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# **VITAMIN D AND TYPE 1 DIABETES**

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ACADEMIC DISSERTATION

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# ABSTRACT

Type 1 diabetes is an autoimmune disease, in which insulin-producing  $\beta$ -cells in the pancreas are destroyed leading to a life-long external insulin-dependency. Finland has the highest incidence of type 1 diabetes in the world, increasing almost 5-fold since the 1950s. The reasons for this increase are not known, therefore it is not possible to delay or prevent the disease. Identifying the factors that can modify the disease risk and interfere with the disease process is highly important, since despite advances in disease management, the life expectancy of patients with type 1 diabetes is estimated as more than 10 years shorter than that of the normal population.

Type 1 diabetes develops as a result of complex interactions between genetic and environmental factors. While more than 50 genetic loci associated with type 1 diabetes risk have been identified the strongest risk is conferred by the human leukocyte antigen (HLA) gene region. The environmental factors, however, remain unknown. Epidemiological evidence suggests an association between vitamin D and type 1 diabetes, but information on the actual vitamin D status (serum 25-hydroxyvitamin D (25OHD) concentration) during pregnancy or childhood prior to diagnosis is scarce. Genes in the metabolic pathway of vitamin D are associated with type 1 diabetes, but the interaction between vitamin D status, vitamin D related genetic factors and type 1 diabetes is poorly known.

We investigated the association between vitamin D and type 1 diabetes by determining maternal vitamin D status in a retrospectively case-control study setting, when it was already known which of the children later developed type 1 diabetes. We utilised a unique sample collection, the Finnish Maternity Cohort (FMC) that collects serum samples of almost every pregnancy in Finland. We evaluated the association between single nucleotide polymorphisms (SNPs) in the metabolic pathway of vitamin D and type 1 diabetes. Furthermore, we investigated the relationship between vitamin D status and the HLA gene region.

No difference in maternal serum 25OHD concentrations emerged between mothers of diabetic and non-diabetic children (43.9 nmol/l and 43.5 nmol/l;  $n=686$ ). Around 70% of all pregnant women were vitamin D deficient (serum 25OHD concentration  $< 50$  nmol/l). We found novel proof of a relationship between maternal genetic factors in the metabolic pathway of vitamin D and type 1 diabetes risk in the child. The genotypes of two SNPs located in the vitamin D receptor (*VDR*) gene showed different genotype distribution between mothers whose children later developed type 1 diabetes and those whose did not. The *VDR* regulates the transcription of hundreds of vitamin D target genes.

We also found for the first time that associations between serum 25OHD concentration and SNPs in the *VDR* and the group-specific component (*GC*) were stronger during pregnancy in mothers whose children later developed type 1 diabetes than in mothers whose children did not. The *GC* gene encodes vitamin D binding protein to which vitamin D is attached during circulation. Furthermore, we found a novel association between serum 25OHD concentration and the HLA gene region. A group of HLA alleles (HLA-B44 supertype) associated with low serum 25OHD concentration.

Our results do not support a direct association between maternal vitamin D status and type 1 diabetes risk in the child. It may be, however, that we were not able to detect a possible protective effect of vitamin D in this population since a considerable proportion of all mothers were vitamin D deficient. Our results demonstrate novel differences in genetic factors related to the metabolic pathway of vitamin D during pregnancy between mothers whose children later developed type 1 diabetes and whose did not. The found relationship between the HLA gene region and vitamin D status indicates an even stronger role for vitamin D in the immune system than previously thought.

Our results suggest that more attention should be paid to the mother and pregnancy, when investigating the early programming of type 1 diabetes.

# TIIVISTELMÄ

Tyypin 1 diabetes on autoimmuunisairaus, jossa insuliinia tuottavat haiman  $\beta$ -solut tuhoutuvat. Tämä johtaa elinikäiseen ulkoisen insuliinin tarpeeseen. Suomessa tyypin 1 diabeteksen ilmaantuvuus on viisinkertaistunut viidessä vuosikymmenessä ja on korkeampi kuin missään muualla maailmassa. Syitä ilmaantuvuuden kasvuun ei tunneta ja täten taudin ehkäisy ei ole vielä mahdollista. Taudin syntyyn vaikuttavien tekijöiden tutkiminen on tärkeää, sillä vaikka tyypin 1 diabeteksen hoito on kehittynyt, potilaiden eliniänodotteen on arvioitu olevan edelleen yli 10 vuotta normaaliväestöä alhaisempi.

Tyypin 1 diabeteksen puhkeamiseen vaikuttaa sekä perinnöllinen alttius, ympäristötekijät että näiden yhteisvaikutus. Taudin riskiin vaikuttavia perinnöllisiä tekijöitä tunnetaan yli 50, joista suurin vaikutus on HLA-geenialueella. Ympäristötekijöitä ei ole vielä pystytty varmuudella tunnistamaan. D-vitamiinin ja tyypin 1 diabeteksen yhteydestä on epidemiologista näyttöä, mutta diagnoosia edeltävistä D-vitamiinitasoista (seerumin 25-hydroksi-D-vitamiini; 25OHD) on vain vähän tietoa. Tiedetyt D-vitamiinin aineenvaihduntaan osallistuvat geenit ovat yhteydessä tyypin 1 diabetekseen. D-vitamiinitason, D-vitamiinin aineenvaihduntaan osallistuvien geenien ja tyypin 1 diabeteksen vuorovaikutus tunnetaan kuitenkin vielä huonosti.

Tavoitteemme oli tutkia äidin raskaudenaikaisen D-vitamiinistatuksen yhteyttä lapsen riskiin sairastua tyypin 1 diabetekseen jälkikäteen, kun jo tiesimme ketkä lapsista myöhemmin sairastuivat ja ketkä eivät. Meillä oli mahdollisuus käyttää ainutlaatuisen Finnish Maternity Cohort (FMC) näytekokelman näytteitä. FMC-seerumipankkiin on kerätty näyte lähes kaikilta raskaana olevilta suomalaisilta vuodesta 1983 alkaen. Tutkimme myös useiden D-vitamiinin aineenvaihduntaan osallistuvien geenien pistemutaatioiden (single nucleotide polymorphisms; SNPs) yhteyttä tyypin 1 diabetekseen. Lisäksi tutkimme HLA-geenialueen ja D-vitamiinistatuksen yhteyttä.

Diabeetikoiden ja terveiden lasten äitien raskaudenaikaisissa seerumin 25OHD-pitoisuuksissa ei ollut eroa (43.9 nmol/l ja 43.5 nmol/l; n=686). Noin 70 %:lla sekä diabeetikoiden että terveiden lasten äideistä oli raskaudenaikainen D-vitamiininpuutos (seerumin 25OHD-pitoisuus < 50 nmol/l). Löysimme uutta tietoa äidin D-vitamiinin aineenvaihduntaan osallistuvien geenien yhteydestä lapsen riskiin sairastua tyypin 1 diabetekseen. Kahden D-vitamiinireseptorissa (*VDR*) sijaitsevan SNP:n genotyyppijakauma oli erilainen diabeetikoiden ja terveiden lasten äideillä. *VDR* välittää D-vitamiinin vaikutukset sen satoihin kohdegeeneihin.

Havaitsimme myös ensimmäistä kertaa että diabeetikoiden äideillä *VDR*- ja *GC*-geeneissä (group-specific component) sijaitsevat SNPt yhdistyivät

voimakkaammin seerumin 25OHD-pitoisuuteen raskauden aikana kuin terveiden lasten äideillä. GC koodaa D-vitamiinin kuljetuksessa tarvittavaa proteiinia (DBP). Löysimme myös uuden yhteyden seerumin 25OHD-pitoisuuden ja HLA-geenialueen välillä. Ryhmä kudostekijöitä (HLA-B44 supertyyppi) yhdistyi matalaan seerumin 25OHD-pitoisuuteen.

Tutkimuksessamme ei löydetty suoraa yhteyttä äidin raskaudenaikaisen D-vitamiinitason ja lapsen diabetesriskin välillä. Saattaa olla, että D-vitamiinin mahdollinen suojavaikutus jäi näkymättä tässä tutkimusjoukossa, koska D-vitamiinin puutos oli huomattavan yleistä kaikilla äideillä. Diabeetikoiden ja terveiden lasten äidit olivat kuitenkin erilaisia tiettyjen D-vitamiinin aineenvaihduntaan osallistuvien geenien osalta. Löydetty yhteys HLA-geenialueen ja D-vitamiinitason välillä korostaa D-vitamiinin merkittävää roolia puolustusjärjestelmän säätelijänä.

Tuloksemme viittaavat siihen, että äitiin ja raskauteen liittyviin tekijöihin tulisi kiinnittää enemmän huomiota kun tutkitaan tyyppi 1 diabeteksen varhaista ohjelmoitumista.





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## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I Miettinen ME, Reinert L, Kinnunen L, Harjutsalo V, Koskela P, Surcel H-M, Lamberg-Allardt C, Tuomilehto J. Serum 25-hydroxyvitamin D level during early pregnancy and type 1 diabetes risk in the offspring. *Diabetologia* 2012 May;55(5):1291-4.
- II Miettinen ME, Smart MC, Kinnunen L, Mathews C, Harjutsalo V, Surcel HM, Lamberg-Allardt C, Tuomilehto J, Hitman GA. Maternal VDR variants rather than 25-hydroxyvitamin D concentration during early pregnancy are associated with type 1 diabetes in the offspring. *Diabetologia*. 2015 Oct;58(10):2278-83.
- III Miettinen ME, Smart MC, Kinnunen L, Harjutsalo V, Reinert-Hartwall L, Ylivinkka I, Surcel H-M, Lamberg-Allardt C, Hitman GA, Tuomilehto J. Genetic determinants of serum 25-hydroxyvitamin D concentration during pregnancy and type 1 diabetes in the child. Submitted to *Plos One*
- IV Miettinen ME, Kinnunen L, Harjutsalo V, Aimonen K, Surcel HM, Lamberg-Allardt C, Tuomilehto J. Association of serum 25-hydroxyvitamin D concentration with HLA-B, -DRB1 and -DQB1 genetic polymorphisms. *Eur J Clin Nutr*. 2017 Jan;71(1):128-131

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

## ABBREVIATIONS

BMI	body mass index
CYP	Cytochrome P450
DBP	Vitamin D binding protein
DHCR7	7-dehydrocholesterol reductase
FDR	False discovery rate
FMC	Finnish Maternity Cohort
GC	Group-specific component
HLA	Human leukocyte antigen
NADSYN1	NAD synthetase 1
NOD	Non-obese diabetic
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
THL	National Institute for Health and Welfare
Th1	T helper cell 1
Th2	T helper cell 2
Th17	T helper cell 17
VDR	Vitamin D receptor
VDRE	Vitamin D response element
25OHD	25-hydroxyvitamin D
1,25OHD	1,25-hydroxyvitamin D

# 1 INTRODUCTION

Before starting this work, I once read that type 1 diabetes is a researcher's nightmare. Back then, a wide selection of both genetic and environmental factors had been introduced to explain the pathogenesis of the disease. Nevertheless, the researchers did not seem to have a clue why the incidence of type 1 diabetes kept increasing especially in the western world.

Now, some years and a lot of research later, we are faced with the same question. While genetic research has now identified over 50 susceptibility loci for type 1 diabetes (Onengut-Gumuscu et al, 2015), we still do not know what initially causes  $\beta$ -cells in the pancreas to be destroyed by an autoimmune reaction. We also still have not been able to identify with certainty the environmental factors that modify the risk. Thus, we still do not have means to delay, stop or reverse the disease process of type 1 diabetes.

When we started to plan the "Vitamin D and Type 1 Diabetes" study the extent of ongoing vitamin D research was a lot smaller. A few high-quality studies existed, however, indicating that the intake of vitamin D from supplements during pregnancy or infancy may modify the risk for type 1 diabetes in the child (Hyppönen et al, 2001; Stene et al, 2000). Since the vitamin D status (serum 25-hydroxyvitamin D; 25OHD concentration) is, in addition to vitamin D supplement use, strongly influenced by the amount of UV radiation and to some extent dietary factors, we thought it would be interesting to measure the actual serum 25OHD concentrations during pregnancy to evaluate the association between maternal vitamin D status and type 1 diabetes risk in the child. We had the privilege of using a unique sample collection, the Finnish Maternity Cohort (FMC) containing serum samples from almost all pregnancies in Finland since 1983. Therefore, we had the possibility to analyse maternal serum 25OHD concentrations retrospectively, when type 1 diabetes development in the children was already known.

When planning the study, existing research suggested that genes in the metabolic pathway of vitamin D also contribute to the inherited risk for type 1 diabetes (Lopez et al, 2004; Pani et al, 2000). The human leukocyte antigen (HLA) gene region was already known to be a strong genetic component in the disease aetiology (Nerup et al, 1974). To investigate not only an epidemiological association between maternal vitamin D status and type 1 diabetes, but also a potential mechanism explaining the possible association, we decided to include genetic analyses in our project.

This thesis evaluates the role of maternal vitamin D status during pregnancy for the risk for type 1 diabetes in the child. It also presents results of the differences in genetic factors in the metabolic pathway of vitamin D between mothers whose children later develop type 1 diabetes and those

whose do not. In addition, this thesis evaluates the relationship between the HLA gene region and vitamin D status.

## **2 REVIEW OF THE LITERATURE**

### **2.1 TYPE 1 DIABETES**

#### **2.1.1 TYPE 1 DIABETES IN FINLAND**

Type 1 diabetes is a complex autoimmune disease, in which the insulin-producing  $\beta$ -cells in the pancreas are destroyed in a predominantly T cell mediated process. Insulin is needed to move sugar from the blood into cells to be used as an energy source. The lack of insulin leads to an increased blood sugar concentration and therefore a life-long exogenous insulin dependency.

Usually around 80% of the insulin-producing  $\beta$ -cells have been destroyed by an autoimmune reaction when the disease is diagnosed (Foulis et al, 1986), but the autoimmune process leading to the clinical disease can last years or even decades before diagnosis (Knip et al, 2010). Type 1 diabetes accounts for approximately 5-10% of all diabetes and is one of the most common chronic diseases in children, but it can be diagnosed at any age (American Diabetes Association, 2017; Gale, 2005).

For the patient or their families, the incurable disease requires constant monitoring and care. The treatment of type 1 diabetes consists of monitoring of the blood sugar concentration, a personal insulin treatment regimen with both rapid-acting and long-acting insulin, as well as diet and exercise planning. The development of continuous glucose monitoring systems, small wearable devices that continuously track blood glucose concentration and notify when the blood sugar is too low or high, have proven to be beneficial for a considerable part of the patients in their disease management (Beck et al, 2017; Lind et al, 2017). Presently, only a small portion of the type 1 diabetic patients in Finland have access to these devices ([www.diabetes.fi/diabetesliitto](http://www.diabetes.fi/diabetesliitto)).

Information on the health care costs that are particular to type 1 diabetes is scarce. It is known that around 10% of all health care costs in Finland during 1998-2007 were related with diabetes (any type of diabetes) (Jarvala et al, 2010).

Inequalities between the developing countries and the western world mean that patients with type 1 diabetes face different challenges in different countries. In the developing countries access to insulin and the technology needed for disease management may be a problem, whereas in the western world the patients may suffer from long-term macrovascular (such as an increased risk for cardiovascular events) and microvascular (such as retinopathy and nephropathy) complications (Atkinson et al, 2014).

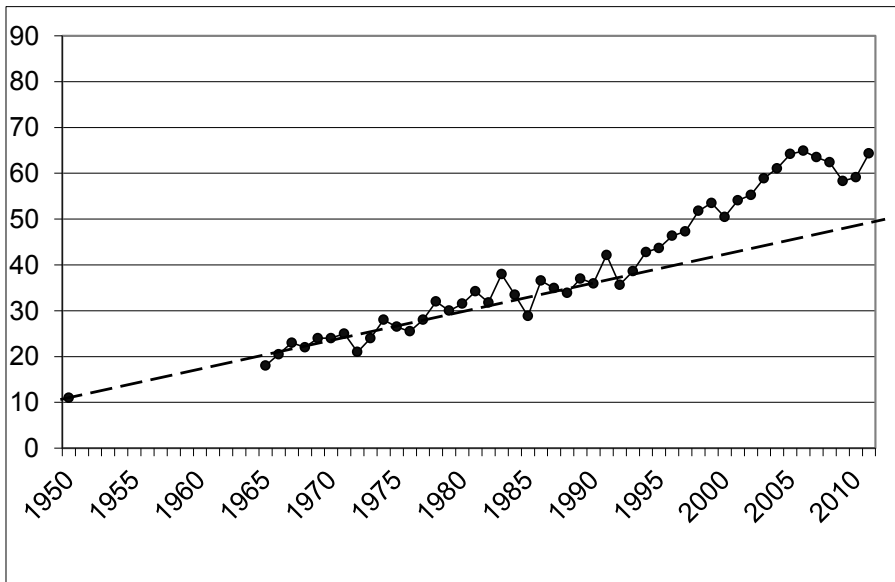
Considering the costs for the health care system and the considerable psychological burden of the disease for the patients and their families



(Hagger et al, 2016; Whittemore et al, 2012), identifying modifiable factors that can interfere with the disease process is extremely important.

Type 1 diabetes occurs worldwide, but differences in incidence are substantial between different countries with the highest reported incidence in Finland (DIAMOND Project Group, 2006; Harjutsalo et al, 2008; Harjutsalo et al, 2013). Countries or geographical regions in close proximity may have substantial differences in the disease incidence. For example, a considerably higher incidence occurs in Finland than in Estonia/Russia and Sardinia and other parts of Italy (Borchers et al, 2010a; DIAMOND Project Group, 2006; Kondrashova et al, 2005; Podar et al, 2001). Some of the geographical variation in type 1 diabetes incidence may result from differences in the predisposing genetic factors, especially differences in the HLA gene region (Rønningen et al, 2001; Tuomilehto, 2013). The presence of predisposing environmental factors and/or the absence of protective environmental factors also contributes (Kondrashova et al, 2007; Rewers and Ludvigsson, 2016).

Type 1 diabetes incidence is increasing in many western countries with around a 2-4% annual increase (Harjutsalo et al, 2008; Patterson et al, 2009; Vehik et al, 2007). In Finland, the first nationwide incidence estimation in the 1950s showed an incidence of 13 per 100 000 persons under 15, whereas in 2011 the incidence was almost 5-fold (Harjutsalo et al 2013; Tuomilehto, 2013) (Figure 1). The reasons for this considerable increase in type 1 diabetes incidence in Finland remain unknown. It has been shown though, that the incidence in the younger age groups in Finland has increased even more than in the older age groups possibly reflecting a shift in the clinical onset of the disease to younger age groups (Harjutsalo et al, 2008). After a peak incidence of 64.9 per 100 000 persons in children younger than 15 in 2006, the increase in the incidence seems to have leveled out (Harjutsalo et al, 2013). It may be too early to draw a conclusion on whether the leveling of the incidence is a temporary phenomenon, or whether it indicates an actual decrease in the disease incidence caused by environmental factors.



**Figure 1.** Incidence of type 1 diabetes in Finland per 100 000 persons under 15 years of age (Harjutsalo et al, 2008; Harjutsalo et al, 2013; Somersalo et al, 1954-5; Tuomilehto et al, 1999).

### 2.1.2 ENVIRONMENTAL TRIGGERS NOT REVEALED

The steady increase in type 1 diabetes incidence especially in the western world indicates an impact of environmental factors in the disease pathogenesis that have changed. Presence of these environmental factors in the disease pathogenesis is evidenced by the observation that migrants acquire a disease risk equal to the population in their new area of residence (Söderström et al, 2012). These environmental triggers act during the foetal period and/or childhood either independently or through interactions with genetic factors, and they determine whether or not the genetically susceptible individuals develop type 1 diabetes.

An increasing incidence of type 1 diabetes means that an increasing proportion of the new patients do not carry a high genetic risk for the disease (Hermann et al, 2003; Steck et al, 2011). Environmental triggers are difficult to identify because there may be an unknown critical time window for a specific environmental exposure. The most studied environmental factors in the pathogenesis of type 1 diabetes are infections, diet and more recently, gut microbiota.

A role for certain viral infections in the disease pathogenesis has been suggested for decades and justified for example based on a finding that viruses can cause diabetes in animals (Yoon et al, 2006).

Enteroviruses have been detected in the pancreatic islets of newly-diagnosed type 1 diabetes patients indicating a role in the destruction of the

$\beta$ -cells (Krogvold et al, 2015). In Finland, the detection of viral RNA in serum or stool is associated with the risk for type 1 diabetes (Hyöty, 2016). An infection of a certain type of enterovirus, coxsackievirus B1, is also associated with an increased risk for the occurrence of autoantibodies that precede the clinical onset of the disease (Laitinen et al, 2014). Increased sanitation and hygiene, along with economic growth, has in many countries gone hand in hand with the increase in the incidence of type 1 diabetes (Bach, 2002). It has been suggested that a decreasing frequency of childhood infections leads to defects in the establishment of immune tolerance, and therefore a higher incidence of type 1 diabetes and other immune system mediated diseases (Bach, 2002). The existing research, however, has been unable to confirm this theory. Though, it is possible that the detected decrease in the enterovirus infections during pregnancy leads to children not receiving maternal protecting antibodies and experiencing their first enterovirus infection later with harmful consequences (Viskari et al, 2005).

Many studies have detected differences in the gut microbiota between type 1 diabetic patients and healthy controls (Bibbò et al, 2016; de Goffau et al, 2014; Giongo et al, 2011). The human gut microbiota has changed considerably during the past decades, resulting from several factors such as changes in the diet, improved hygiene and increased use of antibiotics (Quercia et al, 2014). For decades, there has been an attempt to find means to modify the gut microbiota to improve the health of the host for example by using probiotics and prebiotics. Probiotics are defined as live micro-organisms that confer a health benefit on the host, whereas prebiotics are substances that allow changes in the growth or activity of beneficial host micro-organisms (Roberfroid, 2007; Schlundt, 2012). The prebiotic compounds in breast milk affect the colonisation of the infant gut (Zivkovic et al, 2011). In a recently published study including samples from Finland it was shown that early exposure (0-27 days after birth) to probiotics decreased the risk for autoimmunity by 60% in children with a high genetic risk for type 1 diabetes compared to a later probiotic supplementation or to no supplementation at all (Uusitalo et al, 2016). These studies suggest that the gut microbiota of an infant can be modified and that the modification may decrease type 1 diabetes risk. The studies in the field are at the moment still mainly small and observational by nature, however, and the precise mechanism between the gut microbiota and risk for type 1 diabetes risk is lacking.

Other environmental triggers that have been linked to increased risk for type 1 diabetes relate closely to the gut microbiota, such as breastfeeding, diet, enterovirus infections and antibiotic exposure (Koenig et al, 2011). Therefore, it has been suggested that the associations between these environmental factors and type 1 diabetes may be explained by their effect on the gut microbiota (Gülden et al, 2015).

### **2.1.3 HUMAN LEUKOCYTE ANTIGEN (HLA) GENE REGION ASSOCIATES STRONGLY WITH TYPE 1 DIABETES**

Type 1 diabetes develops as a result of a complex interaction between genetic and environmental factors. Involvement of the genetic factors in the aetiology of type 1 diabetes is evidenced by clustering of the disease in families. Twin studies reveal that the proportion of the twin pairs with both of the twins affected by the disease is considerably higher among identical than non-identical twins acting as further proof of the genetic component in the disease aetiology (Hyttinen et al, 2003). By the age of 60, a majority (65%) of the initially healthy persons with an affected identical twin will develop the disease (Redondo et al, 2008).

Approximately half of the genetic susceptibility is conferred by genes in the HLA gene region on chromosome 6 (Noble 2015). The relationship between the HLA gene region and type 1 diabetes has been known for decades (Nerup et al, 1974) and the HLA gene region is known to also associate with several other autoimmune diseases (Shiina et al, 2009).

The HLA gene region is the most polymorphic region in the human genome with over 9000 HLA alleles now described (Noble, 2015; Robinson et al, 2013). Therefore, studying the HLA gene region, as well as interpreting the HLA genotyping results, can be challenging. Genes in the HLA region can be categorised into class I (HLA-A, -B and -C), class II (HLA-DR, -DQ and -DP) and class III. HLA-A, -B and -C each consist of a single locus, HLA-DR consists of five loci (DRA, DRB1, DRB3, DRB4, DRB5) and HLA-DQ and HLA-DP of two loci (DQA1, DQB1, DPA1, DPB1) (Noble, 2015). Class III contains immunologically relevant genes, but does not encode the classical HLA antigens (Noble and Valdes, 2011). Some of the HLA alleles, for example alleles in the highly polymorphic HLA-B locus, bind similar peptides and can therefore be grouped into supertypes (Sidney et al, 2008).

