has been shown that NK cells may be desensitized by continuous exposure to NKG2D ligand [57,58]. However, other studies have found that the expression of NKG2D ligands decreases MHC class I expression [59], which leads to increased NK cell killing, implying a complex relationship between NKG2D ligand expression and MHC I. In concordance with a study by Maier *et al.* [60], but in contrast to the study by Ogasawara *et al.* [26], we observed no dramatic difference in Rae-1 expression levels between NOD and BALB/c mice, either in intestine, isolated islets or with age. The reason for the difference between our results and those of Ogasawara *et al.* [26] is unknown, but it could be due to an effect of different housing conditions or to dietary differences.

These results help to clarify how the immune status is affected by dietary gluten, which has been reported as an environmental factor in the development of T1D [61] as well as in healthy individuals and individuals reporting to be 'gluten-sensitive' [62]. Our finding that gluten affects immunity both in BALB/c mice and in NOD mice supports the idea that gluten is able to induce cellular changes both in animals predisposed to disease as well as in healthy animals. The effect of gluten on NKG2D and NKG2D ligand expression, as observed in CD patients, is not disease-specific but also present in NOD and BALB/c mice.

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Disclosures

The authors declare that there are no conflicts of interest.

Author contributions

J. C. A. designed, performed, analysed the experiments and wrote the paper. C. W. designed, performed, analysed and wrote about the qPCR experiments. E. A. performed and analysed the histology. J. L. analysed and wrote about the FACS experiments. K. E. participated in the performance of the FACS experiments. D. A. and K. B. supervised and edited the paper.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Natural killer group 2D (NKG2D) and DX5 expression intestinal expression levels at different ages. Expression levels of NKG2D (upper panel) and DX5 (lower panel) normalized to beta actin in intestinal tissue of non-obese diabetic (NOD) mice at 8, 13 and 20 weeks. Asterisks signify significance levels in comparison to expression at 8 weeks, unless directly compared by dotted line.

Paper IV

REGULAR ARTICLE

Perinatal risk factors increase the risk of being affected by both type 1 diabetes and coeliac disease

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Keywords

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ABSTRACT

Aim: This study investigated whether perinatal factors influenced the risk of a double diagnosis of type 1 diabetes and coeliac disease.

Methods: We used multinomial logistic regression models to study the associations between perinatal factors, gender, mode of delivery, season of birth and the risk of type 1 diabetes, coeliac disease or both, in Swedish-born singleton children.

Results: We found that 4327 of the 768 395 children (0.6%) had been diagnosed with type 1 diabetes, 3817 (0.5%) had been diagnosed with coeliac disease and 191 (0.02%) were affected by both diseases. If the children already had type 1 diabetes ($n = 4518$), the absolute risk of being affected by coeliac disease increased to 4.2% ($n = 191$). Children with both type 1 diabetes and coeliac disease were more likely to be female (OR = 1.48, 95% CI = 1.01–1.97), delivered by Caesarean section (OR = 1.60, 95% CI = 1.07–2.39), have native-born Swedish mothers (OR = 4.84, 95% CI = 1.96–11.97) or be born during the summer months (OR = 1.43, 95% CI = 1.07–1.92).

Conclusion: The increased risk of being affected by a double diagnosis of type 1 diabetes and coeliac disease was modulated by perinatal risk factors. This suggests that early life events are important when it comes to children with type 1 diabetes also developing coeliac disease.

INTRODUCTION

Type 1 diabetes and coeliac disease are two autoimmune disorders that affect approximately 0.5% and 3% of the Swedish childhood population, respectively (1,2). Both diseases have a latent phase, when autoantibodies are present before clinical or subclinical indications of the disease manifest themselves.

It is not uncommon for patients to develop both type 1 diabetes and coeliac disease, as people with type 1 diabetes have a higher risk of coeliac disease than the general population, with estimates ranging from 2.4 to 16.4% (3).

Several explanations have been proposed for the occurrence of both coeliac disease and type 1 diabetes. It is well established that the diseases share a common genetic predisposition to the HLA *DQA1*05:01-DQB1*02:01 (DQ2) and, or, DQA1*05:01-DQB1*03:02 (DQ8) haplotypes, but non-HLA loci are also implied in the shared risk (4). However, the sudden rise in the incidence of autoimmune diseases over the past few decades clearly implicates the importance of identifying the common environmental factors that are necessary to trigger type 1 diabetes, coeliac disease or both.