Several alleles in the HLA gene region are associated with either an increased or decreased risk for type 1 diabetes. HLA-DRB1 alleles DRB1\*03 and DRB1\*04, and especially the genotype these alleles form together, create the strongest genetic risk for type 1 diabetes in Caucasians (Johnston et al, 1983; Langholz et al, 1995; Pociot and Lernmark, 2016). As an example of the complexity of the HLA gene region, however, some of the DRB1\*04 subtypes (there are a number of different subtypes of the DRB1\*04 allele) are strongly associated with a decreased risk for type 1 diabetes (Harfouch-Hammoud et al, 1996). In Scandinavia, Europe and North America around 90-95% of the patients with type 1 diabetes carry a DRB1\*03 or DRB1\*04 allele or both (Dib and Gomes, 2009), but the proportion is decreasing along with an increasing disease incidence (Steck et al, 2011).

Although the HLA class II alleles are mainly responsible for the genetic risk for type 1 diabetes within the HLA gene region, class I alleles also show association with the disease. For example, B\*39 associates with an increased risk (Mikk et al, 2014), whereas B\*57 with a decreased risk for type 1 diabetes (Noble et al, 2010).

The main function of the HLA gene region in the immune system is to protect the body from harmful agents. The HLA antigens recognise the foreign agents, bind the antigenic peptides and present them to T cells (Shiina et al, 2009; Noble and Valdes 2011). The disease associations of the HLA gene region are often interpreted to be a result of differences in the repertoire of self-peptides that are presented to T-cells (Tsai and Santamaria, 2013). Only in a few autoimmune diseases, however, the genetic susceptibility is conferred solely to the HLA gene region (Howell 2013; Shiina et al, 2009).

The remaining genetic risk for type 1 diabetes outside the HLA gene region is conferred by over 50 susceptibility loci that have been identified thus far (Onengut-Gumuscu et al, 2015; Pociot and Lernmark, 2016).

#### **2.1.4 PREVENTION OF TYPE 1 DIABETES IS STILL NOT POSSIBLE**

The ultimate goal of type 1 diabetes research is to find means to delay, prevent or reverse the disease process. A pre-clinical phase that can last from months to decades and that is characterised by the appearance of autoantibodies, precedes type 1 diabetes diagnosis (Knip et al, 2010). Primary prevention aims to prevent the initiation of the disease process by preventing the first appearance of the autoantibodies. Secondary prevention aims to prevent the development of the actual clinical disease after autoantibody detection. Tertiary prevention aims to preserve at least a part of the insulin-producing capacity of the  $\beta$ -cells after the disease diagnosis.

There are several ongoing type 1 diabetes prevention trials (Jacobsen et al, 2016), but means to prevent or delay the clinical onset of type 1 diabetes have thus far not been found. These trials have nevertheless revealed new information regarding the pre-clinical phase of type 1 diabetes. The pre-clinical phase starts when the autoantibodies to pancreatic islet antigens are detectable. Autoantibodies to insulin, glutamic acid decarboxylase, insulinoma-associated antigen 2 and zinc transporter-8 are usually measured to investigate the risk for type 1 diabetes or the pre-clinical disease status (Ziegler et al, 2013). The risk for clinical disease is estimated as approximately 10-15% (during 5-10 years) with a single autoantibody and 35-70 % (during 5-10 years) with multiple autoantibodies (Steck et al, 2015; Ziegler et al, 2013). The pre-clinical phase may also be characterised by a weaker insulin response years before the diagnosis, suggesting a decrease in  $\beta$ -cell function or in  $\beta$ -cell mass (Koskinen et al, 2016).

Discussion is ongoing as to whether all children or children with close relatives with type 1 diabetes should be screened for autoantibodies or for a weakened insulin response (Insel et al, 2015; Ziegler et al, 2016). It has been proposed that the asymptomatic autoantibody-positive children should be diagnosed as 'Autoimmune B Cell Disorder' patients, because such a large percentage of autoantibody-positive children do develop the clinical disease over time (Bonifacio et al, 2017). According to this view introducing a

diagnosis to these asymptomatic children will over time change the future scope from the treatment of type 1 diabetes to the treatment and prevention of pre-clinical diabetes (Bonifacio et al, 2017), as well as reduce the prevalence of ketoacidosis (consequence of the body using fat as an energy source instead sugar due to lack of insulin) at diagnosis of clinical type 1 diabetes (Ziegler et al, 2016). This view has been criticised on basis that not all children with autoantibodies develop type 1 diabetes, or the development may take as long as 20 years (Knip et al, 2010). A concern is that labeling these children as patients may cause psychological stress for the children and their parents (Knip et al, 2017), since mothers who are aware of their children's genetically increased risk for type 1 diabetes, experience more anxiety (Roth et al, 2015). It has also been recently shown that reversion of  $\beta$ -cell autoimmunity (autoantibodies that have persistently been detected disappear) is possible, highlighting the fact that not all children with autoantibodies will develop type 1 diabetes, although the reversion is strongly restricted to children with a single autoantibody (Vehik et al, 2016).

According to the "Barker Hypothesis", also referred to as the "Foetal Origin Hypothesis" diseases occurring in different stages of life may have their origin in the foetal or neonatal environment (Barker and Osmond, 1986). While this hypothesis was originally proposed in relation to diseases occurring in later life, the idea of a foetal origin and an early programming of type 1 diabetes have been suggested by many investigators based on the appearance of type 1 diabetes related autoantibodies sometimes only months after birth (Siljander et al, 2009). The development of both the foetal immune system and the pancreas begin during the first weeks of pregnancy, and by 7.5 weeks, insulin-positive cells can be detected in the foetal pancreas (Holt and Jones, 2000; Piper et al, 2004). Mother or pregnancy-related factors may modify this development and may therefore prove to be important in early programming of type 1 diabetes. First study in which pregnancy-related environmental factors will be monitored and that aims for prevention of type 1 diabetes is now being carried out in Australia (Penno et al, 2013) with no reported results yet. In this study for example the gut microbiota, nutritional status and weight gain of the mother during pregnancy will be analysed.

While waiting for successful means of preventing the disease, advances are taking place in the management of an already diagnosed disease. The results of pancreas and islet cell transplantation have improved with advances in surgical techniques and immunosuppression (Niclauss et al, 2016). For example, in Norway, 44% of the patients showed a 50% reduction in insulin requirement 4 years after the initial islet transplantation (Schive et al, 2017). The emerging technologies will also provide some advances for the patients to monitor their disease. For example, with the help of continuous blood sugar monitoring and insulin/glucagon pumps controlled via a smartphone (the first artificial pancreas which has been approved by the FDA in 2016), the prevention or hypo- and hyperglycemias will become

considerably easier. This is highly important, since the life expectancy has been estimated to be over 10 years shorter in type 1 diabetic patients even in the western world (Livingstone et al, 2015). In Finland, for example, no clear advances have occurred during the last decades in the treatment balance of patients with type 1 diabetes (Valle et al, 2010).

## **2.2 VITAMIN D**

Vitamin D has been studied during the past decades with an unseen intensity with a PubMed search now producing 58 847 hits for the term “vitamin D”. Vitamin D has been suggested as a wonder cure for practically every illness and undesired health condition producing even greater hype than antioxidants in the 1990s. Practically all chronic diseases have shown associations with low serum 25OHD concentrations, but the researchers have not been able to define the significance of these various associations let alone to translate this information into clinical practice.

### **2.2.1 VITAMIN D RESEMBLES A HORMONE**

Vitamin D is obtained mainly from the UV light through skin, and to a lesser extent from the diet, where the most important sources are fish and at least in Finland, fortified foods such as milk and margarine. Although it is called a vitamin, the actions of vitamin D in the body resemble that of steroid hormones and in contrast to the traditional definition of a vitamin, it can also be produced in the body. Chemically vitamin D refers to a group of fat-soluble secosteroids. In humans, the most important compounds in this group are vitamin D<sub>3</sub> (also known as cholecalciferol) and vitamin D<sub>2</sub> (ergocalciferol).

Vitamin D is necessary for normal calcium and phosphate metabolism by stimulating the absorption of these minerals from the intestine. In the absence of adequate vitamin D supply, the risk for developing rickets (defective mineralisation of the bones) in children and osteomalasia (softening of the bones) in adults increases.

In Finland, the archiatre Arvo Ylppö already paid attention in 1920s to the high incidence of rickets that he described to be a result of the northern location and low amount of sunlight (Ylppö, 1925). This eventually led to a regular vitamin D supplementation of 100 µg per day for the infants in Finland, which was gradually decreased to the current recommendation of 10 µg per day ([www.thl.fi](http://www.thl.fi)).

## 2.2.2 SEVERAL GENES ARE INVOLVED IN VITAMIN D METABOLISM

Vitamin D synthesis in the body begins in the skin as a result of exposure to UV radiation. In the skin, UV radiation promotes the formation of previtamin D from 7-dehydrocholesterol that is derived from cholesterol (Prabhu et al, 2016). The production of previtamin D in the skin is dependent by the amount of pigmentation (Xiang et al, 2015). Exposure to UV radiation increases the pigmentation in the skin decreasing previtamin D synthesis in the skin.

NAD synthetase 1 (*NADSYN1*) and 7-dehydrocholesterol reductase (*DHCR7*) convert 7-dehydrocholesterol back to cholesterol thus being able to control the amount of available 7-dehydrocholesterol for the previtamin D synthesis (Prabhu et al, 2014). *NADSYN1* and *DHCR7* genes are located near each other and are often referred to as the *NADSYN1/DHCR7* locus.

Previtamin D is converted by non-enzymatic thermal isomerisation to vitamin D (Bouillon et al, 1998). Vitamin D is converted in the liver to 25OHD by cytochromes P450 (CYPs) enzymes, encoded by the *CYP2R1* and *CYP27A1* genes (Schuster, 2011). 25OHD is then transported in the circulation by vitamin D binding protein (DBP), encoded by the group-specific component (*GC*) gene.

Cubilin, encoded by the *CUBN* gene, mediates the uptake of the 25OHD-DBP complex to the kidney (Christensen et al, 2013; Nykjaer et al, 2001). 25OHD is hydroxylated to the metabolically active form of vitamin D, 1,25OHD, by 1- $\alpha$ -hydroxylase encoded by the *CYP27B1* gene mainly in the kidney, but also, for example, in the  $\beta$ -cells and immune cells (Bikle, 2009). Degradation of active vitamin D is initiated by a hydroxylase encoded by the *CYP24A1* gene (Bikle, 2011).

The active form of vitamin D acts in the body through the vitamin D receptor (VDR), encoded by the *VDR* gene (Brown et al, 1999; Issa et al, 1998). VDR is a nuclear transcription factor that belongs to the nuclear hormone receptor superfamily and regulates the transcription of vitamin D target genes (Bouillon et al 1998; Issa et al, 1998). After the VDR is activated via binding the active vitamin D, it heterodimerizes with other nuclear receptors and binds to specific DNA sequences, referred to as vitamin D response elements (VDREs) in the promoter region of vitamin D target genes (Saccone et al, 2015).

VDRs are found in almost all tissues in the human body and they regulate the expression of hundreds of genes. *VDR* gene polymorphisms are associated with type 1 diabetes and other autoimmune diseases, different cancers, severe diabetic retinopathy, and osteoporosis, but also for example with pancreatic insulin secretion (Hitman et al, 1998; Jolliffe et al, 2016; Ogunkolade et al, 2002; Pani et al, 2000).



### 2.2.3 VITAMIN D DEFICIENCY IS DEFINED BY SERUM 25OHD CONCENTRATION

Concentration of 1,25OHD in the blood is efficiently regulated while 25OHD concentration is not. Serum 25OHD concentration therefore provides an indication of vitamin D stores obtained from both synthesis in the skin and dietary intake and is generally accepted as an indicator of vitamin D status in the body (Zerwekh et al, 2004). Although vitamin D can be produced in the skin through UV radiation, it is usually essential for people living far from the equator to receive vitamin D from diet and supplements.

Vitamin D status is also modified by several physiological, life-style and genetic factors. Factors that associate with low serum 25OHD concentration include decreased intestinal absorption and for example smoking, low physical activity and high body mass index (BMI) (Margulies et al, 2015; Miettinen et al, 2014; Skaaby et al, 2016). The biological mechanisms explaining most of these associations are poorly understood.

The heritability of serum 25OHD concentration is estimated to be at least 30% (Hunter et al, 2001; Shea et al, 2009). Several single nucleotide polymorphisms (SNPs) in genes of the metabolic pathway of vitamin D affect serum 25OHD concentration. Previous studies have consistently found associations between serum 25OHD concentration and several SNPs in the *NADSYN1/DHCR7*, *CYP2R1*, *CYP24A1* and *GC* genes or near these genes (Abbas et al, 2008; Ahn et al, 2010; Engelman et al, 2008; Fang et al, 2009; Jolliffe et al, 2016; McGrath et al, 2010; Wang et al, 2010). SNPs located in the *VDR* and *GC* genes influence the response to vitamin D supplementation (Gaffney et al, 2016).

Frequently discussed matters in the field of vitamin D research are the optimal serum 25OHD concentration for health, as well as the threshold for vitamin D deficiency.

For a long time, serum 25OHD concentration of  $\leq 25$  nmol/l was considered indicative of vitamin D deficiency. With the increase of research in this field in the 1990s revealing a number of associations between vitamin D and different diseases, many researchers started to question whether the threshold should be set higher. The Institute of Medicine has in their report (2011) concluded that 50 nmol/l or higher should cover the vitamin D requirements of 97.5% of the population in North America, whereas the Endocrine Society ended up recommending a serum 25OHD concentration of at least 75 nmol/l as sufficient (Holick et al, 2011). This debate has been ongoing in Finland as well with some researchers stating that the recommendations should be set higher (Mäkitie, 2012) and some speculating that the current recommendations may be adequate (Raulio et al, 2016).

It has been argued that the threshold for an adequate vitamin D status should not be set based on the lowest serum 25OHD concentration that prevents symptoms of vitamin D deficiency (such as rickets or osteomalasia), but based on different criteria. For example, it has been suggested that vitamin D status should be equal to that of people living near the equator

(>100 nmol/l) with living habits similar to our ancestors (Luxwolda et al, 2012). It has also been suggested that vitamin D intake should be high enough for a lactating mother to be able to fulfil the vitamin D need of the infant which has been estimated to end up with similar range around 100-150 nmol/l (Heaney, 2014).

Researchers do not agree on the question of toxicity of vitamin D either. The current tolerable upper limit of vitamin D intake is according to the European Food Safety Authority 25 µg per day for children under 10 and 50 µg for older children and adults ([www.efsa.europa.eu](http://www.efsa.europa.eu)). The Institute of Medicine in the United States suggests a maximum of 25 µg per day for ages 0-1, 62.5 µg for ages 1-3, 75 µg for ages 3-8 and 100 µg for age 9 and above (IOM, 2011). The Endocrine Society recommends a maximum of 400 µg per day (Holick et al, 2011; Ross et al, 2011). A threshold for toxic serum 25OHD concentration has been suggested to lie somewhere from 250 to 600 nmol/l, depending on the view (Hathcock, 2007; Holick et al, 2011; Jones 2008).

In Finland, vitamin D deficiency has shown to be common during the past decades in different population groups such as children and adolescents, pregnant women, dark-skinned immigrants and in the general adult population (Cashman et al, 2016; Islam et al, 2012; Miettinen et al, 2014; Munger et al, 2013; Pekkinen et al, 2012). Therefore, nutrition policy strategies have been implemented to improve general vitamin D status in Finland. Vitamin D fortification was started in 2002 with an amount of 0.5 µg of vitamin D per 100 ml of liquid dairy products and 10 µg per 100g of margarine (Ministry of Trade and Industry of Finland, 2002). In 2010 the suggested amount of fortification was doubled (National Nutrition Council, 2010). According to national population-based FINRISK surveys implemented in Finland in 2002, 2007 and 2012, the average vitamin D intake has increased from 2002 to 2012 (Raulio et al, 2012), although the increase results not only from an increased vitamin D intake from dietary sources, but also from increased use of vitamin D containing supplements.

#### **2.2.4 VITAMIN D ASSOCIATES WITH SEVERAL DISEASES**

In addition to direct proven consequences of vitamin D deficiency, rickets in children and osteomalasia in adults, inadequate vitamin D status is associated with a large number of diseases including different types of cancers, cardiovascular and autoimmune diseases. Vitamin D status associates also with the survival of some of these diseases. The inability to define the significance of these associations together with mainly lean results in intervention trials has led to a suggestion that poor vitamin D status is a mere biological marker of ill health rather than a causal agent in the disease process of any of these diseases (Autier et al, 2014).

Some relatively strong evidence of the capacity of vitamin D to control symptoms of a disease, however, exists. Additional vitamin D supplementation decreases the occurrence of severe asthma attacks in

children according to a recent Cochrane review of clinical trials (Martineau, 2016). In a recent randomised control trial it was also shown that the symptoms of children with autism spectrum disorder can be efficiently controlled with additional relatively high dose of vitamin D supplementation (7.5 µg/kg/day) (Saad et al, 2016).

It may be that the amount of vitamin D supplementation in most of the previous intervention trials has been too low to produce notable differences in vitamin D status between the treated and untreated groups, and therefore no protective effect has been seen.

## **2.3 EXISTING EVIDENCE ON THE RELATIONSHIP BETWEEN VITAMIN D AND TYPE 1 DIABETES**

High-quality intervention trials investigating the capacity of vitamin D to prevent or delay type 1 diabetes are lacking, but there is a considerable amount of studies evaluating epidemiological associations.

### **2.3.1 GEOGRAPHICAL AND SEASONAL PATTERNS OF TYPE 1 DIABETES**

The involvement of vitamin D in the pathogenesis of type 1 diabetes has been suggested based on an epidemiological association between geographical location and incidence of type 1 diabetes. Type 1 diabetes is more common in the northern latitudes (EURODIAB ACE Study Group, 2000; Dahlquist and Mustonen, 1994) which has been suggested to reflect the effect of a low supply of vitamin D through UV radiation. It has also been shown that the average daily UV radiation predicts the future incidence of type 1 diabetes (Sloka et al, 2008). There seems to be an overall higher disease incidence in countries with a western life-style, however, compared to lower income countries at the same latitude, for example between the Western and Eastern Europe or between Finland and Russia (EURODIAB ACE Study Group, 2000; Podar et al, 2001; Kondrashova et al, 2005). In Sardinia, the disease incidence is considerably higher compared to the other parts of Italy and southern Europe (Borchers et al, 2010a). Given the high amount of solar UV radiation in Sardinia (~300 days of sunlight per year) (Cocco et al, 2012), the high incidence of type 1 diabetes is inconsistent with the UV radiation hypothesis. Genetic factors have been suggested to at least partly explain the high incidence (Borchers et al, 2010a).

The supply of vitamin D through skin exposure to UV radiation has also been suggested to influence the clinical diagnosis of type 1 diabetes. A seasonal pattern in the clinical diagnosis of type 1 diabetes was presented in a study that used comprehensive data of the World Diabetes Mondiale project

(WHO DiaMond) collected from 53 countries worldwide (Moltchanova et al, 2009). Altogether 42 of the 105 centers included showed significant seasonal variation. The peak in the season of diagnosis varied according to the geographical position with steeper seasonal pattern detected in areas further from the equator. Mainly a peak in the season of diagnosis was detected during the winter months and a trough during the summer months. A seasonal trend in the disease diagnosis has been presented in other studies as well, but with the peak found during different seasons in different studies (Karvonen et al, 1996; Samuelsson et al, 2007; Vaiserman et al, 2007).

Some studies have also demonstrated seasonality in birth of type 1 diabetes patients suggesting an in-utero effect of a seasonal environmental factor. A peak in the season of birth of type 1 diabetes patients has been seen during the spring and summer months in some studies (Kahn et al, 2009; Rothwell et al, 1996), whereas other studies report no seasonal trends (McKinney and the EURODIAB Seasonality of Birth Group, 2001).

While the geographical and seasonal patterns associated with type 1 diabetes have been interpreted to result from differences in the supply of vitamin D, it needs to be considered that in addition to vitamin D synthesis, UV radiation has a variety of other functions in the human body. It is not known whether the other effects of UV radiation contribute in the pathogenesis of type 1 diabetes. Since viral infections are also suspected environmental triggers of type 1 diabetes, the known seasonal trend in viral infections may explain the seasonal trends in type 1 diabetes (Afoke et al, 1991). The seasonal trends in viral infections have been suggested to result at least partly from seasonal variation in UV radiation and thus vitamin D supply (Abhimanyu and Coussens, 2017).

Since the autoimmune process of type 1 diabetes is known to begin years or even decades before the diagnosis (Knip et al, 2010), the detected seasonal pattern in the clinical onset of the disease does not provide information on the environmental triggers that initially start the autoimmune process. It has been discovered though, that also the appearance of the first autoantibodies follows a seasonal pattern with higher appearance of the first autoantibodies during the fall and winter (Kimpimäki et al, 2001; Lynch et al, 2008).

Interestingly, it has also been shown that gene expression follows a seasonal pattern. This novel finding was presented in 2015 with results of a seasonal pattern in mRNA expression levels of more than 4000 genes (Dopico et al, 2015). Importantly, many of these genes encode proteins essential for the function of the immune system, such as interleukin 6 receptor and C-reactive protein. In winter a pro-inflammatory transcriptomic profile can be detected, which may be a consequence of the lack of sunlight (Dopico et al, 2015). These findings create a novel approach in understanding the meaning of the observed seasonal patterns in type 1 diabetes and do not support a theory that the mere restricted supply of vitamin D would explain the findings.

### **2.3.2 STUDIES IN THE NON-OBESE DIABETIC (NOD) MICE**

Non-obese diabetic (NOD) mice provide a useful tool for investigating the role of vitamin D and other environmental factors in the aetiology of type 1 diabetes. NOD mice develop autoimmune diabetes as a result of T-cell mediated destruction of the  $\beta$ -cells in the pancreas usually by 4-6 months of age (Delovitch and Singh, 1997).

It was already shown in the 1990s that treating the NOD mice before the first symptoms of an inflammation in the  $\beta$ -cells with 1,25OHD, delays the disease process (Mathieu et al, 1992). In these early experiments, a vitamin D analogue (KH1060) was also shown to decrease the incidence of type 1 diabetes in NOD mice (Mathieu et al, 1995). Later in the 2000s it was shown that the active form of vitamin D not only delays the disease process but can even completely protect the NOD mice from the disease (Zella et al, 2003).

The effect of vitamin D during the pre-clinical phase has also been studied. First it was shown that although a vitamin D analogue (MC1288) alone could not delay the disease process in the pre-clinical phase of NOD mice, the combination of vitamin D and cyclosporin A (immunosuppressive drug that inhibits signalling from the T-cell receptor) could (Casteels et al, 1998). Later it was shown that another vitamin D analogue (Ro 26-2198) was independently capable of delaying the disease process in the pre-clinical phase of the disease NOD mice (Gregori et al, 2002).

Since different vitamin D analogues can delay or prevent diabetes progression in NOD mice, it was a surprise to find that in the NOD mice genetically lacking the VDR, the disease development did not differ from the control NOD mice (Gysemans et al, 2008). Another later study, however, showed an accelerated type 1 diabetes development in NOD mice lacking the VDR, but interestingly, this acceleration was only seen when the mice were calcium deficient (Drivel et al, 2011). Feeding the mice with a diet high in calcium prevented the accelerated disease process (Driver et al, 2011).

The challenge in these experiments or in testing the effect of these vitamin D analogues in humans is the fact that the positive results have been obtained by using non-physiological vitamin D analogue dosing that leads to increased calcium levels. These studies do, however, provide strong evidence of the capacity of vitamin D analogues to interfere with the disease process in the NOD mice and therefore justify future intervention studies in humans.

### **2.3.3 STUDIES IN HUMANS**

Several human studies have investigated the association between vitamin D intake or vitamin D status and type 1 diabetes. Overall the results are conflicting and well-planned large intervention trials are still missing.

### *Intake of vitamin D during pregnancy and childhood*

Studies evaluating the relationship between type 1 diabetes or autoantibody appearance and intake of vitamin D from dietary sources or supplements during pregnancy or childhood will be described in this section. Three different approaches have been used in these studies, intake of vitamin D from cod-liver oil, from vitamin D containing supplements or from food.

Vitamin D status is influenced by many factors: the amount of UV radiation exposure, diet, intestinal absorption, life-style and genetic factors. Several factors such as sex, baseline vitamin D status and physical activity have also shown to modify the individual response for vitamin D supplementation (Rees et al, 2016). Therefore, studies that have evaluated the association between type 1 diabetes and vitamin D intake based only on the vitamin D supply from dietary sources or supplements, cannot reliably define the actual vitamin D status of the mother or infant. This needs to be considered when evaluating the following evidence.

In the first study in the field, it was shown that maternal cod-liver oil supplementation associated with a lower risk for type 1 diabetes in the offspring in Norway (Stene et al, 2000). The same group, however, could not confirm their findings in a larger study with a similar study setting (Stene et al, 2003). The maternal use of vitamin D supplements associated with a reduced occurrence of autoantibodies in the All Babies In Southeast Sweden (ABIS) study at 1 year of age, but not at 2.5 (Brekke et al, 2007). Later, the ABIS study did not find an association between maternal intake of vitamin D supplements and the risk for type 1 diabetes in children before 14-16 (Granfors et al, 2016). No association was found between maternal vitamin D supplement use and the risk for type 1 diabetes or the occurrence of autoantibodies in Finnish children (Marjamäki et al, 2010). Higher maternal vitamin D intake from food, but not from supplements, associated with a decreased risk for the appearance of autoantibodies in the United States (Fronczak et al, 2003). The authors suggest that this may result, for example, from a better bioavailability of vitamin D from food than from supplements.

Vitamin D supplement use during infancy associated with a considerable reduction in type 1 diabetes risk in Finland (Hyppönen et al, 2001). The children that regularly received vitamin D supplementation had a markedly (88%) reduced risk for developing type 1 diabetes. The same association was shown with data from seven European countries (The EURODIAB Substudy 2 Study Group, 1999). In Norway, no association was found between cod-liver oil or vitamin D supplementation during infancy with the risk for type 1 diabetes in a pilot study, but in a larger study by the same group a couple of years later, the use of cod-liver oil, but not vitamin D supplements, during infancy associated with a reduced risk for type 1 diabetes (Stene et al, 2000; Stene et al, 2003). The intake of vitamin D prior to diagnosis did not associate with the appearance of autoantibodies or the risk for developing type 1 diabetes in the United States (Brekke et al, 2007; Simpson et al, 2011).

Overall these studies present conflicting results. This may be a result of a low vitamin D supplementation recommendation amount, leading to only small differences in the actual vitamin D status of the mothers/infants that took vitamin D supplementation compared to those that did not. An evident exception is the Finnish study by Hyppönen et al (2001) where a daily dose of 50 µg of vitamin D supplementation was recommended for the infants leading presumably to a marked difference in vitamin D status between the supplement users and non-users. This study showed the strongest association between intake of vitamin D and type 1 diabetes risk of all studies reported thus far.

#### *Serum 25OHD concentration during pregnancy and childhood*

Studies evaluating the relationship between type 1 diabetes and serum 25OHD concentration during pregnancy or childhood will be described in this section.

Low serum 25OHD concentration during the last trimester of pregnancy associated with an increased risk for type 1 diabetes in the child in Norway (Sørensen et al, 2012). In a relatively large Danish and small Italian study no association was found between neonatal serum 25OHD concentration in dried blood spots and risk for developing type 1 diabetes (Cadario et al, 2015; Jabobsen et al, 2016). In Norway, the average serum 25OHD concentrations were considerably higher than in the Danish study, although they cannot be directly compared because of the differences in both analytical methods and sample collection.

Serum 25OHD concentrations in children prior to diagnosis did not associate with the appearance of autoantibodies or the development of type 1 diabetes in the Finnish DIPP and DIABIMMUNE studies, and in the DAISY study in the United States (Mäkinen et al, 2016; Reinert-Hartwall et al, 2014; Simpson et al, 2011). In a German study, however, children with multiple autoantibodies were found to have lower serum 25OHD levels than children with no autoantibodies (Raab, 2014). In this study vitamin D deficiency was not, however, associated with faster development of type 1 diabetes in children with multiple autoantibodies. Another study that used samples collected from the military service members in the United States found that lower serum 25OHD concentration associated with an increased risk for developing type 1 diabetes (Gorham et al, 2012).

#### *Serum 25OHD concentration among type 1 diabetic patients*

There seems to be no information available on the possible differences in vitamin D intake between type 1 diabetic patients and controls, but it has been shown that type 1 diabetic patients have lower serum 25OHD concentrations than non-diabetic controls (Littorin et al, 2006; Pozzilli et al, 2006). Given the substantial evidence on lower serum 25OHD concentrations compared to controls in various autoimmune and other chronic diseases, and considering the inability of the current research to

interpret these associations, the clinical significance of these observations remains unclear.

The low serum 25OHD concentrations in type 1 diabetic patients may be explained by the presence of conditions that have an impact on vitamin D metabolism/ supply. Celiac disease is more common among type 1 diabetic patients than in non-diabetic controls; the prevalence of celiac disease is about 1% in the general population but higher (1-16%) in type 1 diabetic patients (Pham-Short et al, 2015). Poorer vitamin D status may therefore result at least partly from decreased intestinal absorption of vitamin D among the patients. Chronic kidney disease, as well as autoimmune thyroid disease, both affect up to 30% of type 1 diabetic patients (González et al, 2004; Hovind et al, 2003). Both of these conditions associate strongly with vitamin D deficiency, but the detailed mechanism explaining the association is not known.

In addition to the classical long-term complications related with type 1 diabetes, such as retinopathy, neuropathy and increased cardiovascular disease risk, the disease also relates with skeletal fragility (Weber and Schwartz, 2016). While it has been suggested that hyperglycemia may be the explaining factor (Neumann et al, 2011), the role of vitamin D has also been discussed due to its central role in the development and maintenance of healthy bones (Thraillkill and Fowlkes, 2013). The ability of the disease process of type 1 diabetes to alter vitamin D metabolism is not known. The increased fracture risk of the type 1 diabetic patients, however, seems interestingly to already be apparent during early stage of the disease (Weber et al, 2015). This raises a question whether there are certain vitamin D metabolism related innate differences in type 1 diabetic patients compared to controls, which may affect not only the formation of the bones but possibly also the risk for type 1 diabetes.

It has been reported that people with an immune-mediated disease such as multiple sclerosis, have a weaker response to vitamin D supplementation (Bhargava et al, 2016). This suggests that the activation of the immune system that follows the onset of an immune-mediated disease increases the need for active vitamin D, mirrored by a decrease in serum 25OHD concentration. It is also possible, though, that the low serum 25OHD concentrations of the patients with type 1 diabetes or other immune-mediated disease are at least partly explained by a decreased intestinal absorption of vitamin D.

Intervention studies investigating the effects of vitamin D supplementation on type 1 diabetic patients are scarce. In one study supplementation with active vitamin D did not show protective effect on the remaining  $\beta$ -cells of the patients (Bizzarri et al, 2010). However, a relatively large amount of vitamin D supplementation (100  $\mu$ g per day) was in another study shown to improve the blood sugar control of type 1 diabetic patients (Bogdanou et al, 2016). The effect of relatively large amount of vitamin D



supplementation during pre-clinical phase of the disease should be investigated.

### 2.3.4 GENETIC FACTORS IN THE METABOLIC PATHWAY OF VITAMIN D

Several genes, or SNPs, in the metabolic pathway of vitamin D have been associated with type 1 diabetes.

The most studied SNPs are *Taq1* (rs731236), *Bsm1* (rs154410), *Apa1* (rs7975232) and *Fok1* (rs2228570) located in the *VDR* gene. These SNPs have also been associated with other autoimmune diseases and osteoporosis. The *VDR* gene encompasses at least 105 kb and the polymorphisms in this large gene have not been extensively studied. In Caucasians, there are five genomic blocks within the *VDR* gene showing high linkage disequilibrium (Fang et al, 2005). Within such a block, SNPs are in strong linkage disequilibrium with each other, but show very little linkage disequilibrium with SNPs outside the block.

A systematic review and meta-analysis published in 2014 concluded that there is no association between the individual SNPs in the *VDR* gene (*Taq1*, *Bsm1*, *Apa1* and *Fok1*) and type 1 diabetes, whereas the haplotypes that some of these SNPs form together were suggested to associate with the disease (Tizaoui et al, 2014). Another meta-analysis from 2012 found an association between type 1 diabetes and *Bsm1* but not with *Taq1*, *Apa1* and *Fok1* (Zhang et al, 2012). When exploring the existing evidence in more detail, it was seen that some studies report clearly different results for the *Bsm1* and *Taq1* SNPs (please see for example Panierakis et al, 2009), whereas others report almost equal associations for these two SNPs (please see for example Orlow et al, 2012).

Looking at this inconsistency a little bit closer, it was noticed that there are two mutations (SNPs) located in the binding site of the *Bsm1* primer that has been extensively used in studies using traditional restriction fragment length polymorphism technique (Figure 2). As it turned out, the inconsistency in the results had been noticed by others as well. Zajícková et al. (2003) experimentally tested the effect of SNPs in the *Bsm1* primer binding site. They found that SNP in the *Bsm1* primer binding site can cause failure of the polymerase chain reaction (PCR) amplification with a drop-out of the *b* allele in heterozygotes, and result in errors in the prevalence of BB genotypes of the *Bsm1* SNP. This may lead to errors in the results and confound genetic findings.

Therefore, the results of a considerable amount of previous studies assessing the association between *VDR* gene polymorphisms and type 1 diabetes may contain errors and should be evaluated with caution. When using primers that do not overlap with the SNPs in the *VDR* gene sequence (Orlow et al, 2012; Figure 2), the *Bsm1* and *Taq1* SNPs produce similar

associations and can be concluded to be in almost complete linkage disequilibrium.

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ccttcaccatggacgacatgtcctggacctgtggcaaccaagactacaagtaccgcgtca
gtgacgtgaccaaaggtatgcctagactccacctcctggggagtcttttcagctcccag
attctggctccaccgctcctggggtttggctccaatcagatacatgggagggttaggc
accaacaggagagaagggcgagggtcagacccatggggttgagggtgggtgggcggctc
ctcagctctgccccagtagctggccattgtctctcacagccggacacagcctggagctg
attgagcccctcatcaagttccagggtgggactgaagaagctgaacttgcagaggaggag
catgtcctgctcatggccatctgcacgtctccccagggtatggggccaggcaggaggag
ctcaggacctggggagcggggagtatgaaggacaaagacctgctgaggccagctgggc
aacctgaaggagacgtagcaaaaggagacacagataaggaaatacctactttgctggtt
tgcagagcccctgtggtgtgtggacgctgaggtgccccctcactgcccttagctctgcctt
gcagagtgtgcaggcgattcgtaggggggattctgaggaactagataagcagggttctg
gggccacagacaggcctgcc1cattccaataactcaggctc2cgctcctgctgaactg3ggc
tcaacattcctgttatttgaggtttcttgc4cggcagggtacaaaactttggagcctgaga
gatggttctgcctatatagtttacctgattgattttggaggcaatgtgcagtga5cccttg
6acctcttccgctggttagaggtgagaagaggagaaaaggccgaagaggaagtattgtg

```

- 1 = rs1544410 (BsmI)
- 2 = rs11574109
- 3 = rs112473013
- 4 = rs11574110
- 5 = rs757343
- 6 = rs11574111

BsmI primers overlapping with two SNPs in the VDR gene sequence (used in several RFLP studies, for example by):

```

caaccaagactacaagtaccgcgtcagtga          BsmI PCR primer 1
cccttcacctcttcgctggtt                   BSM1 PCR primer 2

```

BsmI primers not overlapping any SNPs in the VDR gene sequence (used for example by Orlow et al, 2011):