Several environmental and perinatal risk factors, as well as demographic features, seem to modulate the risk of developing both diseases. These include dietary factors,

such as duration of breastfeeding, intake of gluten and cow's milk, together with exposure to viral infections, prenatal growth and mode of delivery (5–8). Despite this, few studies have been able to identify population-based risk factors that are common for both coeliac disease and type 1 diabetes.

In this study, we set out to investigate if perinatal risk factors were associated with a double diagnosis of type 1 diabetes and coeliac disease in children diagnosed before the age of 14 years, using a population-based multinomial logistic regression model.

Key notes

- This study investigated if perinatal factors increased the risk of having a double diagnosis of type 1 diabetes and coeliac disease.
- We looked at the medical record of 768 395 Swedish-born singletons born in Sweden and found that 4.2% of the children with type 1 diabetes ($n = 4518$) also had coeliac disease.
- Having a double diagnosis was associated with several perinatal risk factors, including being female and being born by Caesarean section.

MATERIALS AND METHODS

Study population

We used the Swedish Medical Birth Registry to identify 811 599 children born in Sweden between 1987 and 1993. The Registry, which was set up in 1973, records data on more than 98% of Swedish births that culminate in delivery. Each person who lives in Sweden has a unique personal identification number and this can be used to link data in this Registry with a number of other national registry databases administered by Statistics Sweden and the National Board of Health and Welfare. These include the National In-patient Registry, the Migration Registry and the National Mortality Registry. Before they sent us the databases, the Swedish authorities encrypted the personal identifiers to ensure the anonymity of the subjects. The Regional Ethics Review Board in southern Sweden approved the use of the database, which has also been used in previous studies (9). For the purpose of this study, 43 203 children were excluded from the original database: 5554 had died before 14 years of age, 19 167 were not singletons, or information on this aspect was missing, and the information on maternal age was missing for one child. A further 18 482 children were excluded because they had emigrated before the age of 14.

Assessment of the outcome variable

We searched the Swedish National In-patient Registry to identify cases of coeliac disease and type 1 diabetes. These were defined using the following codes from the International Classification of Diseases (ICD) 9th and 10th editions: 579.0 (ICD 9) or K90.0 (ICD 10) for coeliac disease and 250 (ICD 9), E14 (ICD 10) or E10 (ICD 10) for type 1 diabetes. We then categorised them into the following groups: (i) no coeliac disease or type 1 diabetes, (ii) only coeliac disease, (iii) only type 1 diabetes, and (iv) both coeliac disease and type 1 diabetes.

Assessing the potential risk factors

Children were identified as small for gestational age if their birthweight in grams was two standard deviations (SD) below the expected birthweight for their gestational age in days. We calculated the expected birthweight using a gender-specific formula previously derived from ultrasonic intrauterine measurements of children born at term without complications (10). Children not born small for gestational age were used as the reference in the analyses. Maternal age at the time of childbirth was categorised as <20 years, 20–24 years, 25–29 years, 30–34 years, 35–39 years and more than 39 years. We used the category of <20 years as the reference in the analyses. The season of birth was categorised as autumn and winter (September to February) or spring and summer (March to August), and we used autumn and winter as the reference category. Children delivered by a Caesarean section were compared with children delivered vaginally.

The mothers were classified according to their smoking habits in early pregnancy, and we compared children who

were born to mothers who smoked with those who did not. We obtained the information about smoking habits from the medical birth register, which had been gathered by a midwife at the first antenatal care visit, between the first 6–12 gestational weeks.

Information about significant congenital malformations was retrieved from the Swedish Medical Birth Registry, using the ICD-9 codes 740–759 and the ICD-10 codes Q00 to Q99, and we compared children with and without significant malformations. A list of these codes is available upon request.

We also used register information to define the mother's educational achievement, using the highest completed level of education in the year of 1990. This variable was categorised into four groups: 9 years spent in elementary school or a lower level school, 12 years culminating in lower secondary school, more than 12 years culminating in higher educational achievement and unknown information. The higher educational achievement category was used as the reference group in the comparisons. We also used the Income and Asset Registry to determine whether or not the parents were receiving welfare benefits the year before the birth of their child, as well as information on the income of the parents the year before and after the birth of their child. Welfare benefits were defined as receiving any level of subsidy, granted by the Swedish Social Service agency if the applicant was unable to provide for themselves. The income variables were categorised in groups by tertiles, with a fourth category for missing information.