```

cctcactgcccttagctctg          BsmI PCR primer 1
ccacagacaggcc                  BsmI PCR extension primer
ctttggagcctgagagatgg         BsmI PCR primer 2

```

**Figure 2.** Location of the single nucleotide polymorphisms (SNPs) in the vitamin D receptor (VDR) gene sequence. Two SNPs (5 and 6) are located in the binding site of the PCR primer often used in studies using restriction fragment length polymorphism technique.

In a recent review covering over 50 gene loci currently known to contribute to the risk for type 1 diabetes (Pociot and Lernmark, 2016) only one SNP association was reported in the central genes in the metabolic pathway of vitamin D (rs4646536 in *CYP27B1*). This association was originally reported in 2007 by Bailey et al. and confirmed by the same group in a study in 2011. In the latter study an association was also found between the SNPs in the *DHCR7* and *CYP2R1* genes and type 1 diabetes (Cooper et al, 2011). Rs4646536 in the *CYP27B1* gene has also been associated with type 1 diabetes related autoantibodies along with rs12785878 in *NADSYN1/DHCR7* gene locus (Frederiksen et al, 2013a).

The results of associations between type 1 diabetes and SNPs located in genes in the metabolic pathway of vitamin D have been partly conflicting. There are several explanations for the inconsistency between the results, including small sample size leading to lack of statistical power, and differences in allele frequencies between different populations. The SNP association may also only be a marker of a causal genetic association meaning, for example, that different populations may have different SNP markers for a specific causal genetic factor (Uitterlinden et al, 2004). Last, genetic association studies without a justified biological mechanism explaining the associations will also always produce false also positive results.

## **2.4 SUGGESTED MECHANISMS EXPLAINING THE RELATIONSHIP BETWEEN VITAMIN D AND TYPE 1 DIABETES**

The evidence of an association between vitamin D and type 1 diabetes is mainly epidemiological without detailed information on the possible mechanisms explaining the association. Some mechanisms have been proposed mainly related to the capacity of vitamin D to modulate the functions of the immune system. Autoimmune diseases are characterised by an inflammation, which is part of the complex reactions of the immune system aimed at removing a harmful substance. A properly working immune system protects the body against invading micro-organisms such as viruses and bacteria, by producing antibodies and lymphocytes. Type 1 diabetes develops as a result of activation of the immune system when the  $\beta$ -cells in the pancreas are incorrectly recognised as foreign and destroyed.

*Vitamin D affects type 1 diabetes or type 1 diabetes affects vitamin D metabolism?*

While the participation of vitamin D deficiency in the development of autoimmune and other immune-mediated diseases has been suggested for decades, it has not been discussed whether the disease process itself and the alterations the disease produces to the physiology of the patient, could modify vitamin D metabolism. An interesting finding pointing towards this interpretation is that type 1 diabetic patients already have an increased fracture risk early in the disease (Weber et al, 2015). Although hyperglycemia has been suggested to contribute to the bone fragility of the patients, at least in the long term, it is not likely to explain the increased fracture risk early in the disease. It may be that the disease process of type 1 diabetes begins very early, possibly during the foetal period, and then modifies the development of vitamin D metabolism. This may be mirrored by an increased fragility of the bones of the patients, but also by lower serum 25OHD concentrations.

*T helper cell cytokine profile*

T helper cells are classified as Th1, Th2 and Th17 cells based on their cytokine profile (Harrington et al, 2005). The disease process of type 1 diabetes has traditionally been described to include a decrease in Th2 cytokine production and an increase in type Th1 cytokine production. Vitamin D, or its analogues, have been shown to decrease the Th1-associated pro-inflammatory cytokine production such as interleukin 2 and interferon- $\gamma$ , and to increase the Th2-associated anti-inflammatory cytokine production such as interleukin 5 and interleukin 10 (Alhassan Mohammed et al, 2016; Boonstra et al, 2001; Lemire et al, 1995).

Interestingly, the risk for developing an autoimmune disease or the risk for relapses of an existing autoimmune disease in women has been shown to be higher during the months following a delivery (Khashan et al, 2013; Vukusic et al, 2004). This has been interpreted to result from the presence of foetal cells in the circulation of the mothers postpartum, as foetal cells could induce a rejection reaction in the mother and therefore activation of the immune system (Borchers et al, 2010b). No consensus on the mechanism has been obtained though. The immune system encounters several changes during pregnancy to prevent the mother's body from rejecting the developing foetus. Pregnancy is characterised by a decrease in Th1 cytokine production and an increase in Th2 cytokine production (Strom et al, 1996), following a shift back after the delivery. Serum 25OHD concentrations have been shown to strongly decrease after the delivery, which has been interpreted by the returning of the vitamin D status back to normal after pregnancy (Luxwolda et al, 2012). The same kind of an immediate drop in serum 25OHD concentrations has, however, also been shown after knee

surgery (Reid et al, 2011) which does not support this interpretation. It can be proposed that a shift from a Th2 to a Th1 pattern that is normal right after delivery together with a drop in serum 25OHD concentrations (increasing this shift) may be the triggering factor for genetically susceptible individuals for an initiation or worsening of an autoimmune disease.

It has been discovered that also Th17 cells are important in the pathogenesis of type 1 diabetes evidenced by an increased production of Th17 cells in correlation with an infection of the  $\beta$ -cells in NOD mice (Martin-Orozco et al, 2009). The active form of vitamin D has been shown to decrease the production of Th17-associated pro-inflammatory cytokines (da Costa et al, 2016), therefore possibly participating in the initiation of the disease process of type 1 diabetes.

### *Vitamin activating enzymes and VDRs in the cells of the immune system*

Immune cells have been shown to express vitamin D activating enzymes, with the ability to locally convert inactive vitamin D to its active form (Sigmundsdottir et al, 2007). VDRs have been detected in practically all cells of the immune system such as B- and T-lymphocytes (Alhassan Mohammed et al, 2016), justifying the important role of vitamin D in the immune system and immune system related diseases.

### *$\beta$ -cells*

VDRs and 1- $\alpha$ -hydroxylase (an enzyme that converts 25OHD to active vitamin D) have been identified in the  $\beta$ -cells of the pancreas (Bland et al, 2004). This suggests a role for vitamin D in controlling the function of the  $\beta$ -cells, evidenced by the presence of VDREs in the promoter region of the human insulin gene (Maestro et al, 2003).

In the 1980s in animal models, it was already shown that vitamin D deficiency inhibits the secretion of insulin and that the secretion can be improved by vitamin D administration (Cade and Norman, 1986; Norman et al, 1980). In a recent study it was found that maternal vitamin D-restricted diet of mice leads to increased blood sugar levels, smaller  $\beta$ -cell mass and lower insulin concentration in the offspring (Maia-Ceciliano et al, 2016), highlighting the importance of adequate vitamin D supply during the developmental processes. Administration of vitamin D decreases the inflammation in the pancreas in a mouse model of type 1 diabetes (Wang et al, 2016).

Human studies have gained mixed results on the capacity of vitamin D to improve the release or sensitivity of insulin (Lips et al, 2016). Future studies will need to distinguish the effect of vitamin D on the  $\beta$ -cells in adults or

children with an existing disease and the effect of vitamin D during the developmental process in-utero for later  $\beta$ -cell function.

### *HLA gene region*

Type 1 diabetes has a strong connection with the HLA gene region (Noble, 2015). It has been shown that VDREs exist in the promoter region of at least certain HLA alleles suggesting that vitamin D has a role in the regulation of HLA gene expression (Cocco et al, 2012; Israni et al, 2009; Livingstone 2009; Ramagopalan et al, 2009).

### *Gut microbiota*

Gut microbiota has been suggested as an environmental trigger for type 1 diabetes (Bibbò et al, 2016). Interestingly, recent novel findings point towards a role for vitamin D in modulating the gut microbiota (Kanhere et al, 2016) and it has been shown that the maternal microbiota in the gut and other body sites changes during pregnancy (Nuriel-Ohayon et al, 2016). Therefore, discovery of a role of vitamin D in modulating gut microbiota may reveal a novel mechanism not presented in the literature to explain the various epidemiological associations between vitamin D and type 1 diabetes.

### *Response to vitamin D supplementation*

People with an immune-mediated disease such as multiple sclerosis, have a weaker response to vitamin D supplementation (Bhargava et al, 2016). This observation does not provide a mechanism for associations between vitamin D and type 1 diabetes, but highlights the fact that a higher amount may be needed to control the disease-associated inflammation. This may translate to a higher tolerance of vitamin D supplementation of individuals with immune mediated diseases, which should be further investigated.

### **3 AIMS**

Our main aim was to compare the serum 25OHD concentrations during pregnancy between mothers whose children later developed type 1 diabetes and mothers whose children did not.

#### **Additional aims**

1. Evaluate the prevalence of vitamin D deficiency in pregnant Finnish women
2. Analyse the association between SNPs located in genes in the metabolic pathway of vitamin D and serum 25OHD concentration
3. Analyse the association between SNPs in the metabolic pathway of vitamin D and type 1 diabetes
4. Analyse the association between HLA genetic polymorphisms and serum 25OHD concentration

## 4 SUBJECTS AND METHODS

In order to study the possible relationship between maternal vitamin D deficiency and the risk for type 1 diabetes in the child, we wanted to measure serum 25OHD concentration during pregnancy. In Finland, serum samples are collected in the maternity clinics from almost all pregnancies (98%) during the end of the first trimester to screen for infectious diseases (The Finnish Maternity Cohort; FMC). Samples have been collected since 1983 and are stored in the Prenatal Serology laboratory in the National Institute for Health and Welfare (THL). These samples were ideal for our study purposes since we could measure maternal serum 25OHD concentration after later development of type 1 diabetes in the children was already known – provided that serum 25OHD would be stable enough to be measured years after the sample collection.

### 4.1 FAMILIES WITH TYPE 1 DIABETIC CHILDREN (CASE FAMILIES)

Since the principal investigator of the “Vitamin D and Type 1 Diabetes” study, Professor Jaakko Tuomilehto, had done intensive type 1 diabetes research for decades, we had a database of families with type 1 diabetic children in THL. From this database we were able to identify suitable families for our study. We decided to also include a non-diabetic sibling of the patient to be able to compare serum 25OHD concentrations in two pregnancies of the same mother with a different outcome (a child with or without type 1 diabetes).

At the time of planning the “Vitamin D and Type 1 Diabetes” study it was known that 25OHD is chemically stable. The exact information on the duration of storage after which serum 25OHD concentration could still be measured reliably, however, was lacking. Earlier we had found that there was no difference in the mean serum 25OHD concentration between samples stored for 15-20 years (mean concentration 37.2 nmol/l; n = 45) and for 9-14 years (mean concentration 37.2 nmol/l; n = 31). Based on this result and based on the decades of experience in vitamin D analytics of the co-principal investigator of our project, Professor Christel Lamberg-Allardt (University of Helsinki), we decided to choose 20 years as the maximum storage time. Thus we chose 1994 as the earliest possible birth year for the patients with type 1 diabetes. Later it was discovered that no significant degradation of 25OHD in serum occurs during at least the first 24 years of storage in -20°C (Agborsangaya et al 2010).



The THL database contained 751 families with type 1 diabetic children born 1994 or later in the database (case families). We confirmed with power calculations that this number was large enough to achieve our main aim: to compare serum 25OHD concentrations in case and control mothers. A case-control study setting was thought to serve our study purposes best and therefore we decided to include control families.

## **4.2 FAMILIES WITH NON-DIABETIC CHILDREN (CONTROL FAMILIES)**

Since our main aim was to compare serum 25OHD concentrations during pregnancies of mothers whose children later developed type 1 diabetes (case mothers) to that of mothers whose children did not, we needed control mothers with non-diabetic offspring. Seasonal variation in serum 25OHD concentrations is considerable in Finland (Virtanen et al, 2011), so we decided to match the case and control mothers according to the serum sample collection time. Serum samples of the control mothers were selected so that the samples of the case and control mother pairs were collected during the same day. To ensure a sufficient sample size, we chose 2 control mothers for each case mother.

## **4.3 INVITING OF THE STUDY SUBJECTS**

In case families, an invitation letter was sent to the parents, to the diabetic child and to a non-diabetic sibling of the patient, if available. If there were several siblings, we invited the oldest one (born 1994 or later). In control families an invitation letter was sent to the parents and the control child. Altogether around 5200 persons were invited to participate. The contact information of the case families was available in the THL database. The contact information of the control families was obtained from the National Population Registry.

## **4.4 SAMPLES**

We asked for written informed consent from all invited mothers to use the FMC serum sample for 25OHD concentration measurement. The FMC serum samples are collected during the end of first trimester of pregnancy and stored in -20°C. The duration of pregnancy is on average 40 weeks. The first

trimester of pregnancy consists of weeks 1-12, the second trimester of weeks 13-28 and the third trimester of weeks 29-40. The exact week of pregnancy of each serum sample was not known in this study.

We asked for a saliva sample for DNA extraction from all participants. We chose to collect saliva samples for DNA extraction instead of blood samples for four reasons; 1) there was a relatively new promising kit available (Oragene® kits, DNA Genotek Inc. Ottawa, Canada) that did not seem to have the same problems with DNA yield and quality than the previous saliva extraction methods; 2) samples could be stored at room temperature for a relatively long time in contrast to blood samples that need to be stored in -20°C; 3) sample collection kits could be sent to the homes of the participants instead of them needing to go to a health centre, which we hoped would increase the participation rate; 4) we had children in our study group and therefore a non-invasive home-based sample collection method was considered more convenient for the children compared to collecting blood samples.

## **4.5 LABORATORY ANALYSES AND THE RELIABILITY OF THE RESULTS**

### *Serum 25OHD concentration measurement*

Serum 25OHD concentrations were measured in the laboratory of Professor Christel Lamberg-Allardt to indicate the vitamin D status of the mothers during their pregnancies. Measurement of this metabolite provides the best estimate of an individuals' vitamin D status because it is not significantly regulated and it has a rather long serum half-life (approximately 3 weeks) thus providing an indication of vitamin D stores obtained from both UV radiation and dietary/supplement intake over long periods (Zerwekh, 2004).

The serum 25OHD concentrations were analysed with enzyme-linked immunosorbent assay method with IDS OCTEIA 25-Hydroxy Vitamin D kits, (Immunodiagnostic Systems Ltd., Boldon, UK). The method is based on the binding of specific antibodies to 25OHD in the samples.

It is important to note that serum 25OHD is relatively difficult to quantitate and therefore the analyses require a strong quality control. In our study, the analyses were done in a laboratory with decades of experience in vitamin D analytics and the quality and accuracy of the serum 25OHD analysis was assured on an ongoing basis by participation in the international vitamin D External Quality Assessment Scheme (DEQAS, Charing Cross Hospital, London UK). DEQAS was established in 1989 to ensure analytical reliability of 25OHD assays (Carter et al 2004). The intra- and inter-assay coefficients of variation were 3.57 % and 3.68 %.

Vitamin D status was classified as follows:

<25 nmol/l	severe vitamin D deficiency
25-49 nmol/l	vitamin D deficiency/insufficiency
50-75 nmol/l	sufficient vitamin D status
>75 nmol/l	optimal vitamin D status

This classification is based on the view that seemed to be universal among vitamin D researchers at the time. Later in 2011, the Institute of Medicine in the United States suggested that the threshold for vitamin D deficiency of at least 50 nmol/l is high enough to cover the requirements of 97.5% of the population in Northern America (IOM, 2011). This report has further strengthened the view of setting 50 nmol/l as the threshold for vitamin D deficiency.

*Vitamin D metabolism related genotyping*

For DNA extraction, 2 ml of saliva was collected from all participants using Oragene® kits (DNA Genotek Inc. Ottawa, Canada) (Rogers et al, 2007). In the instructions that were sent to each participant with the Oragene kit, the participants were asked to rinse their mouth with water to remove food particles and then give 2 ml of saliva to the Oragene sample collection vial and close the cap. Oragene antibacterial solution stabilizes the DNA and enables the sample to be stored and shipped at room temperature. The DNA extraction was done with the reagents included in the Oragene kits with a modification to the kit instructions: we added an extra purification step with 70% ethanol since the DNA extracted with the kit instructions did not work properly in the PCR. The DNA samples were stored in -20°C until analysis.

Genotyping of the SNPs in genes in the metabolic pathway of vitamin D was done with TaqMan assay (Applied Biosystems, Paisley, United Kingdom) in the laboratory of the co-principal investigator of the “Vitamin D and Type 1 Diabetes” study, Professor Graham A. Hitman (Barts and the London School of Medicine and Dentistry). The Hardy-Weinberg equilibrium was tested and 10% of the genotyping was done in duplicate. For part of the samples, the correctness of the results of the children was confirmed based on the genotyping results of the parents. A total of 31 SNPs in genes in the metabolic pathway of vitamin D were initially selected on basis of previously published associations with serum 25OHD concentration.

*HLA genotyping*

HLA genotyping was performed using sequence-specific primers (DynaL AllSet+ SSP, Dynal Biotech Ltd, Bromborough, UK). Dynal® Allset™). HLA-B, -DRB1 and -DQB1 genes were selected for HLA genotyping based on

their previously published associations with type 1 diabetes (Pociot et al, 2010).

The HLA-B is highly polymorphic, and thus the number of participants carrying a certain HLA-B allele was in most cases small. Therefore, HLA-B alleles present in our study population were grouped into supertypes according to Sidney et al (2008) as follows: Supertype B07 (B\*07, B\*35, B\*51, B\*55, B\*56), B08 (B\*8), B27 (B\*14, B\*27, B\*38, B\*39, B\*48), B44 (B\*18, B\*37, B\*40, B\*44), B58 (B\*57, B\*58) and B62 (B\*15). The HLA-B alleles B\*13 and B\*47 do not belong to any of the HLA-B supertypes. Some of the HLA-B supertypes and unclassified B alleles (B58 supertype, n=8; B\*13 allele, n=11; B\*47 allele, n=2), and some of the HLA-DRB1 alleles (DRB1\*10, n=10; DRB1\*12, n=24; DRB1\*14, n=21; DRB1\*16, n=7) were not analysed separately due to small sample size. When possible, the correctness of the results of the children was confirmed based on the genotyping results of the parents.

## 4.6 STATISTICAL ANALYSES

When planning the study, we had to define the difference in serum 25OHD concentrations that we would want to detect between the case and control mothers. No information existed on how large of an increase or decrease in serum 25OHD concentration would cause physiological effects. Therefore, we decided to plan the study so that it would detect a relatively small difference (5 nmol/l) between the case and control mothers. At a two-sided 1% significance level ( $\alpha$ ), and statistical power of 90% ( $\beta$ ), the needed sample size was 540; 270 cases and 270 controls (assuming standard deviation of 15 nmol/l in serum 25OHD concentrations).

Since there were 751 families with type 1 diabetic offspring in the THL database, we needed at least 270/751 (36%) of the invited mothers to participate. Since we needed 270 case-control mother pairs, and not just any 270 participants from the case and control mother groups, we estimated that we would need around 50% of the invited mothers to participate. We did not calculate sample size for the genetic analyses since it was not possible to increase the sample size (only 751 families fulfilling the criteria were available). Considering the genetic analyses, we decided to invite all families fulfilling our inclusion criteria (although the mere serum 25OHD concentration comparison could have been done with a smaller sample size).

Statistical analyses were performed using PASW statistics 18 for Windows (*Released 2009*, Chicago: SPSS Inc.), Intercooled Stata10 for Windows (StataCorp. 2007. *Stata Statistical Software: Release 10*. College Station, TX: StataCorp LP) and SAS 9.3 (SAS Institute Inc., Cary, NC). Since serum 25OHD concentrations are known to have considerable seasonal variation in Finland, all analyses were adjusted for month of sample collection. This was

not, however, needed when we compared maternal serum 25OHD concentrations between case and control mothers since the samples of each case-control pair were matched according to the date of sample collection.

The mean serum 25OHD concentrations were compared with a Student's *t*-test and with Pearson's  $\chi^2$  test. Linear regression modelling was used when adjustment for the month of sample collection was needed. When we tested mother type difference, interaction term between case and control mothers was incorporated into the models.

*P* values  $< 0.05$  were considered statistically significant. Multiple testing was controlled for using the false discovery rate (FDR) method (step-up procedure described by Benjamini and Hochberg (Benjamini and Hochberg, 1995). If the original *p* value was smaller than the Benjamini-Hochberg critical value, the difference in genotype distribution was considered statistically significant.

## 4.7 IDENTIFYING CRITICAL STEPS AND SOURCES OF ERROR

Before initiating the "Vitamin D and Type 1 Diabetes" study, we tried to identify the critical steps and sources of error. The risks related to the reliability or quality control of the laboratory analyses and their solutions were described in a previous chapter (please see 4.5 for more information). In addition, we identified the following risks:

1. The saliva sample may not be from the person it was supposed to be

*Solution:* The saliva samples were collected by the participants themselves in their homes. To decrease the probability that the saliva sample collection tubes would switch by mistake between the invited family members, we decided to pack the study information including the tubes individually to each family member. We also wrote the first name of each family member with large letters to the sample collection tubes. Though, a possibility that a part of the sample collection tubes have been switched still exists. We estimated that the proportion of switched samples would be low and would therefore have only a minor impact on the results.

2. The number of the participants would not be sufficient

*Solution:* According to the power calculations, we would need around 50% of the invited mothers to participate. We thought this was achievable and considered the risk for not being able to recruit enough participants low. We did not know, however, if the sample size would be large enough to perform the genetic analyses (SNP and HLA). Therefore, we planned alternative

manners to perform the analyses. We decided to group HLA alleles into larger groups (supertypes) if the sample size would be too small to analyse HLA alleles separately.

3. Some of the children classified as non-diabetic control children or non-diabetic siblings may in fact be type 1 diabetes patients. They may not be included in our database of families with type 1 diabetic children, or the non-diabetic children may have developed type 1 diabetes after the last update of our database.

*Solution:* We included a short questionnaire in the study, where the disease status was asked from all participants. We excluded those control children and non-diabetic siblings that had type 1 diabetes. An uncertainty, however, remained as to whether some of the control children and non-diabetic siblings would develop type 1 diabetes during the study, but we estimated that this would only have a minor impact on the results.

4. The requested serum sample may not be available in the FMC.

*Solution:* We were told by the Prenatal Serology laboratory (THL) that is in charge of the FMC samples, that usually over 90% of samples are available for any research project done with the FMC samples. Since we selected two control families for each case family, we considered that this risk would concern mainly the samples of the case mothers.

5. The FMC serum samples may have been thawed several times after the collection and initial storage.

*Solution:* We contacted the Prenatal Serology laboratory (THL) and asked whether it was likely that the samples had been thawed several times, which could possibly cause either evaporation of the samples or degradation of 25OHD, or both. We were told that it is unlikely that a significant proportion of the samples would have been used or that the samples would have been thawed multiple times. Although we did not have the exact information on whether the samples had been thawed or not, we estimated this risk low and decided to accept it.

6. The DNA quality and quantity in the saliva samples

*Solution:* The fact that there would be no health care personnel collecting the samples but they would be collected at homes of the participants, could affect whether the instructions were followed correctly and therefore the quality

and quantity of the samples. We included figures of different steps of the sample collection in the instructions to decrease the risk for incorrect sample collection.

Although the quality reports from the manufacturer (DNA Genotek Inc.) of the saliva kit seemed impressive, we did not know whether the DNA quality would be good enough for our purposes. Therefore, we performed a pilot testing of the Oragene kits. We considered both the DNA yield and quality satisfactory.

## **4.8 ETHICAL CONSIDERATIONS**

Informed written consent was collected from all participants. The consents of the children were signed by their legal guardians. There was no benefit for the participants to take part in the study and no compensation was paid. The study subjects and families were assigned a study-ID numbers according to the prevailing practice in THL. The genetic information was not given to the participants. The study plan was approved by the Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa (168/Eo/2006).





## **5 RESULTS AND THEIR EVALUATION**

### **5.1 STUDY PARTICIPANTS AND SAMPLES (I-IV)**

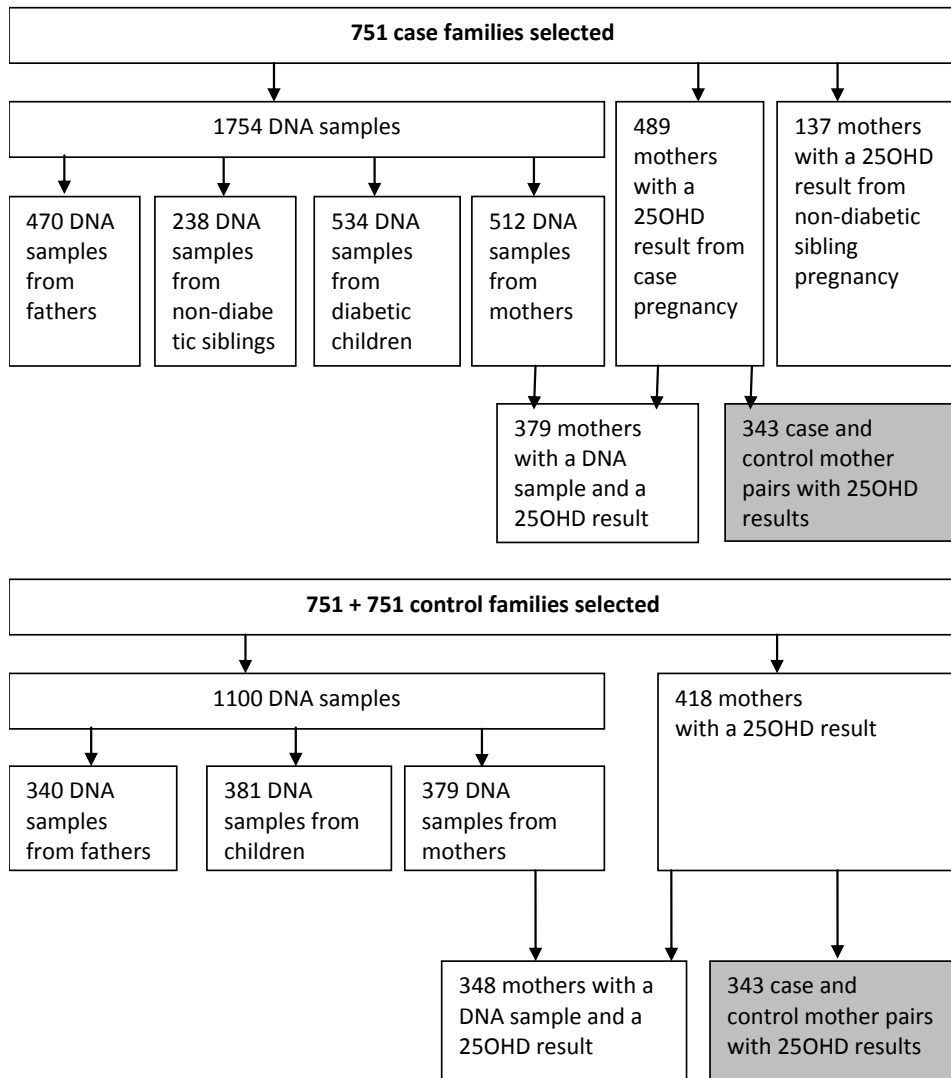
The samples collected in the “Vitamin D and Type 1 Diabetes” -study were used in all the individual studies of this thesis (I-IV). Initially, 751 families with type 1 diabetic children (case families) were selected from the THL database. We pre-selected two control families (families with a non-diabetic child) for each case family. If the first control family did not participate, we invited the other. The final number of DNA samples received and serum 25OHD concentration results obtained are presented in figure 3. The total number of serum 25OHD concentration results was 1044 and DNA samples 2854. The number of successful SNP/HLA analysis results varied according to the SNP/HLA allele.

The main reason for a missing sample was that the invited persons did not react to our invitation or reminder letter. Over 90% of the mothers that participated in our study had a serum sample available in the FMC. Over 99% of the serum 25OHD concentration analyses were successful. Over 95% of the DNA extractions and over 90% of the SNP and HLA analyses were successful. The main reason for unsuccessful DNA extractions or genetic analyses was a poor quality of the saliva sample or DNA sample derived from the saliva sample.

Serum 25OHD concentration was measured and SNP analyses were done in all samples. Due to high costs, HLA genotyping was only performed in part of the DNA samples (~40% of all samples).

In the end, 343 case-control pairs (46% of the selected case-control pairs) with a successful serum 25OHD concentration measurement were included. According to our power calculations 270 case-control pairs were needed. The sample collection was therefore evaluated successful.

A higher percentage of the invited case families participated than of the control families, even though we pre-selected two control families for each case family. This is likely to result from higher motivation among families with a diabetic child. Since we had a case-control study setting, possible differences between the participants and non-participants can be assumed to be similar among the case and control families and therefore not likely to cause biases.



**Figure 3.** Participants of the "Vitamin D and Type 1 Diabetes" study. Final number of DNA samples and serum 25-hydroxyvitamin D (25OHD) concentration results separately for case and control families.

*Final number of participants/samples in the individual studies:*

I) 343 case and control mother pairs with serum 25OHD concentration results, 137 case mothers with a serum 25OHD result from a pregnancy of a non-diabetic sibling of the diabetic child.

II) 474 case and 348 control mothers with both a serum 25OHD concentration result and a DNA sample available. DNA samples from 512 case mothers, 470 case fathers, 534 type 1 diabetic children, 238 non-diabetic siblings, 379 control mothers, 340 control fathers, 381 control children.

III) 474 case and 348 control mothers with both a serum 25OHD concentration result and a DNA sample available.

IV) 395 mothers (216 case and 179 control mothers) with a HLA-B result, 501 mothers (264 case and 237 control mothers) with a HLA-DRB1 result and 475 mothers (236 case and 239 control mothers) with a HLA-DQB1 result available.

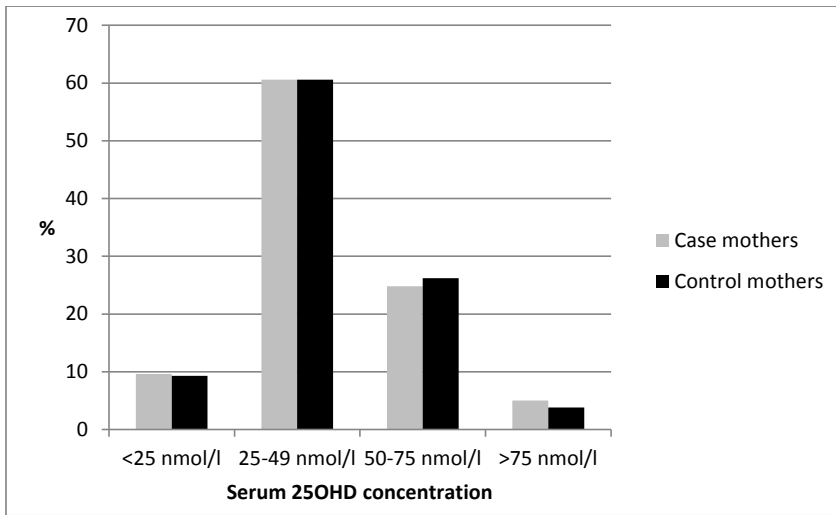
## **5.2 VITAMIN D DEFICIENCY IN PREGNANT FINNISH WOMEN (I-IV)**

In the “Vitamin D and Type 1 Diabetes” study, a serum 25OHD concentration result was available from 1044 pregnancies. Serum samples were collected during the first trimester of pregnancy with diabetic children, non-diabetic siblings and control children.

Only 5.4% of the pregnant women had a serum 25OHD concentration that is considered optimal and more than two thirds (68.9%) of the mothers presented with vitamin D deficiency or insufficiency (Figure 4).

The mean serum 25OHD concentration in all samples was 44.4 nmol/l. The lowest serum 25OHD concentration detected was 14.3 nmol/l and the highest 173.1 nmol/l.

Studies on vitamin D status during pregnancy in Finland are scarce, but similar results to ours have been found. When evaluating results obtained in different studies, it needs to be remembered that serum 25OHD is relatively difficult to quantitate and therefore the absolute serum 25OHD concentrations obtained with different methods cannot be directly compared (Lips et al, 1999). For example, one study analysed the proportion of vitamin D deficiency in the same set of samples using three different methods. The results were 8%, 22% and 43% depending on the method (Snellman et al, 2010).



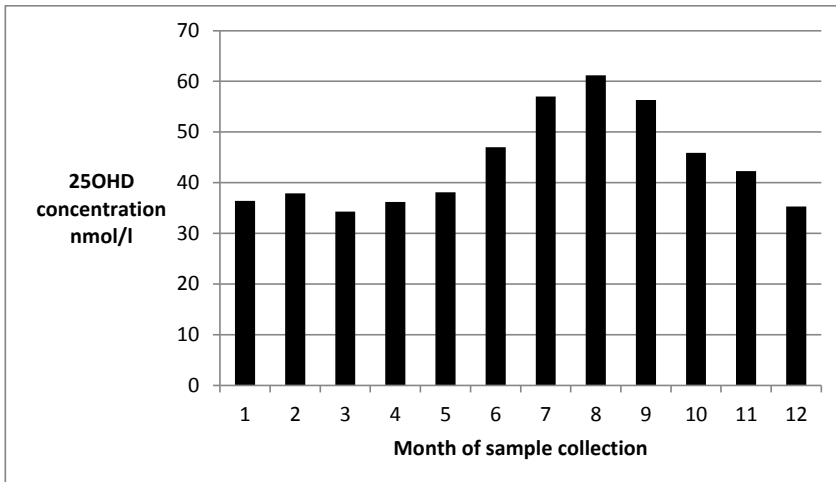
**Figure 4.** The percentage of samples of the “Vitamin D and type 1 diabetes” study in each category describing vitamin D status (n=1044)

Three Finnish studies using samples that were collected during approximately the same time period as our samples found serum 25OHD concentrations of 26-44 nmol/l in pregnant women (Kuoppala et al 1986; Munger et al 2016; Toriola et al 2009). Since then, there have been some changes that may have improved the vitamin D status in Finland. The fortification of milk and margarine with vitamin D started in 2003 (Ministry of Trade and Industry of Finland, 2002) and the general vitamin D related awareness has increased considerably leading to a higher consumption of vitamin D supplements (Raulio et al, 2016). A study that used FMC samples collected in 2007, however, did not show an improved vitamin D status during pregnancy (Viljakainen et al 2010). In other population groups an improvement in the vitamin D status has been shown. The mean serum 25OHD concentrations in Finland have increased both in children and adults (Ministry of Social Affairs and Health, 2006; Raulio et al, 2016). Unfortunately, recent studies on vitamin D status in pregnant Finnish women are lacking.

A recent systematic review provided a global summary of the vitamin D status of pregnant women (Saraf et al, 2016). It was found that in general, the highest maternal serum 25OHD concentrations are found in Africa (mean 92 nmol/l), the Americas (60 nmol/l) and the Western Pacific (57 nmol/l), and the lowest in the Eastern Mediterranean (20 nmol/l), Europe (30 nmol/l) and South-East Asia (30 nmol/l), although the differences in the methods used affect the results.

### 5.3 SEASONAL VARIATION IN SERUM 25OHD CONCENTRATIONS (I-IV)

Using all serum samples we received in the “Vitamin D and Type 1 Diabetes” study (n=1044), we calculated the mean serum 25OHD concentrations in samples collected during the different months of the year (Figure 5). A clear and expected seasonal variation was seen (figure 5). Figure 5 shows that the effect of higher UV radiation exposure during the summer months extends to the autumn months. This highlights the need for vitamin D supplementation especially during the winter and spring months.



**Figure 5.** Serum 25-hydroxyvitamin D (25OHD) concentrations collected during different months in all samples of the “Vitamin D and type 1 diabetes” study (n=1044)

The seasonal variation in serum 25OHD concentrations in Finland is a well-known fact, documented in several studies (Agborsangaya et al 2010; Holmlund-Suila et al, 2013; Virtanen et al, 2011). The same kind of seasonal pattern also exists, for example, in Sweden (Klingberg et al, 2015) and Norway (Jorde et al, 2010), but even in the southern parts of Europe, like Portugal, a clear difference between the serum 25OHD concentrations during the winter and summer months can be seen (Bettencourt et al, 2016).

## 5.4 SERUM 25OHD CONCENTRATIONS SIMILAR DURING PREGNANCY IN CASE AND CONTROL MOTHERS (I)

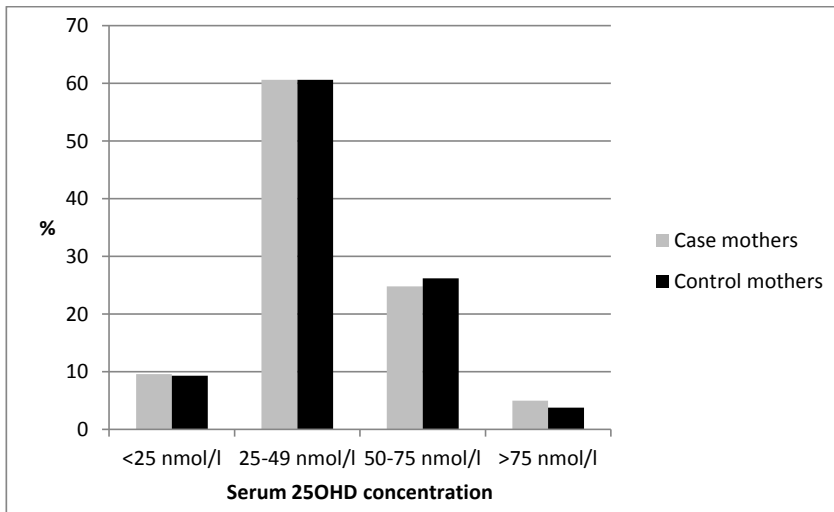
Serum 25OHD concentration was available from 343 case-control mother pairs. We compared the serum 25OHD concentrations of case and control mothers in samples collected during the first trimester of pregnancy. There was no difference in the mean serum 25OHD concentrations between the case and control mothers. The mean serum 25OHD concentration during the pregnancy of the case mothers was 43.9 nmol/l (n=343; SD=16.9) and 43.5 nmol/l (n=343; SD=16.6) for the control mothers (p=0.70).

We also compared the proportions of samples from case and control mothers belonging to each category that describes vitamin D status. The comparison between proportion of case and control mothers with severe vitamin D deficiency (<25 nmol/l), insufficiency (25-49 nmol/l), with a sufficient vitamin D status (50-75 nmol/l) and with an optimal vitamin D status (>75 nmol/l) is illustrated in figure 6. No difference was found between case and control mothers in the proportions in each category (p=0.88).

Two studies investigating the association of maternal/neonatal vitamin D status with type 1 diabetes risk in the child have published their results after we started our study.

In a Norwegian study, with a very similar study setting to ours, an association was found between maternal serum 25OHD concentration and type 1 diabetes risk in the child (Sørensen et al, 2011). The difference is that the serum samples were mainly collected during the last trimester of pregnancy. The mean serum 25OHD concentrations were also considerably higher (65.8 nmol/l in case and 73.1 nmol/l in control mothers). The results, however, cannot be directly compared since the measurements were done with different methods.

In a recent Danish study, serum 25OHD concentrations were measured in neonatal dried blood spots from children that later developed type 1 diabetes and from non-diabetic control children (Jacobsen et al, 2016). No association between neonatal vitamin D status and type 1 diabetes risk was found. The median serum 25OHD concentrations were low (<30 nmol/l), but cannot be directly compared since the samples were from children and the sample collection method (dried blood spots) was different from ours and the Norwegian study.



**Figure 6.** The proportion of serum 25-hydroxyvitamin D (25OHD) concentration results in each category defining the vitamin D status in case and control mothers (343 case-control pairs).

In older studies assessing the relationship between vitamin D and type 1 diabetes, the use of vitamin D supplements or cod-liver oil during pregnancy and during the first years of life have in some studies been associated with a lower the risk for type 1 diabetes in the child (The EURODIAB Substudy 2 Study Group, 1999; Hyppönen et al, 2001; Stene et al, 2000). This association has, however, not been seen in all studies. Comparing our results to these studies, where information on the vitamin D intake is based only on the supplement use, is difficult, since vitamin D status is influenced by several other factors as well such as exposure to UV radiation, BMI, physical activity and smoking (Miettinen et al 2014, Skaaby et al, 2016). We had the possibility of measuring the actual serum 25OHD concentration, which results from all these factors.

Considering the discrepancy in the results of all studies in the field, it is justified to assume that the possible association between vitamin D and type 1 diabetes is not likely to be as straightforward as has been initially suggested.

## 5.5 SNPs IN THE METABOLIC PATHWAY OF VITAMIN D ASSOCIATE WITH SERUM 25OHD CONCENTRATION IN FINNISH WOMEN (II)

We selected 31 SNPs in 8 genes based on previously found associations with serum 25OHD concentration, type 1 diabetes, or both. All the SNPs were in Hardy–Weinberg equilibrium except SNP rs7975232 localised to the *VDR* gene, which was removed from further analyses. Of the genotypes, 100% were concordant in 120 duplicate samples.

First we wanted to test which of the selected SNPs associated with serum 25OHD concentration in our study, since it had not been previously studied in the Finnish population. In order to test this, we selected all persons that had both serum 25OHD concentration and SNP genotyping results available (822 persons). Since the serum 25OHD concentrations were only available from the mothers, the 822 persons were all either case or control mothers. We found a nominal association ( $p \leq 0.05$ ) between serum 25OHD concentration and 13 SNPs in four genes (*NADSYN1/DHCR7*, *VDR*, *GC* and *CYP27A1*; table 1).

**Table 1.** Association between SNPs in the metabolic pathway of vitamin D and serum 25-hydroxyvitamin D (25OHD) concentration.

SNP	Gene	<i>p</i>
rs4945008	<i>NADSYN1/DHCR7</i>	0.03
rs12785878	<i>NADSYN1/DHCR7</i>	0.02
rs4944957	<i>NADSYN1/DHCR7</i>	0.05
rs7944926	<i>NADSYN1/DHCR7</i>	0.02
rs3794060	<i>CYP27A11/DHCR7</i>	0.02
rs12800438	<i>NADSYN1/DHCR7</i>	0.02
rs4516035	<i>VDR</i>	0.02
rs731236	<i>VDR</i>	0.03
rs1544410	<i>VDR</i>	0.03
rs10783219	<i>VDR</i>	0.02
rs12512631	<i>GC</i>	0.03
rs4588	<i>GC</i>	0.05
rs17470271	<i>CYP27A1</i>	0.03



Since our analyses, several studies have evaluated the genetic determinants of serum 25OHD concentration in the Finnish population. These studies have confirmed, for example, the associations of rs12785878 (*NADSYN1/DHCR7*) and rs4588 (*GC*) with serum 25OHD concentration (Pekkinen et al; 2014; Voipio et al 2015). In other populations, the role of genes as determinants of serum 25OHD concentration has been shown in several studies (Abbas et al, 2008; Ahn et al, 2010; Engelman et al, 2008; Fang et al, 2009; Jolliffe et al, 2016; McGrath et al, 2010; Wang et al, 2010).

## **5.6 GENOTYPES OF SNPs IN THE VITAMIN D RECEPTOR (VDR) GENE DISTRIBUTE DIFFERENTLY IN THE CASE AND CONTROL MOTHERS (II)**

Altogether we received 2854 DNA samples from the case and control families to study the role of SNPs in the metabolic pathway of vitamin D as determinants of type 1 diabetes risk in the child (DNA samples of 512 case mothers, 470 case fathers, 534 type 1 diabetic children, 238 non-diabetic siblings, 379 control mothers, 340 control fathers, 381 control children).

After confirming an association between certain SNPs in the metabolic pathway of vitamin D with serum 25OHD concentration, we wanted to test a possible difference in the genotype distribution of these SNPs between the case and control families. Our main interest was comparing the type 1 diabetic and control children, but we included all family members in the analyses. We compared the case and control mothers, the case and control fathers as well as the type 1 diabetic children and their non-diabetic siblings.

We did not find any difference in the genotype or allele distribution between the type 1 diabetic children and control children. We did, however, find a difference in the genotype distribution of three SNPs between the case and control mothers (table 2). All three SNPs were localised to the *VDR* gene (rs1544410,  $p=0.007$ ; rs731236,  $p=0.003$ ; and rs4516035,  $p=0.015$ ) (table 2). Two SNPs (rs1544410 and rs731236) remained statistically significant after correction for multiple testing with FDR.

No differences in the genotype or allele distributions emerged between the case and control fathers, or between the type 1 diabetic children and their non-diabetic siblings. In the case families, no allele of the 13 SNPs studied was preferentially transmitted to the type 1 diabetic children.

Our finding suggests that maternal genetic factors modify type 1 diabetes risk in the child, independent on the child's genotype, although the overall effect may not be strong given the multifactorial nature of the disease. To our knowledge the finding is novel and no previous studies exist. Thus far the focus in type 1 diabetes related genetic studies has been on the affected child

and the idea of an independent role of maternal genetic factors has not been presented.

**Table 2.** Genotype distributions of SNPs in the vitamin D receptor (*VDR*) in case and control mothers.

<sup>a</sup>multiple testing correction with false discovery rate (FDR) approach (Benjamini-Hochberg step-up procedure). If the original *p* value was less than the Benjamini-Hochberg critical value, it was considered statistically significant

\*statistically significant after multiple testing correction with FDR approach.

SNP	Case mothers	Control mothers	OR (95% CI)	<i>p</i>	Benjamini-Hochberg critical value <sup>a</sup>
rs1544410 ( <i>VDR</i> )				0.007*	0.008
Genotypes <i>n</i> (%)					
AA	48 (10.2)	42 (12.0)	0.85 (0.58-1.26)	0.41	
AG	227 (48.1)	130 (37.0)	1.30 (1.10-1.53)	0.0016	
GG	197 (41.7)	179 (51.0)	0.81 (0.70-0.94)	0.0062	
rs731236 ( <i>VDR</i> )				0.003*	0.004
Genotypes <i>n</i> (%)					
CC	49 (9.9)	43 (12.3)	0.80 (0.55-1.18)	0.26	
CT	243 (49.0)	130 (37.2)	1.32 (1.12-1.55)	0.0007	
TT	204 (41.1)	176 (50.4)	0.82 (0.70-0.95)	0.0075	
rs4516035 ( <i>VDR</i> )				0.015	0.012
Genotypes <i>n</i> (%)					
CC	124 (25.1)	63 (18.4)	1.37 (1.04-1.79)	0.0215	
CT	225 (45.5)	189 (55.1)	0.83 (0.72-0.95)	0.0066	
TT	145 (29.4)	91 (26.5)	1.11 (0.89-1.38)	0.37	

The phenomenon that maternal genetic factors affect the disease risk or the development of the child independent on the child's genotypes, however, has been presented outside the field of type 1 diabetes research. The maternal genetic factors have previously been shown to associate with the child's brain morphology and cognitive development (Pilsner et al, 2010; van der Knaap, 2014) and risk for several diseases, for example autism and atopic dermatitis (Esparza-Gordillo et al, 2015; James et al 2010). One previous study covers results related with the *VDR* gene. The allele frequency of SNP rs2228570 in

the *VDR* gene was shown to differ between mothers with a preterm birth and those with a full-term birth (Manzon et al, 2014).

The mechanism explaining the relationship between certain maternal *VDR* genotypes and type 1 diabetes risk in the child is not clear and can only be speculated at this point. Type 1 diabetes is a disease that can develop early in life. Type 1 diabetes-associated autoantibodies that precede the diagnosis of type 1 diabetes can appear within months of birth, which suggests a foetal programming of the disease (Siljander et al, 2009). It is possible that the variation in the maternal *VDR* gene modifies the various effects of vitamin D (through the action of its receptor). This may be especially apparent when the amount of circulating 25OHD is not adequate. In a meta-analysis it was suggested that the association between the polymorphism of the *VDR* gene (of the patient) and type 1 diabetes is influenced by the vitamin D status (Tizaoui et al, 2014).

The effects of the *VDR* gene are also dependent on other genes. The Diabetes Autoimmunity Study in the Young (DAISY) study showed that interaction between the *VDR* and protein tyrosine phosphatase, non-receptor type 2 gene (*PTPN2*), affects the risk for type 1 diabetes (Frederiksen et al, 2013b). In this study an association of certain maternal SNPs of the *VDR* gene with the risk for type 1 diabetes in the child was seen. It is possible that other SNPs in the metabolic pathway of vitamin D not included in our study, however, affect the risk for the disease as well, either independently or interacting with other SNPs or environmental factors.

The *VDR* is a transcription factor with more than 500 target genes. We did not see any difference in the genotype distributions of the SNPs in the *VDR* gene between the diabetic patients and the non-diabetic control children. This may result from the fact that we only selected a few SNPs of the *VDR* gene. Our results suggest that the *VDR* gene associates with the in-utero environment and the developmental processes in the foetus. Further studies are needed but ours and previous studies suggest an important role for the *VDR* in defining the relationship between vitamin D and type 1 diabetes.

## **5.7 GENETIC DETERMINANTS OF SERUM 25OHD CONCENTRATION ARE DIFFERENT IN THE CASE AND CONTROL MOTHERS (III)**

In our earlier study (II), we showed that certain genotypes in the *VDR* distribute differently in the case and control mothers, suggesting a role for the maternal *VRD* in the development of type 1 diabetes in the child. We decided to re-analyse our data on the association between SNPs in the

metabolic pathway of vitamin D and serum 25OHD concentrations separately for the case and control mothers to detect other vitamin D genetics related differences.

Earlier, we confirmed a statistically significant association between 13 SNPs in genes in the metabolic pathway of vitamin D and serum 25OHD concentration (II). Afterwards, we noticed that six SNPs in *NADSYN1/DHCR7* and two SNPs in the *VDR* (rs731236 and rs1544410) were in strong linkage disequilibrium and thus had similar associations with serum 25OHD concentration. When re-analysing the data separately for the case and control mothers, we randomly selected only one SNP of these SNP groups resulting in the final number of 7 SNPs in the analyses.

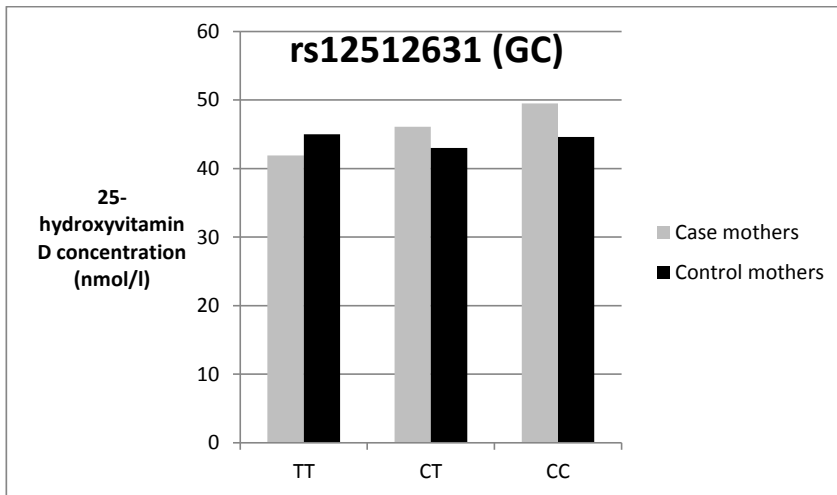
We detected a difference in the association of SNPs with serum 25OHD concentration between the case and control mothers. An interaction test indicated that association of two SNPs with serum 25OHD concentration differed between case and control mothers (table 3). Rs12512631 in the *GC* gene and rs4516035 in the promoter region of the *VDR* gene associated stronger with serum 25OHD concentration in case ( $p=0.002$  and  $0.0002$ ) versus control ( $p=0.64$  and  $0.83$ ) mothers ( $p$  interaction= $0.02$  and  $0.03$ ) (figure 7a and 7b).