Statistical and epidemiological analysis

Multinomial logistic regression models were applied to estimate the odds ratios (OR) with 95% confidence intervals (CI) of having type 1 diabetes, coeliac disease or both diseases in relation to factors previously known to be associated with coeliac disease. These were maternal age, small for gestational age, delivery by caesarean section, maternal smoking, preterm birth, gender, season of birth, maternal birth country and congenital malformations (7,11–15). All statistical analyses were performed using the Statistical Package for Social Sciences version 20.0.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

Characteristics of population in relation to type 1 diabetes and coeliac disease

Figure 1 shows the cross-tabulation between cases of type 1 diabetes and coeliac disease. Of the 768 395 children in the study cohort, 4327 (0.6%) had type 1 diabetes, 3817 (0.5%) had coeliac disease and 191 (0.02%) had both diseases (Table 1). Children who had both type 1 diabetes and coeliac disease were diagnosed with type 1 diabetes at a median age of 2.6 years, which was much younger than the children who were just diagnosed with type one diagnosis, at a median age of 4.7 years (Table 1). The absolute risk of coeliac disease in the population with type 1 diabetes

(n = 4518) was 4.2% (n = 191). Of the total study cohort of 763 877, 0.5% (n = 3817) just had coeliac disease (p < 0.0001) and 0.6% (n = 4518) just had type 1 diabetes in (p < 0.0001).

Being born small for gestational age was associated with just having coeliac disease and having a congenital malformation or being female were also more common in children with coeliac disease, regardless of their type 1 diabetes status (Table 3). Caesarean delivery and being born in summer were associated with a double diagnosis of type 1 diabetes and coeliac disease, and there was also an association between being born in summer and having just coeliac disease (Table 2). The risk of having coexisting type 1 diabetes and coeliac disease was higher for children born to native Swedish mothers. Mothers who gave birth to

children with both coeliac disease and type 1 diabetes were older than mothers whose children were not affected by coeliac disease or type 1 diabetes (Table 3).

Association between perinatal factors known to increase the risk of coeliac disease and developing type 1 diabetes
 Preterm birth and length of maternal age slightly increased the risk of type 1 diabetes and, in contrast to having just coeliac disease, female gender was slightly protective. Maternal smoking decreased the risk of type 1 diabetes, but did not influence the risk of coeliac disease. Children born to native Swedish women faced a higher risk of having coeliac disease, type 1 diabetes or both diseases. Socio-economic factors were only weakly associated with an increased risk of coeliac disease and type 1 diabetes (Table 3).

		Coeliac disease		
		Yes	No	
Type 1 diabetes	Yes	191	4327	4518
	No	3817	760060	763877
		4008	764387	768395
		Absolute risk of coeliac disease in type 1 diabetes =		0042
		Absolute risk of coeliac disease without type 1 diabetes =		0005
		RR (coeliac disease in type 1 diabetes) =		8460

Figure 1 Cross table showing absolute risks of coeliac disease and of coeliac disease affected by type 1 diabetes.

DISCUSSION

The absolute risk of having a double diagnosis of type 1 diabetes and coeliac disease was just over 4% in Swedish children, which is eight times higher than the risk in the country's general population. We also found that children affected by both type 1 diabetes and coeliac disease were predominantly female, delivered by Caesarean section, born during the summer and born to native Swedish mothers. This indicates that early life events modulate the risk of developing both of these autoimmune disorders.

Our finding that females faced an increased risk of early and symptomatic coeliac disease, as well as an increased risk of being affected by both coeliac disease and type 1 diabetes, is in line with previous studies (16,17) and reflects the importance of gender modulating the risk of autoimmune disorders. The reason for this increased risk in females is still unclear, but it could be attributed to genetic

Table 1 Child characteristics of the study population of singleton children born in Sweden between 1987 and 1993