**Table 3.** Association between serum 25-hydroxyvitamin D (25OHD) concentration and SNPs in genes in the metabolic pathway of vitamin D in the case and control mothers, and according to the presence of the effect allele (EA).

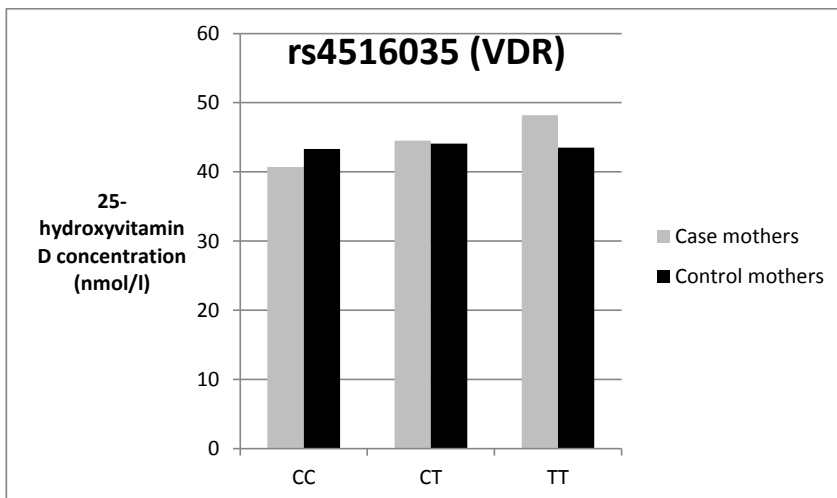
\*The effect allele (EA) is the allele that increases the 25-hydroxyvitamin D concentration

\*\*Statistically significant interaction

SNP (gene)	Case	Control	Effect of presence of the effect allele			
	mothers	mothers	EA*	$p$ cases	$p$ controls	$p$ interaction
	$p$ (n)	$p$ (n)				
rs4945008 ( <i>NADSYN1/DHCR7</i> )	0.20 (445)	0.054 (325)	G	0.093	0.034	0.89
rs4516035 ( <i>VDR</i> )	0.0038 (445)	0.93 (321)	T	0.0002	0.83	0.031**
rs1544410 ( <i>VDR</i> )	0.093 (427)	0.32 (326)	A	0.043	0.20	0.58
rs10783219 ( <i>VDR</i> )	0.037 (437)	0.55 (317)	T	0.0076	0.35	0.23
rs12512631 ( <i>GC</i> )	0.021 (435)	0.67 (324)	C	0.0020	0.64	0.023**
rs4588 ( <i>GC</i> )	0.051 (419)	0.65 (322)	C	0.019	0.50	0.26
rs17470271 ( <i>CYP27A1</i> )	0.052 (428)	0.49 (325)	T	0.028	0.86	0.21



**Figure 7a.** In the case mothers the presence of a C allele is associated with an average increase of 3.9 nmol/l in serum 25-hydroxyvitamin D (25OHD) concentration ( $p=0.002$ ) while in the control mothers such an association was not found ( $p=0.64$ ) ( $p$  interaction= $0.02$ ).



**Figure 7b.** In the case mothers the presence of a T allele is associated with an average increase of 4.06 nmol/l in serum 25-hydroxyvitamin D (25OHD) concentration ( $p=0.0002$ ) while in the control mothers such an association was not found ( $p=0.83$ ) ( $p$  interaction= $0.03$ ).

The finding is novel and no previous studies exist. It has, however, been shown that associations between SNPs and an outcome are influenced by environmental factors such as age and gender. For example, several SNPs associate more strongly with fat distribution in women than in men (Heid et al, 2010). Several SNPs have also shown stronger effects on BMI in younger than older people (Winkler et al, 2015). Smoking can also drastically alter the effects of SNPs. Certain SNPs have been found to associate with low BMI in smokers, but with high BMI in non-smokers (Taylor et al, 2014). Recently it was shown that sunlight exposure may modify the association between SNP in the *VDR* and serum 25OHD concentration so that the lowering effect of the SNP in the *VDR* on serum 25OHD concentration is smaller when the sunlight exposure is increased (Livingstone et al, 2016). It seems that certain SNPs only associate with serum 25OHD in men, and that the associations between SNPs in genes in the metabolic pathway of vitamin D and serum 25OHD concentrations are weaker with increasing age (Miettinen et al, unpublished results).

We showed a difference in the strength of associations between certain SNPs and serum 25OHD concentration in case and control mothers. This difference can be interpreted as a marker of possibly several other differences in vitamin D genetics and metabolism between the case and control mothers. It is possible that these differences lead to an altered supply of vitamin D from the mother to the foetus, or to an altered regulation of the genes in the *VDR* controls, or both. The fact that we detected this difference in two out of four gene loci highlights the importance of the finding.

It is possible that since pregnancy causes various physiological changes, it may prove to be one of the factors that modify associations between SNPs and outcome. This, however, may not explain the finding, since both case and control mothers were pregnant.

The mechanism and the clinical meaning of this finding are beyond the data of the present study and therefore further studies are needed.

## **5.8 HLA GENE REGION ASSOCIATES WITH SERUM 25OHD CONCENTRATION (IV)**

Since both the HLA gene region and vitamin D have been associated with type 1 diabetes, we wanted to study the possible relationship between these two factors. Of the mothers that had serum 25OHD concentrations available, successful HLA genotyping was performed as follows: HLA-B of 395 mothers, HLA-DRB1 of 501 mothers and HLA-DQB1 of 475 mothers. Due to high polymorphism of the HLA-B region, HLA-B alleles were grouped into supertypes (please see 4.5 for more information).

The case and control mothers were analysed as one group. In our earlier study (III) we, however, observed a difference between the case and control mothers in the strength of associations between SNPs and serum 25OHD concentration, therefore we checked for possible interaction according to the mother type. No interaction was found.

An association was found between serum 25OHD concentration and HLA-B44 supertype ( $p=0.009$ ) (table 4). The HLA-B44 supertype associated with a lower serum 25OHD concentration. In these data, we could not identify an association between serum 25OHD concentration and individual HLA-B alleles that form the B44 supertype. We did not find an association between HLA-DRB1 or -DQB1 alleles and serum 25OHD concentration. Multiple testing was addressed using the FDR approach (II).

**Table 4.** Association of human leukocyte antigen (HLA) B superotypes, HLA-DRB1 and HLA-DQB1 alleles with serum 25-hydroxyvitamin D (25OHD) concentration

<sup>a</sup>multiple testing correction with false discovery rate (FDR) approach (Benjamini-Hochberg step-up procedure). If the original  $p$  value was less than the Benjamini-Hochberg critical value, it was considered statistically significant

\*statistically significant after multiple testing correction with FDR approach.

HLA-B supertype/HLA-DRB1/HLA-DQB1 allele	n	serum 25OHD concentration (nmol/l)	$p$	Benjamini-Hochberg critical value <sup>a</sup>
B07 supertype	241	44.3	0.96	
B08 supertype	87	46.0	0.40	
B27 supertype	93	42.6	0.18	
B44 supertype	160	41.0	0.009*	0.02
B62 supertype	113	42.4	0.29	
DR1	140	43.0	0.52	
DR3	130	44.0	1.00	
DR4	209	43.6	0.60	
DR7	47	41.2	0.10	
DR8	91	43.0	0.43	
DR9	35	48.5	0.24	
DR11	38	44.3	0.87	
DR13	107	45.1	0.64	
DR15	96	44.7	0.89	
DQ2	150	44.2	0.92	
DQ3	270	44.1	0.92	
DQ4	86	43.0	0.51	
DQ5	159	43.5	0.83	
DQ6	181	45.4	0.15	

A trend was detected in the serum 25OHD concentrations according to how many B44 supertype alleles (0,1, or 2) the participants had ( $p$  for trend=0.05; table 5). Having two HLA-B44 supertype alleles associated with the lowest serum 25OHD concentrations.

**Table 5.** Association of HLA B44 supertype and serum 25-hydroxyvitamin D (25OHD) concentration in participants carrying 0, 1 or 2 B44 supertype alleles.

Number of B44 supertype alleles	n	serum 25OHD concentration (nmol/l)	$p$ for trend
0	235	45.7	0.05
1	142	41.8	
2	18	37.3	

Our finding is novel and suggests an even stronger role for vitamin D in the functions of the immune system than previously proposed. It also indicates that the HLA gene region is one of the genetic determinants of serum 25OHD concentration, which has not been shown before.

HLA polymorphism has been known for decades to associate with type 1 diabetes and several other autoimmune diseases (Shiina et al, 2009). Since vitamin D has also been long suspected to have a role in the development of type 1 diabetes and other autoimmune diseases, the lack of previous studies on the relationship between vitamin D status and HLA alleles / superotypes is surprising. It may be that in type 1 diabetes studies, often only HLA-DRB1 and HLA-DQB1 alleles are genotyped and thus no association has been found. As for studies researching the genetic determinants of serum 25OHD concentrations, the high polymorphism of the HLA gene region may have prevented the traditional GWAS to detect any associations. The relatively high costs of traditional HLA genotyping may also have influenced the possibilities to include HLA genotyping.

The development of next-generation techniques in GWAS will also enable the inclusion of the HLA gene region in the analyses. This will considerably help evaluate the importance of the HLA polymorphisms in other diseases in addition to autoimmune diseases. They will also help to evaluate the association between vitamin D status and the HLA gene region more closely.

Our initial aim was to study if HLA alleles that associate with type 1 diabetes, would associate with lower serum 25OHD concentration thus providing a suggestion of the mechanism behind the putative association between vitamin D and type 1 diabetes. No association was found between the serum 25OHD concentrations and the HLA alleles that associate the strongest with type 1 diabetes (HLA-DRB1\*3 and HLA-DRB1\*4).



HLA-B\*18, B\*37, B\*40, B\*44 genes that form the HLA-B44 supertype, have been associated with both low and high risk for type 1 diabetes (and with other diseases). Of these, B\*18 gene has previously been associated with an increased risk for type 1 diabetes, whereas the B\*44 gene with a decreased risk (Howson et al, 2009; Noble et al, 2010). Thus, our finding does not suggest a causal relationship between low serum 25OHD concentration and type 1 diabetes risk, but further strengthens the role of vitamin D in the functions of the immune system. It is known, for example, that cells in the immune system are able to synthesise active vitamin D (1,25OHD<sub>2</sub>) (Aranow, 2011; Guillot et al, 2010). Due to the small sample size, we could not analyse the association of serum 25OHD concentration with individual HLA-B alleles.

The mechanism explaining our finding is not clear, but the fact that VDREs exist in the promoter region of at least certain HLA alleles (Cocco et al 2012; Israni et al 2009; Ramagopalan et al, 2009), indicates a role for vitamin D in the regulation of the HLA gene region related expression. At least some HLA-B genes are also associated with increased levels on serum inflammation markers (Lenna et al, 2015). It is possible that a low serum 25OHD concentration reflects a higher “consumption” of circulating vitamin D as a response to an increased susceptibility for inflammation in individuals carrying certain HLA alleles.



## 6 DISCUSSION

### 6.1 FINDINGS

Our main discovery was that maternal genes in the metabolic pathway of vitamin D seem to modify type 1 diabetes risk in the child. Especially the *VDR* gene seems to have an important role. We did not find proof to support a direct relationship between maternal vitamin D status and type 1 diabetes risk in the child. Our study, however, showed an even more diverse role for vitamin D as part of the immune system through an association between the HLA gene region and vitamin D status.

Our findings related with the possible role of maternal genes in early programming of type 1 diabetes risk in the child are novel. Our results indicate that the association between vitamin D and type 1 diabetes is more complex than previously thought. The central role of the *VDR* gene may relate to the fact that the *VDR* controls hundreds of genes, and in the presence of vitamin D deficiency, the function of the receptor may be altered.

### 6.2 IMPORTANCE AND INTERPRETATION OF THE FINDINGS

In this study, we had the strong advantage of being able to retrospectively identify mothers, whose children later developed type 1 diabetes by using the FMC samples that are being collected from practically all pregnant Finnish women. We had therefore an ideal study setting to search for vitamin D related differences between the case and control mothers that may have contributed to the disease process.

Only one similar study exists (Sørensen et al, 2012). The lack of studies is understandable due to difficulties in implementing these kinds of studies. One approach would be to recruit a large group of pregnant women/children without any specific inclusion criteria, collect samples during pregnancy and infancy, and wait for a type 1 diabetes diagnosis in a sufficient number of children. This approach would require massive resources as well as demand a long period of time. The second approach would be to recruit a large group of pregnant women/children with genetically increased risk for type 1 diabetes. This may be done by recruiting healthy siblings of the diagnosed type 1 diabetic patients or by screening for high-risk HLA alleles. Inclusion criteria based on the high-risk HLA alleles has been used, for example, in the Finnish Diabetes Prediction and Prevention (DIPP) study. This approach will provide a sufficient number of patients more quickly, but demands considerable

resources as well. The third option is the one we were able to utilise; to use existing samples that have been collected for other purposes, and to analyse the samples after knowing which of the children developed type 1 diabetes.

The Norwegian study (Sørensen et al, 2012) found an association between maternal serum 25OHD concentration and type 1 diabetes risk in the child whereas we did not. Since the maternal serum 25OHD concentrations in Norway were considerably higher than in our study, it needs to be considered whether a considerably better maternal vitamin D status than in our study could protect the child from type 1 diabetes. Indeed, the most promising results of vitamin D intervention studies have been obtained in studies using relatively high dose vitamin D supplementation, such as the recent study that showed an effect of vitamin D in controlling the symptoms of autism spectrum disorder (Saad et al, 2016).

Our findings and the suggested role of maternal genes in the metabolic pathway of vitamin D as determinants of type 1 diabetes risk in the child are novel. While the phenomenon of maternal genetic factors modifying a disease risk in the child, independent on the child's corresponding factors, is not new, the idea has not been suggested in the literature of type 1 diabetes research before. It is unclear whether the genetic differences between the case and control mothers were visible in our study because the mean serum 25OHD concentrations were so low in all mothers. It may be, for example, that particular forms of the *VDR* gene lead to differentiated overall function of the VDR only when there is a lack of circulating 25OHD to be converted to active vitamin D (which binds to the VDR and is needed for an adequate function of the VDR).

The immune system of the foetus develops throughout the pregnancy (Holt and Jones, 2000) and insulin-positive cells can already be detected in the foetal pancreas during the first trimester of pregnancy (Piper et al, 2004). Since type 1 diabetes can develop at a young age, it is justified to assume that the development of the autoimmune process may already begin during the foetal period. No information exists, however, on the time window during the foetal development that would be critical for the pathogenesis of the disease.

Serum 25OHD concentrations have been shown to rise through pregnancy (Bärebring et al, 2016; Davies-Tuck et al, 2015), but it is unclear whether this increase reflects actual physiological changes or for example a more frequent use of vitamin D containing supplements during late pregnancy. In mice (Shahbazi et al, 2011), however, the expression of the *VDR* markedly increases in the late pregnancy suggesting a role for vitamin D in the foetal development during late pregnancy.

The fact that associations between a certain SNP and an outcome can vary between individuals has been shown in many studies outside type 1 diabetes research, but the phenomenon is still somewhat new and its meaning has not been fully understood. Our findings suggested that SNPs in the metabolic pathway of vitamin D associate differently with vitamin D status between the case and control mothers. Although in recent years a huge number of genetic

variants that associate with complex traits and diseases have been identified by GWAS studies, they have so far explained a surprisingly small proportion of the disease susceptibility. Several explanations have been suggested for the “missing heritability”. It seems that some of the heritability can be hidden in GWAS because the study populations consist of distinct sub-populations in which the variants have different effects. Thus, it would be important to analyse genetic determinants of a health outcome separately for different sub-populations, stratified according to relevant environmental factors or host characteristics, given that a justification for forming these sub-populations exist.

The focus of genetic studies related with type 1 diabetes has mainly been on the patient, and the effect of maternal genes independent of the corresponding genes in the child, has not previously been proposed as a determinant of type 1 diabetes risk in the child. Our findings suggest a new window for prevention of diabetes, the target being the mother and the pregnancy instead of the child. Maternal genes in the metabolic pathway of vitamin D seem, on the basis of our results, to associate with type 1 diabetes risk in the child, but at present there is no evidence to support a hypothesis that additional vitamin D supplementation would change these genetic effects (although no evidence excludes this hypothesis either).

Vitamin D supplementation would be an attractive means for preventing type 1 diabetes, since the prevention would not require, for example, genetic or autoantibody screening. These screenings are expensive and difficult to implement at a population level, whereas increasing the average serum 25OHD concentrations through, for example, increased vitamin D fortification would be a cost-effective action that is easily put into practice. If vitamin D has the potential to prevent or delay type 1 diabetes, we should be able to see a decrease in type 1 diabetes incidence in Finland due to an increase in the use of vitamin D supplements (Raulio et al, 2016) and due to starting of vitamin D fortification (Ministry of Trade and Industry of Finland, 2002). This has, in fact, already been investigated and it was found that an increase in serum 25OHD concentrations preceded a plateau in type 1 diabetes incidence in Finland (Mäkinen et al, 2014). This is an epidemiological association that will need to be confirmed in well-designed intervention studies.

Our finding of an association between vitamin D status and the HLA gene region suggests an even stronger role for vitamin D as part of the immune system. The mechanism of this association is beyond our study, but it can be speculated that certain people may be more prone to an inflammation (due for example to their HLA and other genetic profile, regardless of any inflammation-linked disease), and thus may “consume” more vitamin D, which is mirrored by a weaker vitamin D status. An interesting example of the “consumption” of vitamin D as a result of a reaction of the immune system is when a healthy person has surgery or delivers a baby; the serum

25OHD concentrations drop drastically within hours to be then normalised slowly within the next few months (Luxwolda et al, 2012).

Even though the association between vitamin D and type 1 diabetes is not clear at present, it is important to remember that vitamin D has many important functions in the foetal development including the development and the mineral density of the bone (Viljakainen et al, 2010) and that the foetus is entirely dependent on the mother for an adequate vitamin D supply. In Finland, very little sunlight is available from the autumn to the spring. It has been suggested that due to evolutionary strain, to increase the vitamin D synthesis the light colour of the skin has evolved in the northern parts of the world to compensate for the low supply of vitamin D through UV radiation (Juzeniene et al, 2009). This can be regarded as proof of the crucial role of vitamin D in the body. Without any supplementation, vitamin D deficiency is a problem for pregnant women and to the developing foetus.

Our findings suggest a role for the mother or pregnancy in the initiation of the disease process of type 1 diabetes, and therefore underline the need to investigate this relationship further.

### **6.3 CRITICAL EVALUATION OF THE STUDY**

We chose to use saliva samples in our study to increase the number of participants. It is difficult to determine an average percentage of participants in Finnish medical or genetic studies, since the study settings and sample collection methods vary considerably. It is therefore difficult to conclude whether using saliva samples instead of blood samples actually increased the number of participants since we had more problems with the quality of the samples than we usually have with blood samples. In some cases there was too little saliva or the DNA that was extracted from the saliva was of poor quality. We, however, got enough participants and usable samples when considering our power calculations and thus consider the method of sample collection satisfactory. Since we also had children in our study population, giving a saliva sample can be considered to cause less inconvenience and thus a more ethical choice.

We did not have the possibility to choose any other stage of pregnancy or a time-point during childhood to define vitamin D status, since the FMC samples are only collected during the first trimester of pregnancy in Finland. The development of the foetal immune system and pancreas start during the first trimester of pregnancy, but continue throughout the pregnancy. It is therefore not possible with the present knowledge to determine the adequate time window for early programming of type 1 diabetes. Being able to define the vitamin D status only during the first trimester of pregnancy is a limitation in our study, but there is no reason to believe it would cause biases or errors to the results.

The results obtained through laboratory analyses are often automatically seen as objective, accurate or as the “truth”, compared to data collected through, for example, questionnaires or other methods. This is an impression that needs to be considered in a critical manner. The laboratory analyses without a careful quality control and understanding of the nature of the analyses can lead to substantial errors. These errors are extremely difficult to notice, if the results are evaluated by a person without the appropriate education or if the quality control has been inadequately described, for example, in a scientific publication. In the manuscript review process, the reviewers may concentrate more on the presented results and their significance without questioning the justification to rely on the presented results in the first place. Serum 25OHD concentration analytics is a good example in this context due to the relative complexity of the analysis. We had a relatively strong quality control in our laboratory analyses, nevertheless it is important to understand that the exact numbers of serum 25OHD concentrations do not represent the “truth” and that our results cannot be compared to other results obtained with different methods.

There are number of genes related to vitamin D metabolism and a number of SNPs in each of these genes. In this study only a small selection of SNPs in some of these genes were analysed. It is possible that if we had selected the SNPs differently, we would have received different results. The extent of alternatives when planning the study increased our need to create grounds for selecting the SNPs. We chose to select those SNPs in the metabolic pathway of vitamin D that had been associated with vitamin D status, type 1 diabetes or both. Although some interesting findings were made with this particular selection of SNPs, it is useful to take into account that the findings present only a small part of the overall picture of the relationship between genes in the metabolic pathway of vitamin D and type 1 diabetes. Our findings can be considered as markers of several other possible differences and as a justification to further studies in the field.

In genetic association studies but also in epidemiological association studies in general, there is a possibility of false positive findings when testing several associations. This problem needs to be addressed and many researchers do this by using some of the various multiple testing correction methods described in the literature. Using multiple testing correction is not a specific procedure but simply means setting a stricter significance threshold for a finding than the commonly used  $p$  value of lower than 0.05. Different multiple testing correction methods accept distinct level of uncertainty when reporting the true findings. When hundreds or thousands of associations are tested without a pre-specified biologically justified hypothesis, the usefulness of using these corrections may be easy to understand. When there is a biologically justified hypothesis, the question is more complex. In our study, all of our association analyses were based on at least some kind of physiological relationship or a proposed mechanism, explaining why we chose to do just these particular analyses. Nevertheless, since our findings