	No CD or T1D	CD	T1D	CD and T1D
Study population (number)	760 060	3817	4327	191
Median age (years) at diagnosis				
Type 1 diabetes (T1D)	NA	NA	4.7	2.6
Coeliac disease (CD)	NA	1.3	NA	7.5
Child characteristics				
Small for gestational age*	2.6 (19 990)	4.5 (172)	2.2 (94)	2.1 (4)
Female	48.6 (369 538)	58.7 (2240)	45.1 (1950)	58.1 (111)
Significant malformation	1.5 (11 669)	3.1 (119)	1.7 (72)	2.6 (5)
Caesarean birth	10.3 (78 626)	10.4 (395)	11.3 (487)	15.7 (30)
Birth year:				
1987–1988	26.6 (201 949)	30.4 (1161)	26.4 (1141)	27.2 (52)
1989–1990	29.3 (222 086)	29.8 (1136)	28.4 (1230)	26.7 (51)
1991–1992	30.0 (228 132)	25.9 (987)	31.3 (1354)	28.8 (55)
1993	14.2 (107 893)	14.0 (533)	13.9 (602)	17.3 (33)
Preterm birth†	5.0 (37 777)	4.6 (177)	5.9 (255)	5.8 (11)
Born during summer	53.5 (406 347)	56.8 (2168)	54.4 (2353)	62.3 (119)

*Missing values in small for gestational age: no Coeliac disease (CD) or Type 1 diabetes (T1D) 0.28% (2127), CD 0.29 (11), T1D 0.18 (8).

†Missing values in preterm birth: no CD or T1D 0.18% (1355), CD 0.1 (4), T1D 0.12 (5).

Table 2 Maternal characteristics of the study population of singleton children born in Sweden between 1987 and 1993

	No CD or T1D	CD	T1D	CD and T1D
Study population (number)	760 060	3817	4327	191
Median age (years) at diagnosis				
Type 1 diabetes (T1D)	NA	NA	4.7	2.6
Coeliac disease (CD)	NA	1.3	NA	7.5
Maternal characteristics				
Born in Sweden				
No	11.4 (86 797)	8.8 (334)	7.8 (336)	2.6 (5)
Yes	87.7 (666 630)	90.7 (3463)	91.9 (3977)	96.9 (185)
Unknown	0.9 (6633)	0.5 (20)	0.3 (14)	0.5 (1)
Smoking				
No	70.5 (532 523)	68.8 (2626)	74.9 (3239)	73.3 (140)
Yes	23.2 (176 044)	25.2 (960)	19.6 (847)	21.5 (41)
Unknown	6.4 (48 793)	6.1 (231)	5.6 (241)	5.2 (10)
Primiparity	57.9 (440 260)	59.0 (2253)	57.7 (2498)	56.0 (107)
Age				
<20	2.2 (21 217)	3.3 (127)	2.2 (95)	2.1 (4)
20–24	22.8 (173 530)	27.2 (1037)	22.7 (980)	22.0 (42)
25–29	37.4 (283 851)	39.4 (1503)	36.8 (1591)	37.7 (72)
30–34	25.3 (192 613)	21.9 (837)	26.4 (1142)	24.1 (46)
35–39	9.8 (74 520)	7.1 (269)	10.1 (436)	10.0 (19)
>39	1.9 (14 329)	1.2 (44)	1.9 (86)	4.2 (8)
Social allowance				
No	89.7 (681 373)	89.1 (3402)	92.2 (3988)	93.2 (178)
Yes	8.8 (67 178)	10.1 (385)	7.3 (317)	6.3 (12)
Unknown	1.5 (11 509)	0.8 (30)	0.5 (22)	0.5 (1)
Education				
Low	60.0 (456 418)	65.3 (2494)	60.9 (2636)	62.3 (119)
Middle	25.6 (194 412)	23.7 (904)	27.2 (1177)	23.6 (45)
High	9.0 (68 284)	4.2 (161)	8.4 (362)	8.9 (17)
Unknown	5.4 (40 946)	4.2 (159)	3.5 (152)	5.2 (10)
Income				
Low	32.7 (248 505)	34.0 (1297)	31.1 (1347)	31.4 (60)
Middle	32.9 (249 846)	34.2 (1307)	32.7 (1416)	38.7 (74)
High	32.7 (248 802)	30.9 (1178)	35.5 (1538)	29.3 (56)
Unknown	1.7 (12 907)	0.9 (35)	0.6 (26)	0.5 (1)

and conditional effects, or a combination of both, and needs to be further explored.

In line with previous studies (11,18,19), our research also demonstrated that mode of delivery was a risk factor for both type 1 diabetes and coeliac disease. However, the finding that being born by Caesarean section was associated with an increased risk needs to be interpreted with caution, as it can be associated with factors that are, in turn, associated with the risk of coeliac disease. These include factors related to the pregnancy itself, such as foetal distress and dystocia, maternal factors, such as lifestyle and previous Caesarean sections, and physician attitudes, such as defensive medicine (20). The finding that children with a double diagnosis of type 1 diabetes and coeliac disease were more likely to be born during the summer is in agreement with previous reports, which showed a seasonal effect on disease risks and suggested that these could be explained by a seasonal variation of exposure to viruses (21,22).

The most striking risk for being affected by a double diagnosis of type 1 diabetes and coeliac disease in the present study was being born to a native Swedish mother. This increased risk is likely to be because there are higher proportion of DQ2 and/or DQ8 HLA haplotype carriers in Sweden than other populations. However, previous studies have found that acculturation appears to condition the risk of coeliac disease in offspring born in Sweden (23). Interestingly, we recently demonstrated that mothers who gave birth to children with coeliac disease demonstrated altered serum cytokine levels during early pregnancy, which suggests that mothers exposed to environmental events modulate the risk of autoimmunity in their offspring (24). Hypothetically, it suggests that various risk factors may trigger autoimmunity during pregnancy and that exposures of environmental antigens may differ between populations.

In this study, children were diagnosed younger when they had coeliac disease, with or without type 1 diabetes, than just diagnosed type 1 diabetes. Another study also found that age

Table 3 Unadjusted odds ratios (OR) and 95% confidence intervals (CI) reflecting the association between factors previously related to coeliac disease (CD) and mutually exclusive groups of either coeliac disease, type 1 disease (T1D) or both diseases. The study population were singleton children born in Sweden between 1987 and 1993 who were alive and living in Sweden at the age of 14 years

Variables	Coeliac disease	Type 1 diabetes	Coeliac disease and type 1 diabetes
Child characteristics			
Small for gestational age	1.78 (1.52–2.08)	0.82 (0.67–1.01)	0.69 (0.25–1.89)
Female	1.50 (1.41–1.60)	0.87 (0.82–0.92)	1.48 (1.01–1.97)
Significant malformation	2.13 (1.77–2.56)	1.06 (0.84–1.34)	1.70 (0.96–4.41)
Caesarean	1.03 (0.92–1.14)	1.08 (0.98–1.20)	1.60 (1.07–2.39)
Birth year			
1987–1988	REF	REF	REF
1989–1990	0.89 (0.82–0.96)	0.96 (0.89–1.05)	0.93 (0.63–1.37)
1991–1992	0.75 (0.69–0.82)	1.02 (0.94–1.11)	1.00 (0.67–1.48)
1993	0.76 (0.77–0.96)	0.97 (0.87–1.07)	1.26 (0.80–1.99)
Preterm birth	0.86 (0.74–1.01)	1.20 (1.06–1.37)	1.05 (0.56–1.97)
Born during summer	1.14 (1.07–1.22)	1.03 (0.97–1.09)	1.43 (1.07–1.92)
Maternal characteristics			
Born in Sweden			
No	REF	REF	REF
Yes	1.30 (1.16–1.46)	1.44 (1.29–1.62)	4.84 (1.96–11.97)
Unknown	1.36 (0.73–2.55)	1.11 (0.54–2.25)	0.82 (0.43–1.57)
Smoking			
No	REF	REF	REF
Yes	1.02 (0.95–1.10)	0.79 (0.73–0.86)	0.86 (0.60–1.23)
Unknown	0.95 (0.83–1.09)	0.85 (0.75–0.97)	0.82 (0.43–1.57)
Primiparity	1.16 (1.08–1.25)	0.94 (0.88–1.01)	0.92 (0.66–1.27)
Age			
<20	REF	REF	REF
20–24	0.91 (0.75–1.10)	1.17 (0.95–1.46)	1.22 (0.43–3.44)
25–29	0.78 (0.64–0.95)	1.15 (0.92–1.42)	1.31 (0.46–3.71)
30–34	0.63 (0.52–0.77)	1.24 (0.99–1.55)	1.31 (0.45–3.82)
35–39	0.52 (0.42–0.66)	1.25 (0.98–1.58)	1.44 (0.76–4.48)
>39	0.44 (0.31–0.63)	1.25 (0.92–1.70)	3.09 (0.88–10.86)
Social allowance			
No	REF	REF	REF
Yes	1.14 (1.02–1.28)	0.96 (0.85–1.09)	0.82 (0.44–1.51)
Unknown	1.05 (0.35–3.16)	0.76 (0.24–2.35)	–
Education			
Low	1.2 (1.07–1.40)	1.23 (1.09–1.38)	1.15 (0.67–1.97)
Middle	1.13 (0.98–1.30)	1.20 (1.06–1.35)	0.95 (0.54–1.68)
High	REF	REF	REF
Unknown	1.06 (0.87–1.31)	0.98 (0.80–1.20)	1.58 (0.69–3.62)
Income			
Low	REF	REF	REF
Middle	1.10 (1.01–1.20)	1.01 (0.93–1.09)	1.12 (0.78–1.62)
High	1.11 (1.01–1.22)	1.09 (1.00–1.20)	0.81 (0.53–1.25)
Unknown	0.62 (0.23–1.67)	0.68 (0.25–1.82)	–