were novel and at least partly unexpected, we felt a need to confirm the reliability of the results and to simplify the evaluation of our results to anyone exploring our work. Therefore, we decided to correct for multiple testing and reported only those associations that remained significant after the correction. It is important, however, to note that using multiple testing correction will not guarantee true meaningful associations nor does it mean that the findings that are not significant after the correction are always false positives. It is possible that a strict demand to use multiple testing correction will translate into the researchers not reporting all associations tested (Schulz and Grimes, 2005) and therefore, the careful evidence-based interpretation of the results by the researcher should be the most important demonstration or proof for the significance of the results, whether or not a correction has been used.



## **7 CONCLUSIONS AND FUTURE PERSPECTIVES**

1. No association was found between maternal vitamin D status during the first trimester of pregnancy and the risk for type 1 diabetes in the child.
2. Vitamin D deficiency was common among pregnant women.
3. Maternal genes in the metabolic pathway of vitamin D associated with type 1 diabetes in the child, independent of the corresponding factors in the child.
4. The HLA gene region associated with serum 25OHD concentration.

### **FUTURE RECOMMENDATIONS**

1. The effect of higher (but safe) vitamin D supplementation for pregnant women or infants on the type 1 diabetes risk should be evaluated.
2. The current vitamin D status of the pregnant women in Finland should be investigated.
3. It should be evaluated whether children with autoantibodies have a weaker response to vitamin D supplementation than control children with no autoantibodies. This would suggest that vitamin D takes part in the ongoing autoimmune process of these children.
4. It should be investigated whether the genetic differences in vitamin D metabolism between mothers of diabetic and non-diabetic children are reflected as differences in the vitamin D status of the newborn, for example by using blood samples from the umbilical cord
5. It should be investigated whether the genotype differences between the case and control mothers seen in our study are dependent on the vitamin D status e.g seen only in vitamin D deficient subjects.

6. It should be clarified whether HLA-B44 supertype alleles associate with a higher predisposition to inflammation and thus explain the association between serum 25OHD and HLA-B44 supertype.



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## REFERENCES

Abbas S, Linseisen J, Slinger T, Kropp S, Mutschelknauss EJ, Flesch-Janys D, Chang-Claude J. The Gc2 allele of the vitamin D binding protein is associated with a decreased postmenopausal breast cancer risk, independent of the vitamin D status. *Cancer Epidemiol Biomarkers Prev.* 2008 Jun;17(6):1339-43.

Abhimanyu, Coussens AK. The role of UV radiation and vitamin D in the seasonality and outcomes of infectious disease. *Photochem Photobiol Sci.* 2017 Mar 16;16(3):314-338.

Afoke A, Ludvigsson J, Hed J, Lindblom B. Raised IgG and IgM in "epidemic" IDDM suggest that infections are responsible for the seasonality of type I diabetes. *Diabetes Res.* 1991 Jan;16(1):11-7.

Agborsangaya C, Toriola AT, Grankvist K, Surcel HM, Holl K, Parkkila S, Tuohimaa P, Lukanova A, Lehtinen M. The effects of storage time and sampling season on the stability of serum 25-hydroxy vitamin D and androstenedione. *Nutr Cancer.* 2010;62(1):51-7.

Ahn J, Yu K, Stolzenberg-Solomon R, Simon KC, McCullough ML, Gallicchio L, Jacobs EJ, Ascherio A, Helzlsouer K, Jacobs KB, Li Q, Weinstein SJ, Purdue M, Virtamo J, Horst R, Wheeler W, Chanock S, Hunter DJ, Hayes RB, Kraft P, Albanes D. Genome-wide association study of circulating vitamin D levels. *Hum Mol Genet.* 2010 Jul 1;19(13):2739-45.

Alhassan Mohammed H, Saboor-Yaraghi AA, Mirshafiey A, Vahedi H, Shiri-Shahsavari MR, Mousavi Nasl Khameneh A. Immunomodulatory and Immunosuppressive Roles of 1 $\alpha$ ,25(OH) $_2$ D $_3$  in Autoimmune Diseases. *Scand J Immunol.* 2017 Feb;85(2):95-103.

American Diabetes Association. 2. Classification and Diagnosis of Diabetes. *Diabetes Care.* 2017 Jan;40(Suppl 1):S11-S24.

Aranow C. Vitamin D and the Immune System. *J Investig Med.* 2011 Aug;59(6):881-6.

Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. *Lancet.* 2014 Jan 4;383(9911):69-82.

Autier P, Boniol M, Pizot C, Mullie P. Vitamin D status and ill health: a systematic review. *Lancet Diabetes Endocrinol.* 2014 Jan;2(1):76-89.

Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med.* 2002 Sep 19;347(12):911-20.

Bailey R, Cooper JD, Zeitels L, Smyth DJ, Yang JH, Walker NM, Hyppönen E, Dunger DB, Ramos-Lopez E, Badenhop K, Nejentsev S, Todd JA. Association of the vitamin D metabolism gene CYP27B1 with type 1 diabetes. *Diabetes.* 2007 Oct;56(10):2616-21.

Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet.* 1986 May 10;1(8489):1077-81.

Beck RW, Riddlesworth T, Ruedy K, Ahmann A, Bergenstal R, Haller S, Kollman C, Kruger D, McGill JB, Polonsky W, Toschi E, Wolpert H, Price D; DIAMOND Study Group. Effect of Continuous Glucose Monitoring on Glycemic Control in Adults With Type 1 Diabetes Using Insulin Injections: The DIAMOND Randomized Clinical Trial. *JAMA.* 2017 Jan 24;317(4):371-378.

Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J Roy Stat Soc Ser B* 1995; 57: 289-300

Bettencourt A, Boleixa D, Reis J, Oliveira JC, Mendonça D, Costa PP, Silva BM, Marinho A, Silva AM. Serum 25-hydroxyvitamin D levels in a healthy population from the North of Portugal. *J Steroid Biochem Mol Biol*. 2016 Nov 5. pii: S0960-0760(16)30298-9.

Bhargava P, Steele SU, Waubant E, Revirajan NR, Marcus J, Dembele M, Cassard SD, Hollis BW, Crainiceanu C, Mowry EM. Multiple sclerosis patients have a diminished serologic response to vitamin D supplementation compared to healthy controls. *Mult Scler*. 2016 May;22(6):753-60.

Bibbò S, Dore MP, Pes GM, Delitala G, Delitala AP. Is there a role for gut microbiota in type 1 diabetes pathogenesis? *Ann Med*. 2017 Feb;49(1):11-22.

Bikle D. Nonclassic actions of vitamin D. *J Clin Endocrinol Metab*. 2009 Jan;94(1):26-34.

Bikle DD. Vitamin D metabolism and function in the skin. *Mol Cell Endocrinol*. 2011 Dec 5;347(1-2):80-9.

Bizzarri C, Pitocco D, Napoli N, Di Stasio E, Maggi D, Manfrini S, Suraci C, Cavallo MG, Cappa M, Ghirlanda G, Pozzilli P; IMDIAB Group. No protective effect of calcitriol on beta-cell function in recent-onset type 1 diabetes: the IMDIAB XIII trial. *Diabetes Care*. 2010 Sep;33(9):1962-3.

Bland R, Markovic D, Hills CE, Hughes SV, Chan SL, Squires PE, Hewison M. Expression of 25-hydroxyvitamin D3-1alpha-hydroxylase in pancreatic islets. *J Steroid Biochem Mol Biol*. 2004 May;89-90(1-5):121-5.

Bogdanou D, Penna-Martinez M, Filmann N, Chung TL, Moran-Auth Y, Wehrle J, Cappel C, Huenecke S, Herrmann E, Koehl U, Badenhop K. T-lymphocyte and glycemic status after vitamin D treatment in type 1 diabetes: A randomized controlled trial with sequential crossover. *Diabetes Metab Res Rev*. 2017 Mar;33(3).

Bonifacio E, Mathieu C, Nepom GT, Ziegler AG, Anhalt H, Haller MJ, Harrison LC, Hebrok M, Kushner JA, Norris JM, Peakman M, Powers AC, Todd JA, Atkinson MA. Rebranding asymptomatic type 1 diabetes: the case for autoimmune beta cell disorder as a pathological and diagnostic entity. *Diabetologia*. 2017 Jan;60(1):35-38.

Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J Immunol*. 2001 Nov 1;167(9):4974-80.

Borchers AT, Naguwa SM, Keen CL, Gershwin ME. The implications of autoimmunity and pregnancy. *J Autoimmun*. 2010b May;34(3):J287-99.

Borchers AT, Uibo R, Gershwin ME. The geoepidemiology of type 1 diabetes. *Autoimmun Rev*. 2010a Mar;9(5):A355-65.

Bouillon R, Carmeliet G, Daci E, Segaert S, Verstuyf A. 1998. Vitamin D Metabolism and Action. *Osteoporos Int* 8(suppl.):13-19.

Brekke HK, Ludvigsson J. Vitamin D supplementation and diabetes-related autoimmunity in the ABIS study. *Pediatr Diabetes*. 2007 Feb;8(1):11-4.

Brown AJ, Dusso A, Slatopolsky E. 1999. Vitamin D. *Am J Physiol Renal Physiol* 277: 157-175.

Bärebring L, Schoenmakers I, Glantz A, Hulthén L, Jagner Å, Ellis J, Bärebring M, Bullarbo M, Augustin H. Vitamin D Status during Pregnancy in a Multi-Ethnic Population-Representative Swedish Cohort. *Nutrients*. 2016 Oct 22;8(10). pii: E655.

Cadario F, Savastio S, Pagliardini V, Bagnati M, Vidali M, Cerutti F, Rabbone I, Fontana F, Lera R, De Donno V, Valori A, Gruden G, Bona G, Bruno G. Vitamin D levels at birth and risk of type 1 diabetes in childhood: a case-control study. *Acta Diabetol*. 2015 Dec;52(6):1077-81.

Cade C, Norman AW. Vitamin D<sub>3</sub> improves impaired glucose tolerance and insulin secretion in the vitamin D-deficient rat in vivo. *Endocrinology*. 1986 Jul;119(1):84-90.

Carter GD, Carter CR, Gunter E, Jones J, Jones G, Makin HL, Sufi S. Measurement of Vitamin D metabolites: an international perspective on methodology and clinical interpretation. *J Steroid Biochem Mol Biol*. 2004 May;89-90(1-5):467-71.

Cashman KD, Dowling KG, Škrabáková Z, Gonzalez-Gross M, Valtueña J, De Henauw S, Moreno L, Damsgaard CT, Michaelsen KF, Mølgaard C, Jorde R, Grimnes G, Moschonis G, Mavrogianni C, Manios Y, Thamm M, Mensink GB, Rabenberg M, Busch MA, Cox L, Meadows S, Goldberg G, Prentice A, Dekker JM, Nijpels G, Pilz S, Swart KM, van Schoor NM, Lips P, Eiriksdottir G, Gudnason V, Cotch MF, Koskinen S, Lamberg-Allardt C, Durazo-Arvizu RA, Sempos CT, Kiely M. Vitamin D deficiency in Europe: pandemic? *Am J Clin Nutr*. 2016 Apr;103(4):1033-44.

Christensen EI, Nielsen R, Birn H. From bowel to kidneys: the role of cubilin in physiology and disease. *Nephrol Dial Transplant*. 2013 Feb;28(2):274-81.

Cocco E, Meloni A, Murru MR, Corongiu D, Tranquilli S, Fadda E, Murru R, Schirru L, Secci MA, Costa G, Asunis I, Cuccu S, Fenu G, Loreface L, Carboni N, Mura G, Rosatelli MC, Marrosu MG. Vitamin D responsive elements within the HLA-DRB1 promoter region in Sardinian multiple sclerosis associated alleles. *PLoS One* 2012; 7: e41678.

Cooper JD, Smyth DJ, Walker NM, Stevens H, Burren OS, Wallace C, Greissl C, Ramos-Lopez E, Hyppönen E, Dunger DB, Spector TD, Ouwehand WH, Wang TJ, Badenhop K, Todd JA. Inherited variation in vitamin D genes is associated with predisposition to autoimmune disease type 1 diabetes. *Diabetes*. 2011 May;60(5):1624-31.

da Costa DS, Hygino J, Ferreira TB, Kasahara TM, Barros PO, Monteiro C, Oliveira A, Tavares F, Vasconcelos CC, Alvarenga R, Bento CA. Vitamin D modulates different IL-17-secreting T cell subsets in multiple sclerosis patients. *J Neuroimmunol*. 2016 Oct 15;299:8-18.

Dahlquist G, Mustonen L. 1994. Childhood onset diabetes – time trends and climatological factors. *Int J Epidemiol* 23:1234-1241.

Davies-Tuck M, Yim C, Knight M, Hodges R, Doery JC, Wallace E. Vitamin D testing in pregnancy: Does one size fit all? *Aust N Z J Obstet Gynaecol*. 2015 Apr;55(2):149-55.

de Goffau MC, Fuentes S, van den Bogert B, Honkanen H, de Vos WM, Welling GW, Hyöty H, Harmsen HJ. Aberrant gut microbiota composition at the onset of type 1 diabetes in young children. *Diabetologia*. 2014 Aug;57(8):1569-77.

Delovitch TL, Singh B. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity*. 1997 Dec;7(6):727-38

DIAMOND Project Group. Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999. *Diabet Med*. 2006 Aug;23(8):857-66.

Dib SA, Gomes MB. Etiopathogenesis of type 1 diabetes mellitus: prognostic factors for the evolution of residual beta cell function. *Diabetol Metab Syndr*. 2009 Dec 4;1(1):25.

Dopico XC, Evangelou M, Ferreira RC, Guo H, Pekalski ML, Smyth DJ, Cooper N, Burren OS, Fulford AJ, Hennig BJ, Prentice AM, Ziegler AG, Bonifacio E, Wallace C, Todd JA. Widespread seasonal gene expression reveals annual differences in human immunity and physiology. *Nat Commun*. 2015 May 12;6:7000.



Driver JP, Lamont DJ, Gysemans C, Mathieu C, Serreze DV. Calcium insufficiency accelerates type 1 diabetes in vitamin D receptor-deficient nonobese diabetic (NOD) mice. *Endocrinology*. 2011 Dec;152(12):4620-9.

Engelman CD, Fingerlin TE, Langefeld CD, Hicks PJ, Rich SS, Wagenknecht LE, Bowden DW, Norris JM. Genetic and environmental determinants of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels in Hispanic and African Americans. *J Clin Endocrinol Metab*. 2008 Sep;93(9):3381-8.

Esparza-Gordillo J, Matanovic A, Marenholz I, Bauerfeind A, Rohde K, Nemat K, Lee-Kirsch MA, Nordenskjöld M, Winge MC, Keil T, Krüger R, Lau S, Beyer K, Kalb B, Niggemann B, Hübner N, Cordell HJ, Bradley M, Lee YA. Maternal filaggrin mutations increase the risk of atopic dermatitis in children: an effect independent of mutation inheritance. *PLoS Genet*. 2015 Mar 10;11(3):e1005076.

EURODIAB ACE Study Group. Variation and trends in incidence of childhood diabetes in Europe. *Lancet*. 2000 Mar 11;355(9207):873-6.

EURODIAB Substudy 2 Study Group (1999) Vitamin D supplement in early childhood and risk for Type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 42:51-54.

Fang Y, van Meurs JB, Arp P, van Leeuwen JP, Hofman A, Pols HA, Uitterlinden AG. Vitamin D binding protein genotype and osteoporosis. *Calcif Tissue Int*. 2009 Aug;85(2):85-93.

Fang Y, van Meurs JB, d'Alesio A, Jhamai M, Zhao H, Rivadeneira F, Hofman A, van Leeuwen JP, Jehan F, Pols HA, Uitterlinden AG. Promoter and 3'-untranslated-region haplotypes in the vitamin d receptor gene predispose to osteoporotic fracture: the rotterdam study. *Am J Hum Genet*. 2005 Nov;77(5):807-23.

Foulis AK, Liddle CN, Farquharson MA, Richmond JA, Weir RS. 1986. The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. *Diabetologia*. 1986 May;29(5):267-74.

Frederiksen BN, Kroehl M, Fingerlin TE, Wong R, Steck AK, Rewers M, Norris JM. Association between vitamin D metabolism gene polymorphisms and risk of islet autoimmunity and progression to type 1 diabetes: the diabetes autoimmunity study in the young (DAISY). *J Clin Endocrinol Metab*. 2013a Nov;98(11):E1845-51.

Frederiksen B, Liu E, Romanos J, Steck AK, Yin X, Kroehl M, Fingerlin TE, Erlich H, Eisenbarth GS, Rewers M, Norris JM. Investigation of the vitamin D receptor gene (VDR) and its interaction with protein tyrosine phosphatase, non-receptor type 2 gene (PTPN2) on risk of islet autoimmunity and type 1 diabetes: the Diabetes Autoimmunity Study in the Young (DAISY). *J Steroid Biochem Mol Biol*. 2013 Jan;133:51-7.

Fronczak CM, Barón AE, Chase HP, Ross C, Brady HL, Hoffman M, Eisenbarth GS, Rewers M, Norris JM. In utero dietary exposures and risk of islet autoimmunity in children. *Diabetes Care*. 2003 Dec;26(12):3237-42.

Gaffney-Stomberg E, Lutz LJ, Shcherbina A, Ricke DO, Petrovick M, Cropper TL, Cable SJ, McClung JP. Association Between Single Gene Polymorphisms and Bone Biomarkers and Response to Calcium and Vitamin D Supplementation in Young Adults Undergoing Military Training. *J Bone Miner Res*. 2017 Mar;32(3):498-507.

Gale EA. Type 1 diabetes in the young: the harvest of sorrow goes on. *Diabetologia*. 2005 Aug;48(8):1435-8.

Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, Drew JC, Ilonen J, Knip M, Hyöty H, Veijola R, Simell T, Simell O, Neu J, Wasserfall CH, Schatz D, Atkinson MA, Triplett EW. Toward defining the autoimmune microbiome for type 1 diabetes. *ISME J*. 2011;5(1):82-91.

González EA, Sachdeva A, Oliver DA, Martin KJ. Vitamin D insufficiency and deficiency in chronic kidney disease: a single center observational study. *American Journal of Nephrology*. 2004;24(5):503–510.

Gorham ED<sup>1</sup>, Garland CF, Burgi AA, Mohr SB, Zeng K, Hofflich H, Kim JJ, Ricordi C. Lower prediagnostic serum 25-hydroxyvitamin D concentration is associated with higher risk of insulin-requiring diabetes: a nested case-control study. *Diabetologia*. 2012;55(12):3224–3227.

Granfors M, Augustin H, Ludvigsson J, Brekke HK. No association between use of multivitamin supplement containing vitamin D during pregnancy and risk of Type 1 Diabetes in the child. *Pediatr Diabetes*. 2016 Nov;17(7):525–530.

Gregori S, Giarratana N, Smioldo S, Uskokovic M, Adorini L. 2002. A 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> Analog Enhanced Regulatory T-Cells and Arrests Autoimmune Diabetes in NOD Mice. *Diabetes* 51:1367-1374.

Guillot X, Semerano L, Saidenberg-Kermanac'h N, Falgarone G, Boissier MC. Vitamin D and inflammation. *Joint Bone Spine* 2010; 77: 552-7.

Gülden E, Wong FS, Wen L. The gut microbiota and Type 1 Diabetes. *Clin Immunol*. 2015 Aug;159(2):143-53.

Gysemans C, van Etten E, Overbergh L, Giulietti A, Eelen G, Waer M, Verstuyf A, Bouillon R, Mathieu C. Unaltered diabetes presentation in NOD mice lacking the vitamin D receptor. *Diabetes* 2008; 57: 269–275.

Hagger V, Hendrieckx C, Sturt J, Skinner TC, Speight J. Diabetes Distress Among Adolescents with Type 1 Diabetes: a Systematic Review. *Curr Diab Rep*. 2016 Jan;16(1):9.

Harfouch-Hammoud E, Timsit J, Boitard C, Bach JF, Caillat-Zucman S. Contribution of DRB1\*04 variants to predisposition to or protection from insulin dependent diabetes mellitus is independent of dq. *J Autoimmun*. 1996 Jun;9(3):411-4.

Harjutsalo V, Sjöberg L, Tuomilehto J. Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. *Lancet*. 2008 May 24;371(9626):1777-82.

Harjutsalo V, Sund R, Knip M, Groop PH. Incidence of type 1 diabetes in Finland. *JAMA* 24;310 (4):427-8, 2013

Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. Interleukin 17-producing CD4<sup>+</sup> effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005;6:1123–32.

Hathcock JN, Shao A, Vieth R, Heaney R. Risk assessment for vitamin D. *Am J Clin Nutr* 2007 85(1):6-18.

Heaney RP. Toward a physiological referent for the vitamin D requirement. *J Endocrinol Invest*. 2014 Nov;37(11):1127-30.

Heid IM, Jackson AU, Randall JC, Winkler TW, Qi L, Steinhorsdottir V, Thorleifsson G, Zillikens MC, Speliotes EK, Mägi R, Workalemahu T, White CC, Bouatia-Naji N, Harris TB, Berndt SI, Ingelsson E, Willer CJ, Weedon MN, Luan J, Vedantam S, Esko T, Kilpeläinen TO, Kutalik Z, Li S, Monda KL, Dixon AL, Holmes CC, Kaplan LM, Liang L, Min JL, Moffatt MF, Molony C, Nicholson G, Schadt EE, Zondervan KT, Feitosa MF, Ferreira T, Lango Allen H, Weyant RJ, Wheeler E, Wood AR; MAGIC, Estrada K, Goddard ME, Lettre G, Mangino M, Nyholt DR, Purcell S, Smith AV, Visscher PM, Yang J, McCarroll SA, Nemesh J, Voight BF, Absher D, Amin N, Aspelund T, Coin L, Glazer NL, Hayward C, Heard-Costa NL, Hottenga JJ, Johansson A, Johnson T, Kaakinen M, Kapur K, Ketkar S, Knowles JW, Kraft P, Kraja AT, Lamina C, Leitzmann MF, McKnight B, Morris AP, Ong KK, Perry JR, Peters MJ, Polasek O, Prokopenko I, Rayner NW, Ripatti S, Rivadeneira F, Robertson NR, Sanna S, Sovio U, Surakka I, Teumer A, van Wingerden S, Vitart V, Zhao JH, Cavalcanti-Proença C, Chines PS, Fisher E, Kulzer JR, Lecoœur C, Narisu

N, Sandholt C, Scott LJ, Silander K, Stark K, Tammesoo ML, Teslovich TM, Timpson NJ, Watanabe RM, Welch R, Chasman DI, Cooper MN, Jansson JO, Kettunen J, Lawrence RW, Pellikka N, Perola M, Vandenput L, Alavere H, Almgren P, Atwood LD, Bennett AJ, Biffar R, Bonnycastle LL, Bornstein SR, Buchanan TA, Campbell H, Day IN, Dei M, Dörr M, Elliott P, Erdos MR, Eriksson JG, Freimer NB, Fu M, Gaget S, Geus EJ, Gjesing AP, Grallert H, Grässler J, Groves CJ, Guiducci C, Hartikainen AL, Hassanali N, Havulinna AS, Herzig KH, Hicks AA, Hui J, Igl W, Jousilahti P, Jula A, Kajantie E, Kinnunen L, Kolcic I, Koskinen S, Kovacs P, Kroemer HK, Krzelj V, Kuusisto J, Kvaloy K, Laitinen J, Lantieri O, Lathrop GM, Lokki ML, Luben RN, Ludwig B, McArdle WL, McCarthy A, Morken MA, Nelis M, Neville MJ, Paré G, Parker AN, Peden JF, Pichler I, Pietiläinen KH, Platou CG, Pouta A, Ridderstråle M, Samani NJ, Saramies J, Sinisalo J, Smit JH, Strawbridge RJ, Stringham HM, Swift AJ, Teder-Laving M, Thomson B, Usala G, van Meurs JB, van Ommen GJ, Vatn V, Volpato CB, Wallaschofski H, Walters GB, Widen E, Wild SH, Willemsen G, Witte DR, Zgaga L, Zitting P, Beilby JP, James AL, Kähönen M, Lehtimäki T, Nieminen MS, Ohlsson C, Palmer LJ, Raitakari O, Ridker PM, Stumvoll M, Tönjes A, Viikari J, Balkau B, Ben-Shlomo Y, Bergman RN, Boeing H, Smith GD, Ebrahim S, Froguel P, Hansen T, Hengstenberg C, Hveem K, Isomaa B, Jørgensen T, Karpe F, Khaw KT, Laakso M, Lawlor DA, Marre M, Meitinger T, Metspalu A, Midthjell K, Pedersen O, Salomaa V, Schwarz PE, Tuomi T, Tuomilehto J, Valle TT, Wareham NJ, Arnold AM, Beckmann JS, Bergmann S, Boerwinkle E, Boomsma DI, Caulfield MJ, Collins FS, Eiriksdottir G, Gudnason V, Gyllenstein U, Hamsten A, Hattersley AT, Hofman A, Hu FB, Illig T, Iribarren C, Jarvelin MR, Kao WH, Kaprio J, Launer LJ, Munroe PB, Oostra B, Penninx BW, Pramstaller PP, Psaty BM, Quertermous T, Rissanen A, Rudan I, Shuldiner AR, Soranzo N, Spector TD, Syvanen AC, Uda M, Uitterlinden A, Völzke H, Vollenweider P, Wilson JF, Witteman JC, Wright AF, Abecasis GR, Boehnke M, Borecki IB, Deloukas P, Frayling TM, Groop LC, Haritunians T, Hunter DJ, Kaplan RC, North KE, O'Connell JR, Peltonen L, Schlessinger D, Strachan DP, Hirschhorn JN, Assimes TL, Wichmann HE, Thorsteinsdottir U, van Duijn CM, Stefansson K, Cupples LA, Loos RJ, Barroso I, McCarthy MI, Fox CS, Mohlke KL, Lindgren CM. Meta-analysis identifies 13 new loci associated with waist-hip ratio and reveals sexual dimorphism in the genetic basis of fat distribution. *Nat Genet* 2010; 42: 949-60.

Hermann R, Knip M, Veijola R, Simell O, Laine AP, Akerblom HK, Groop PH, Forsblom C, Pettersson-Fernholm K, Ilonen J; FinnDiane Study Group. Temporal changes in the frequencies of HLA genotypes in patients with Type 1 diabetes--indication of an increased environmental pressure? *Diabetologia*. 2003 Mar;46(3):420-5.

Hitman GA, Mannan N, McDermott MF, Aganna E, Ogunkolade BW, Hales CN, Boucher BJ. 1998. Vitamin D Receptor Gene Polymorphisms Influence Insulin Secretion in Bangladeshi Asians. *Diabetes* 47:688-690.

Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM; Endocrine Society. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. 2011 Jul;96(7):1911-30.

Holmlund-Suila E, Koskivirta P, Metso T, Andersson S, Mäkitie O, Viljakainen HT. Vitamin D deficiency in children with a chronic illness--seasonal and age-related variations in serum 25-hydroxy Vitamin D concentrations. *PLoS One*. 2013 Apr 9;8(4):e60856.

Holt PG, Jones CA. The development of the immune system during pregnancy and early life. *Allergy*. 2000 Aug;55(8):688-97.

Hovind P, Tarnow L, Rossing K, Rossing P, Eising S, Larsen N, Binder C, Parving HH. Decreasing incidence of severe diabetic microangiopathy in type 1 diabetes. *Diabetes Care* 2003; 26: 1258–1264

Howell WM. HLA and disease: guilt by association. *Int J Immunogenet.* 2014 Feb;41(1):1-12.

Howson JM, Walker NM, Clayton D, Todd JA; Type 1 Diabetes Genetics Consortium. Confirmation of HLA class II independent type 1 diabetes associations in the major histocompatibility complex including HLA-B and HLA-A. *Diabetes Obes Metab* 2009; 11 :31-45.

Hunter D, De Lange M, Snieder H, MacGregor AJ, Swaminathan R, Thakker RV, Spector TD. Genetic contribution to bone metabolism, calcium excretion, and vitamin D and parathyroid hormone regulation. *J Bone Miner Res.* 2001 Feb;16(2):371-8.

Hyppönen E, Läärä E, Reunanen A, Järvelin MR, Virtanen SM. Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. *Lancet.* 2001 Nov 3;358(9292):1500-3.

Hyttinen V, Kaprio J, Kinnunen L, Koskenvuo M, Tuomilehto J. Genetic liability of type 1 diabetes and the onset age among 22,650 young Finnish twin pairs: a nationwide follow-up study. *Diabetes.* 2003 Apr;52(4):1052-5.

Hyöty H. Viruses in type 1 diabetes. *Pediatr Diabetes.* 2016 Jul;17 Suppl 22:56-64.

Insel RA, Dunne JL, Atkinson MA, Chiang JL, Dabelea D, Gottlieb PA, Greenbaum CJ, Herold KC, Krischer JP, Lernmark Å, Ratner RE, Rewers MJ, Schatz DA, Skyler JS, Sosenko JM, Ziegler AG. Staging presymptomatic type 1 diabetes: a scientific statement of JDRF, the Endocrine Society, and the American Diabetes Association. *Diabetes Care.* 2015 Oct;38(10):1964-74.

Institute of Medicine, Food and Nutrition Board (2011) Dietary reference intakes for calcium and vitamin D. Washington, DC: National Academy Press.

Islam MZ, Viljakainen HT, Kärkkäinen MU, Saarnio E, Laitinen K, Lamberg-Allardt C. Prevalence of vitamin D deficiency and secondary hyperparathyroidism during winter in pre-menopausal Bangladeshi and Somali immigrant and ethnic Finnish women: associations with forearm bone mineral density. *Br J Nutr.* 2012 Jan;107(2):277-83.

Israni N, Goswami R, Kumar A, Rani R. Interaction of vitamin D receptor with HLA DRB1 0301 in type 1 diabetes patients from North India. *Plos One* 2009;4: e8023.

Issa LL, Leong GM, Eisman JA. 1998. Molecular mechanism of vitamin D receptor action. *Inflamm Res* 47:451-475.

Jacobsen L, Schatz D. Current and future efforts toward the prevention of type 1 diabetes. *Pediatr Diabetes.* 2016 Jul;17 Suppl 22:78-86.

Jacobsen R, Thorsen SU, Cohen AS, Lundqvist M, Frederiksen P, Pipper CB, Pociot F, Thygesen LC, Ascherio A, Svensson J, Heitmann BL. Neonatal vitamin D status is not associated with later risk of type 1 diabetes: results from two large Danish population-based studies. *Diabetologia.* 2016 Sep;59(9):1871-81.