at the onset of type 1 diabetes was inversely associated with the risk of coeliac disease (25), although this was not confirmed by other studies (26). Whether age at diagnosis is relevant for having any or both of the diseases, or just indicates different phenotypes, needs to be determined.

It is well known that combinations of HLA genotypes affect the clinical presentation of coeliac disease and that individuals carrying two copies of HLA-DR3-DQ2 face the highest risk of developing coeliac disease at an early age

(27). Interestingly, one of our previous studies found that a higher proportion of DR3-DQ2 homozygous children with type 1 diabetes developed coeliac disease autoimmunity than type 1 diabetes children with other HLA genotypes (28). We speculate that the children in our cohort that suffered from both type 1 diabetes and coeliac disease and presented at an early age may have had a different HLA genotype than the type 1 diabetes children who were diagnosed at an older age.

The notably low age at diagnosis in those with just coeliac disease confirms the findings from the National Swedish Childhood Coeliac Disease Registry, which included children born during a period between 1985 and 1994 when Sweden experienced an increased incidence of coeliac disease in very young children. At that time, the average incidence rate of coeliac disease in children younger than 2 years of age was 189 cases per 100 000 person years, compared to 48 cases per 100 000 person years between 2004 and 2009 (29). Interestingly, the steep increase and fall in incidence coincided with changes to national infant feeding practices, when the gluten content in commercially available milk cereal drinks and porridges increased and then, shortly afterwards, decreased again. This highlighted the important role that gluten intake during infancy has on the risk of coeliac disease in children. It is tempting to speculate that the ingestion of gluten lowers the gut barrier and increases the risk of being exposed to other antigens involved in the pathogenesis of type 1 diabetes. However, this speculation does not tie in with the fact that the incidence of coeliac disease and type 1 diabetes did not follow the same pattern during the high incidence years (30).

Our study has several strengths. It is based on a large population of children and prospectively collected information gathered from national registries. In contrast to most other studies in this field, we also had access to perinatal demographic and medical information. However, the limitations included using the Swedish National In-Patient Registry to identify coeliac disease cases, as this might have led to some individuals being missed if they had not been admitted to hospital and just were taken care of in an outpatient clinic. Although there is a risk that individuals with coeliac disease who have been identified through a hospital-based register have a more severe form of the disease than the average patient, many of the patients in this study were diagnosed at a young age, when hospital admission was common in those undergoing small-bowel biopsies and other gastrointestinal investigations.

Recent studies have revealed that the majority of coeliac disease cases would be missed without population-based screening, and this suggests that the prevalence of coeliac disease in our cohort was probably underestimated (1). If we assume that two-thirds of all coeliac disease cases would have been detected by screening, this suggests that approximately another 8000 cases with undiagnosed coeliac disease would have been identified in addition to the cases identified by our study. It is also possible that 200 of the coeliac disease cases in our study were reported as a result of previously known Swedish screenings performed during parts of our study period (1). This, in turn, would have influenced the findings on coeliac disease, but are unlikely to have affected the figures on children and adolescents with both diseases as paediatric cases of type 1 diabetes always receive hospital treatment in Sweden (6).

Our analyses suffer from low statistical power due to the low number of cases suffering from both type 1 diabetes and coeliac disease. Furthermore, we have no information on

breastfeeding, gluten consumption and intrauterine and perinatal infections. All of these unmeasured residual confounders may, therefore, affect our observations. Also, the information reported by the midwives on the mothers' smoking habits during pregnancy should be interpreted with caution, due to the risk of under-reporting.

In conclusion, we found that the risk of developing coeliac disease was significantly higher among children with type 1 diabetes than in the general Swedish population. The increased risk of having a double diagnosis of type 1 diabetes and coeliac disease was associated with being female, having a native Swedish mother, being born by Caesarean sections and being born in summer.

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CONFLICTS OF INTEREST

None to declare.

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