James SJ, Melnyk S, Jernigan S, Pavliv O, Trusty T, Lehman S, Seidel L, Gaylor DW, Cleves MA. A functional polymorphism in the reduced folate carrier gene and DNA hypomethylation in mothers of children with autism. *Am J Med Genet B Neuropsychiatr Genet.* 2010 Sep;153B(6):1209-20.

Jarvala T, Raitanen J, Rissanen P. Diabeteksen kustannukset Suomessa 1998-2007. DEHKO ja Suomen Diabetesliitto, 2010 (in Finnish).

Johnston C, Pyke DA, Cudworth AG, Wolf E. HLA-DR typing in identical twins with insulin-dependent diabetes: difference between concordant and discordant pairs. *Br Med J (Clin Res Ed).* 1983 Jan 22;286(6361):253-5.

Jolliffe DA, Walton RT, Griffiths CJ, Martineau AR. Single nucleotide polymorphisms in the vitamin D pathway associating with circulating concentrations of vitamin D metabolites and non-skeletal health outcomes: Review of genetic association studies. *J Steroid Biochem* 2016 Nov;164:18-29.

Jones G. Pharmacokinetics of vitamin D toxicity. *Am J Clin Nutr* 2008 88(2):582S-586S.

Jorde R, Sneve M, Emaus N, Figenschau Y, Grimnes G. Cross-sectional and longitudinal relation between serum 25-hydroxyvitamin D and body mass index: the Tromsø study. *Eur J Nutr*. 2010 Oct;49(7):401-7.

Kahn HS, Morgan TM, Case LD, Dabelea D, Mayer-Davis EJ, Lawrence JM, Marcovina SM, Imperatore G; SEARCH for Diabetes in Youth Study Group. Association of type 1 diabetes with month of birth among U.S. youth: The SEARCH for Diabetes in Youth Study. *Diabetes Care*. 2009 Nov;32(11):2010-5.

Kanhere M, Chassaing B, Gewirtz AT, Tangpricha V. Role of vitamin D on gut microbiota in cystic fibrosis. *J Steroid Biochem Mol Biol*. 2016 Nov 3. pii: S0960-0760(16)30296-5.

Karvonen M, Tuomilehto J, Virtala E, Pitkaniemi J, Reunanen A, Tuomilehto-Wolf E, Akerblom HK. Seasonality in the clinical onset of insulin-dependent diabetes mellitus in Finnish children. Childhood Diabetes in Finland (DiMe) Study Group. *Am J Epidemiol*. 1996 Jan 15;143(2):167-76.

Khashan AS, Kenny LC, Laursen TM, Mahmood U, Mortensen PB, Henriksen TB, O'Donoghue K. Pregnancy and the risk of autoimmune disease. *PLoS One*. 2011;6(5):e19658.

Kimpimäki T, Kupila A, Hämäläinen AM, Kukko M, Kulmala P, Savola K, Simell T, Keskinen P, Ilonen J, Simell O, Knip M. The first signs of beta-cell autoimmunity appear in infancy in genetically susceptible children from the general population: the Finnish Type 1 Diabetes Prediction and Prevention Study. *J Clin Endocrinol Metab*. 2001 Oct;86(10):4782-8.

Klingberg E, Oleröd G, Konar J, Petzold M, Hammarsten O. Seasonal variations in serum 25-hydroxy vitamin D levels in a Swedish cohort. *Endocrine*. 2015 Aug;49(3):800-8.

Klinke DJ 2nd. Extent of beta cell destruction is important but insufficient to predict the onset of type 1 diabetes mellitus. *PLoS One*. 2008 Jan 2;3(1):e1374.

Knip M, Korhonen S, Kulmala P, Veijola R, Reunanen A, Raitakari OT, Viikari J, Akerblom HK. Prediction of type 1 diabetes in the general population. *Diabetes Care*. 2010 Jun;33(6):1206-12.

Knip M, Selvenius J, Siljander H, Veijola R. Reclassification of asymptomatic beta cell autoimmunity: a critical perspective. *Diabetologia*. 2017 Jan;60(1):39-42.

Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE. Succession of microbial consortia in the developing infant gut microbiome. *Proc Natl Acad Sci U S A*. 2011 Mar 15;108 Suppl 1:4578-85.

Kondrashova A, Reunanen A, Romanov A, Karvonen A, Viskari H, Vesikari T, Ilonen J, Knip M, Hyöty H. A six-fold gradient in the incidence of type 1 diabetes at the eastern border of Finland. *Ann Med*. 2005;37(1):67-72.

Kondrashova A, Viskari H, Kulmala P, Romanov A, Ilonen J, Hyöty H, Knip M. Signs of beta-cell autoimmunity in nondiabetic schoolchildren: a comparison between Russian Karelia with a low incidence of type 1 diabetes and Finland with a high incidence rate. *Diabetes Care*. 2007 Jan;30(1):95-100. 1.

Koskinen MK, Helminen O, Matomäki J, Aspholm S, Mykkänen J, Mäkinen M, Simell V, Vähä-Mäkilä M, Simell T, Ilonen J, Knip M, Veijola R,

Toppari J, Simell O. Reduced  $\beta$ -cell function in early preclinical type 1 diabetes. *Eur J Endocrinol*. 2016 Mar;174(3):251-9.

Krogvold L, Edwin B, Buanes T, Frisk G, Skog O, Anagandula M, Korsgren O, Undlien D, Eike MC, Richardson SJ, Leete P, Morgan NG, Oikarinen S, Oikarinen M, Laiho JE, Hyöty H, Ludvigsson J, Hanssen KF, Dahl-Jørgensen K. Detection of a low-grade enteroviral infection in the islets of langerhans of living patients newly diagnosed with type 1 diabetes. *Diabetes*. 2015 May;64(5):1682-7.

Kuoppala T, Tuimala R, Parviainen M, Koskinen T, Ala-Houhala M. Serum levels of vitamin D metabolites, calcium, phosphorus, magnesium and alkaline phosphatase in Finnish women throughout pregnancy and in cord serum at delivery. *Hum Nutr Clin Nutr*. 1986 Jul;40(4):287-93.

Laitinen OH, Honkanen H, Pakkanen O, Oikarinen S, Hankaniemi MM, Huhtala H, Ruokoranta T, Lecouturier V, André P, Harju R, Virtanen SM, Lehtonen J, Almond JW, Simell T, Simell O, Ilonen J, Veijola R, Knip M, Hyöty H. Coxsackievirus B1 is associated with induction of  $\beta$ -cell autoimmunity that portends type 1 diabetes. *Diabetes*. 2014 Feb;63(2):446-55.

Lemire JM, Archer DC, Beck L, Spiegelberg HL. Immunosuppressive actions of 1,25-dihydroxyvitamin D<sub>3</sub>: preferential inhibition of Th1 functions. *J Nutr*. 1995 Jun;125(6 Suppl):1704S-1708S.

Lenna S, Assassi S, Farina GA, Mantero JC, Scorza R, Lafyatis R, Farber HW, Trojanowska M. The HLA-B\*35 allele modulates ER stress, inflammation and proliferation in PBMCs from Limited Cutaneous Systemic Sclerosis patients. *Arthritis Res Ther* 2015; 17: 363.

Lind M, Polonsky W, Hirsch IB, Heise T, Bolinder J, Dahlqvist S, Schwarz E, Ólafsdóttir AF, Frid A, Wedel H, Ahlén E, Nyström T, Hellman J. Continuous Glucose Monitoring vs Conventional Therapy for Glycemic Control in Adults With Type 1 Diabetes Treated With Multiple Daily Insulin Injections: The GOLD Randomized Clinical Trial. *JAMA*. 2017 Jan 24;317(4):379-387.

Lips P, Chapuy MC, Dawson-Hughes B, Pols HA, Holick MF. An international comparison of serum 25-hydroxyvitamin D measurements. *Osteoporos Int*. 1999;9(5):394-7.

Lips P, Eekhoff M, van Schoor N, Oosterwerff M, de Jongh R, Krul-Poel Y, Simsek S. Vitamin D and type 2 diabetes. *J Steroid Biochem Mol Biol*. 2016 Dec 5. pii: S0960-0760(16)30338-7.

Littorin B, Blom P, Schölin A, Arnqvist HJ, Blohmé G, Bolinder J, Ekblom-Schnell A, Eriksson JW, Gudbjörnsdóttir S, Nyström L, Ostman J, Sundkvist G. Lower levels of plasma 25-hydroxyvitamin D among young adults at diagnosis of autoimmune type 1 diabetes compared with control subjects: results from the nationwide Diabetes Incidence Study in Sweden (DISS). *Diabetologia* 2006; 49: 2847–2852.

Livingstone KM, Celis-Morales C, Hoeller U, Lambrinou CP, Moschonis G, Macready AL, Fallaize R, Baur M, Roos FF, Bendik I, Grimaldi K, Navas-Carretero S, San-Cristobal R, Weber P, Drevon CA, Manios Y, Traczyk I, Gibney ER, Lovegrove JA, Saris WH, Daniel H, Gibney M, Martinez JA, Brennan L, Hill TR, Mathers JC; Food4Me Study. Weekday sunlight exposure, but not vitamin D intake, influences the association between vitamin D receptor genotype and circulating concentration 25-hydroxyvitamin D in a pan-European population: the Food4Me study. *Mol Nutr Food Res*. 2017 Feb;61(2).

Livingstone SJ, Levin D, Looker HC, Lindsay RS, Wild SH, Joss N, Leese G, Leslie P, McCrimmon RJ, Metcalfe W, McKnight JA, Morris AD, Pearson DW, Petrie JR, Philip S, Sattar NA, Traynor JP, Colhoun HM; Scottish Diabetes Research Network epidemiology group; Scottish Renal Registry.

Estimated life expectancy in a Scottish cohort with type 1 diabetes, 2008-2010. *JAMA*. 2015 Jan 6;313(1):37-44.

Lopez ER, Regulla K, Pani MA, Krause M, Usadel KH, Badenhop K. CYP27B1 polymorphisms variants are associated with type 1 diabetes mellitus in Germans. *J Steroid Biochem Mol Biol*. 2004 May;89-90(1-5):155-7.

Luxwolda MF, Kuipers RS, Kema IP, Dijck-Brouwer DA, Muskiet FA. Traditionally living populations in East Africa have a mean serum 25-hydroxyvitamin D concentration of 115 nmol/l. *Br J Nutr*. 2012 Nov 14;108(9):1557-61.

Lynch KF, Lernmark B, Merlo J, Cilio CM, Ivarsson SA, Lernmark A; Diabetes Prediction in Skåne (DiPiS) Study Group. Cord blood islet autoantibodies and seasonal association with the type 1 diabetes high-risk genotype. *J Perinatol*. 2008 Mar;28(3):211-7.

Maestro B, Dávila N, Carranza MC, Calle C. Identification of a Vitamin D response element in the human insulin receptor gene promoter. *J Steroid Biochem Mol Biol*. 2003 Feb;84(2-3):223-30.

Maia-Ceciliano TC, Barreto-Vianna AR, Barbosa-da-Silva S, Aguila MB, Faria TS, Mandarim-de-Lacerda CA. Maternal vitamin D-restricted diet has consequences in the formation of pancreatic islet/insulin-signaling in the adult offspring of mice. *Endocrine*. 2016 Oct;54(1):60-69.

Manzon L, Altarescu G, Tevet A, Schimmel MS, Elstein D, Samueloff A, Grisaru-Granovsky S. Vitamin D receptor polymorphism FokI is associated with spontaneous idiopathic preterm birth in an Israeli population. *Eur J Obstet Gynecol Reprod Biol*. 2014 Jun;177:84-8.

Margulies SL, Kurian D, Elliott MS, Han Z. Vitamin D deficiency in patients with intestinal malabsorption syndromes--think in and outside the gut. *J Dig Dis*. 2015 Nov;16(11):617-33.

Marjamäki L, Niinistö S, Kenward MG, Uusitalo L, Uusitalo U, Ovaskainen ML, Kronberg-Kippilä C, Simell O, Veijola R, Ilonen J, Knip M, Virtanen SM. Maternal intake of vitamin D during pregnancy and risk of advanced beta cell autoimmunity and type 1 diabetes in offspring. *Diabetologia*. 2010 Aug;53(8):1599-607.

Martineau AR, Cates CJ, Urashima M, Jensen M, Griffiths AP, Nurmatov U, Sheikh A, Griffiths CJ. Vitamin D for the management of asthma. *Cochrane Database Syst Rev*. 2016 Sep 5;9:CD011511. [Epub ahead of print]

Martin-Orozco N, Chung Y, Chang SH, Wang YH, Dong C. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. *Eur J Immunol*. 2009 Jan;39(1):216-24.

Mathieu C, Casteels K, Waer M, Laureys J, Valckx D, Bouillon R. Prevention of diabetes recurrence after syngeneic islet transplantation in NOD mice by analogues of 1,25(OH)<sub>2</sub>D<sub>3</sub> in combination with cyclosporin A: mechanism of action involves an immune shift from Th1 to Th2. *Transplant Proc*. 1998 Mar;30(2):541.

Mathieu C, Laureys J, Sobis H, Vandeputte M, Waer M, Bouillon R. 1,25 Dihydroxyvitamin D<sub>3</sub> prevents insulinitis in NOD mice. *Diabetes*. 1992 Nov;41(11):1491-5.

Mathieu C, Waer M, Casteels K, Laureys J and Bouillon R. Prevention of Type 1 Diabetes in NOD Mice by Nonhypercalcemic Doses of a New Structural Analog of 1,25-Dihydroxyvitamin D<sub>3</sub>, KH1060. *Endocrinology*. 1995 Mar;136(3):866-72.

McGrath JJ, Saha S, Burne TH, Eyles DW. A systematic review of the association between common single nucleotide polymorphisms and 25-hydroxyvitamin D concentrations. *J Steroid Biochem Mol Biol*. 2010 Jul;121(1-2):471-7.

McKinney PA and EURODIAB Seasonality Of Birth Group. Europe and Diabetes. Seasonality of birth in patients with childhood Type I diabetes in 19 European regions. *Diabetologia*. 2001 Oct;44 Suppl 3:B67-74.

Miettinen ME, Kinnunen L, Leiviskä J, Keinänen-Kiukaanniemi S, Korpi-Hyövälti E, Niskanen L, Oksa H, Saaristo T, Tuomilehto J, Vanhala M, Uusitupa M, Peltonen M. Association of serum 25-hydroxyvitamin D with lifestyle factors and metabolic and cardiovascular disease markers: population-based cross-sectional study (FIN-D2D). *PLoS One*. 2014 Jul 7;9(7):e100235.

Mikk ML, Kiviniemi M, Laine AP, Härkönen T, Veijola R, Simell O, Knip M, Ilonen J; Finnish Paediatric Diabetes Register. The HLA-B\*39 allele increases type 1 diabetes risk conferred by HLA-DRB1\*04:04-DQB1\*03:02 and HLA-DRB1\*08-DQB1\*04 class II haplotypes. *Hum Immunol*. 2014 Jan;75(1):65-70.

Ministry of Social Affairs and Health. Follow-up study on the vitamin D status in the Finnish population 2002 and 2004. Reports of the Ministry of Social Affairs and Health 2006:9(2/26/2014).

Ministry of Trade and Industry of Finland. 917/2002 (in Finnish).

Moltchanova EV, Schreier N, Lammi N, Karvonen M. Seasonal variation of diagnosis of Type 1 diabetes mellitus in children worldwide. *Diabet Med*. 2009 Jul;26(7):673-8.

Munger KL, Äivo J, Hongell K, Soilu-Hänninen M, Surcel HM, Ascherio A. Vitamin D Status During Pregnancy and Risk of Multiple Sclerosis in Offspring of Women in the Finnish Maternity Cohort. *JAMA Neurol*. 2016 May 1;73(5):515-9.

Mäkinen M, Mykkänen J, Koskinen M, Simell V, Veijola R, Hyöty H, Ilonen J, Knip M, Simell O, Toppari J. Serum 25-Hydroxyvitamin D Concentrations in Children Progressing to Autoimmunity and Clinical Type 1 Diabetes. *J Clin Endocrinol Metab*. 2016 Feb;101(2):723-9.

Mäkinen M, Simell V, Mykkänen J, Ilonen J, Veijola R, Hyöty H, Knip M, Simell O, Toppari J, Hermann R. An increase in serum 25-hydroxyvitamin D concentrations preceded a plateau in type 1 diabetes incidence in Finnish children. *J Clin Endocrinol Metab*. 2014 Nov;99(11):E2353-6.

Mäkitie O. Muuttuiko mikään uusien D-vitamiini-suositusten myötä? Pääkirjoitus. *Lääketieteellinen Aikakauskirja Duodecim* 2011;127(12):1181-3 (in Finnish).

National Nutrition Council. Report of vitamin D working group (Valtion ravitsemusneuvottelukunta. D-vitamiiniyöryhmän raportti). 2010 Apr (in Finnish).

Nerup J, Platz P, Andersen OO, Christy M, Lyngsoe J, Poulsen JE, Ryder LP, Nielsen LS, Thomsen M, Svejgaard A. HL-A antigens and diabetes mellitus. *Lancet*. 1974 Oct 12;2(7885):864-6.

Neumann T, Sämann A, Lodes S, Kästner B, Franke S, Kiehnopf M, Hemmelmann C, Lehmann T, Müller UA, Hein G, Wolf G. Glycaemic control is positively associated with prevalent fractures but not with bone mineral density in patients with Type 1 diabetes. *Diabet Med*. 2011 Jul;28(7):872-5.

Niclauss N, Meier R, Bédat B, Berishvili E, Berney T. Beta-Cell Replacement: Pancreas and Islet Cell Transplantation. *Endocr Dev*. 2016;31:146-62.

Noble JA. Immunogenetics of type 1 diabetes: A comprehensive review. *J Autoimmun*. 2015 Nov;64:101-12

Noble JA, Valdes AM. Genetics of the HLA region in the prediction of type 1 diabetes. *Curr Diab Rep*. 2011 Dec;11(6):533-42

Noble JA, Valdes AM, Varney MD, Carlson JA, Moonsamy P, Fear AL, Lane JA, Lavant E, Rappner R, Louey A, Concannon P, Mychaleckyj JC, Erlich HA; Type 1 Diabetes Genetics Consortium. HLA class I and genetic



susceptibility to type 1 diabetes: results from the Type 1 Diabetes Genetics Consortium. *Diabetes*. 2010 Nov;59(11):2972-9.

Norman AW, Frankel JB, Heldt AM, Grodsky GM. Vitamin D deficiency inhibits pancreatic secretion of insulin. *Science*. 1980 Aug 15;209(4458):823-5.

Nuriel-Ohayon M, Neuman H, Koren O. Microbial Changes during Pregnancy, Birth, and Infancy. *Front Microbiol*. 2016 Jul 14;7:1031.

Nykjaer A, Fyfe JC, Kozyraki R, Leheste JR, Jacobsen C, Nielsen MS, Verroust PJ, Aminoff M, de la Chapelle A, Moestrup SK, Ray R, Gliemann J, Willnow TE, Christensen EI. Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D(3). *Proc Natl Acad Sci U S A*. 2001 Nov 20;98(24):13895-900.

Ogunkolade BW, Boucher BJ, Prah J, Bustin SA, Burrin JM, Noonan K, North BV, Mannan N, McDermott MF, DeLuca HF, Hitman GA. 2002. Vitamin D receptor (VDR) mRNA and VDR protein levels in relation to vitamin D status insulin secretory capacity, and VDR genotype in Bangladeshi Asians. *Diabetes* 51:2294-2300.

Onengut-Gumuscu S, Chen WM, Burren O, Cooper NJ, Quinlan AR, Mychaleckyj JC, Farber E, Bonnie JK, Szpak M, Schofield E, Achuthan P, Guo H, Fortune MD, Stevens H, Walker NM, Ward LD, Kundaje A, Kellis M, Daly MJ, Barrett JC, Cooper JD, Deloukas P; Type 1 Diabetes Genetics Consortium, Todd JA, Wallace C, Concannon P, Rich SS. Fine mapping of type 1 diabetes susceptibility loci and evidence for colocalization of causal variants with lymphoid gene enhancers. *Nat Genet*. 2015 Apr;47(4):381-6.

Orlow I, Roy P, Reiner AS, Yoo S, Patel H, Paine S, Armstrong BK, Krickler A, Marrett LD, Millikan RC, Thomas NE, Gruber SB, Anton-Culver H, Rosso S, Gallagher RP, Dwyer T, Kanetsky PA, Busam K, From L, Begg CB, Berwick M; GEM Study Group. Vitamin D receptor polymorphisms in patients with cutaneous melanoma. *Int J Cancer*. 2012 Jan 15;130(2):405-18.

Pani MA, Knapp M, Donner H, Braun J, Baur MP, Usadel KH, Badenhop K. Vitamin D receptor allele combinations influence genetic susceptibility to type 1 diabetes in Germans. *Diabetes*. 2000 Mar;49(3):504-7.

Panierakis C, Goulielmos G, Mamoulakis D, Petraki E, Papavasiliou E, Galanakis E. Vitamin D receptor gene polymorphisms and susceptibility to type 1 diabetes in Crete, Greece. *Clin Immunol*. 2009 Nov;133(2):276-81.

Patterson CC, Dahlquist GG, Gyürüs E, Green A, Soltész G; EURODIAB Study Group. Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. *Lancet*. 2009 Jun 13;373(9680):2027-33.

Pekkinen M, Saarnio E, Viljakainen HT, Kokkonen E, Jakobsen J, Cashman K, Mäkitie O, Lamberg-Allardt C. Vitamin D binding protein genotype is associated with serum 25-hydroxyvitamin D and PTH concentrations, as well as bone health in children and adolescents in Finland. *PLoS One*. 2014 Jan 30;9(1):e87292.

Pekkinen M, Viljakainen H, Saarnio E, Lamberg-Allardt C, Mäkitie O. Vitamin D is a major determinant of bone mineral density at school age. *PLoS One*. 2012;7(7):e40090.

Penno MAC, Couper JJ, Craig ME, Colman PG, Rawlinson WD, Cotterill AM, Jones TW, Harrison LC. Environmental determinants of islet autoimmunity (ENDIA): a pregnancy to early life cohort study in children at-risk of type 1 diabetes. *BMC Pediatr*. 2013 Aug 14;13:124.

Pham-Short A, Donaghue KC, Ambler G, Phelan H, Twigg S, Craig ME. Screening for celiac disease in type 1 diabetes: a systematic review. *Pediatrics*. 2015;136:e170-e176.

Pilsner JR, Hu H, Wright RO, Kordas K, Ettinger AS, Sánchez BN, Cantonwine D, Lazarus AL, Cantoral A, Schnaas L, Téllez-Rojo MM,

Hernández-Avila M. Maternal MTHFR genotype and haplotype predict deficits in early cognitive development in a lead-exposed birth cohort in Mexico City. *Am J Clin Nutr.* 2010 Jul;92(1):226-34.

Piper K, Brickwood S, Turnpenny LW, Cameron IT, Ball SG, Wilson DI, Hanley NA. Beta cell differentiation during early human pancreas development. *J Endocrinol.* 2004 Apr;181(1):11-23.

Pociot F, Akolkar B, Concannon P, Erlich HA, Julier C, Morahan G, Nierras CR, Todd JA, Rich SS, Nerup J. Genetics of type 1 diabetes: what's next? *Diabetes.* 2010 Jul;59(7):1561-71.

Pociot F, Lernmark Å. Genetic risk factors for type 1 diabetes. *Lancet.* 2016 Jun 4;387(10035):2331-9.

Podar T, Solntsev A, Karvonen M, Padaiga Z, Brigis G, Urbonaite B, Viik-Kajander M, Reunanen A, Tuomilehto J. Increasing incidence of childhood-onset Type 1 diabetes in 3 Baltic countries and Finland 1983-1998. *Diabetologia.* 2001 Oct;44 Suppl 3:B17-20.

Pozzilli P, Manfrini S, Crinò A, Picardi A, Leomanni C, Cherubini V, Valente L, Khazrai M, Visalli N; IMDIAB group. Low levels of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 in patients with newly diagnosed type 1 diabetes. *Horm Metab Res.* 2005 Nov;37(11):680-3.

Prabhu AV, Luu W, Li D, Sharpe LJ, Brown AJ. DHCR7: A vital enzyme switch between cholesterol and vitamin D production. *Prog Lipid Res.* 2016 Oct;64:138-151.

Prabhu AV, Sharpe LJ, Brown AJ. The sterol-based transcriptional control of human 7-dehydrocholesterol reductase (DHCR7): Evidence of a cooperative regulatory program in cholesterol synthesis. *Biochim Biophys Acta.* 2014 Oct;1842(10):1431-9.

Quercia S, Candela M, Giuliani C, Turrone S, Luiselli D, Rampelli S, Brigidi P, Franceschi C, Bacalini MG, Garagnani P, Pirazzini C. From lifetime to evolution: timescales of human gut microbiota adaptation. *Front Microbiol.* 2014 Nov 4;5:587.

Raab J, Giannopoulou EZ, Schneider S, Warncke K, Krasmann M, Winkler C, Ziegler AG. Prevalence of vitamin D deficiency in pre-type 1 diabetes and its association with disease progression. *Diabetologia* 2014;57(5):902-908.

Ramagopalan SV, Maugeri NJ, Handunnetthi L, Lincoln MR, Orton SM, Dymment DA, Deluca GC, Herrera BM, Chao MJ, Sadovnick AD, Ebers GC, Knight JC. Expression of the multiple sclerosis-associated MHC class II Allele HLA-DRB1\*1501 is regulated by vitamin D. *PLoS Genet* 2009; 5: e1000369.

Raulio S, Erlund I, Männistö S, Sarlio-Lähteenkorva S, Sundvall J, Tapanainen H, Vartiainen E, Virtanen SM. Successful nutrition policy: improvement of vitamin D intake and status in Finnish adults over the last decade. *The European Journal of Public Health* DOI: <http://dx.doi.org/10.1093/eurpub/ckw154> ckw154 First published online: 10 October 2016

Redondo MJ, Jeffrey J, Fain PR, Eisenbarth GS, Orban T. Concordance for islet autoimmunity among monozygotic twins. *N Engl J Med.* 2008 Dec 25;359(26):2849-50.

Rees JR, Mott LA, Barry EL, Baron JA, Bostick RM, Figueiredo JC, Bresalier RS, Robertson DJ, Peacock JL. Lifestyle and Other Factors Explain One-Half of the Variability in the Serum 25-Hydroxyvitamin D Response to Cholecalciferol Supplementation in Healthy Adults. *J Nutr.* 2016 Nov;146(11):2312-2324.

Reid D, Toole BJ, Knox S, Talwar D, Harten J, O'Reilly DS, Blackwell S, Kinsella J, McMillan DC, Wallace AM. The relation between acute changes in the systemic inflammatory response and plasma 25-hydroxyvitamin D

concentrations after elective knee arthroplasty. *Am J Clin Nutr.* 2011 May;93(5):1006-11.

Reinert-Hartwall L, Honkanen J, Härkönen T, Ilonen J, Simell O, Peet A, Tillmann V, Lamberg-Allardt C, Virtanen SM, Knip M, Vaarala O; DIABIMMUNE Study Group. No association between vitamin D and  $\beta$ -cell autoimmunity in Finnish and Estonian children. *Diabetes Metab Res Rev.* 2014 Nov;30(8):749-60.

Rewers M, Ludvigsson J. Environmental risk factors for type 1 diabetes. *Lancet.* 2016 Jun 4;387(10035):2340-8

Roberfroid M. Prebiotics: the concept revisited. *J Nutr.* 2007 Mar;137(3 Suppl 2):830S-7S.

Robinson J, Halliwell JA, McWilliam H, Lopez R, Parham P, Marsh SG. The IMGT/HLA database. *Nucleic Acids Res.* 2013 Jan;41(Database issue):D1222-7.

Rogers NL, Cole SA, Lan HC, Crossa A, Demerath EW. New saliva DNA collection method compared to buccal cell collection techniques for epidemiological studies. *Am J Hum Biol.* 2007 May-Jun;19(3):319-26.

Roldán MB, Alonso M, Barrio R. Thyroid autoimmunity in children and adolescents with Type 1 diabetes mellitus. *Diabetes Nutr Metab.* 1999 Feb;12(1):27-31.

Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, Jones G, Kovacs CS, Mayne ST, Rosen CJ, Shapses SA. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab.* 2011 Jan;96(1):53-8.

Roth R, Lynch K, Lernmark B, Baxter J, Simell T, Smith L, Swartling U, Ziegler AG, Johnson SB; TEDDY Study Group. Maternal anxiety about a child's diabetes risk in the TEDDY study: the potential role of life stress, postpartum depression, and risk perception. *Pediatr Diabetes.* 2015 Jun;16(4):287-98.

Rothwell PM, Staines A, Smail P, Wadsworth E, McKinney P. Seasonality of birth of patients with childhood diabetes in Britain. *BMJ.* 1996 Jun 8;312(7044):1456-7.

Rønningen KS, Keiding N, Green A. Correlations between the incidence of childhood-onset type I diabetes in Europe and HLA genotypes. *Diabetologia.* 2001;44 Suppl 3:B51-9.

Saad K, Abdel-Rahman AA, Elserogy YM, Al-Atram AA, El-Houfey AA, Othman HA, Bjørklund G, Jia F, Urbina MA, Abo-Elela MG, Ahmad FA, Abd El-Baseer KA, Ahmed AE, Abdel-Salam AM. Randomized controlled trial of vitamin D supplementation in children with autism spectrum disorder. *J Child Psychol Psychiatry.* 2016 Nov 21. doi: 10.1111/jcpp.12652. [Epub ahead of print]

Saccone D, Asani F, Bornman L. Regulation of the vitamin D receptor gene by environment, genetics and epigenetics. *Gene.* 2015 May 1;561(2):171-80.

Samuelsson U, Carstensen J, Löfman O, Nordfeldt S. Seasonal variation in the diagnosis of type 1 diabetes in south-east Sweden. *Diabetes Res Clin Pract.* 2007 Apr;76(1):75-81.

Saraf R, Morton SM, Camargo CA Jr, Grant CC. Global summary of maternal and newborn vitamin D status - a systematic review. *Matern Child Nutr.* 2016 Oct;12(4):647-68

Schive SW, Foss A, Sahraoui A, Kloster-Jensen K, Hafsahl G, Kvalheim G, Lundgren T, von Zur-Mühlen B, Felldin M, Rafael E, Lempinen M, Korsgren O, Jenssen TG, Mishra V, Scholz H. Cost and clinical outcome of islet transplantation in Norway 2010-2015. *Clin Transplant.* 2017 Jan;31(1).

Schlundt J. "Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria". Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. FAO / WHO. 2012.

Schuster I. Cytochromes P450 are essential players in the vitamin D signaling system. *Biochim Biophys Acta*. 2011 Jan;1814(1):186-99.

Shahbazi M, Jeddi-Tehrani M, Zareie M, Salek-Moghaddam A, Akhondi MM, Bahmanpoor M, Sadeghi MR, Zarnani AH. Expression profiling of vitamin D receptor in placenta, decidua and ovary of pregnant mice. *Placenta*. 2011 Sep;32(9):657-64.

Shea MK, Benjamin EJ, Dupuis J, Massaro JM, Jacques PF, D'Agostino RB Sr, Ordovas JM, O'Donnell CJ, Dawson-Hughes B, Vasan RS, Booth SL. Genetic and non-genetic correlates of vitamins K and D. *Eur J Clin Nutr*. 2009 Apr;63(4):458-64.

Shiina T, Hosomichi K, Inoko H, Kulski JK. The HLA genomic loci map: expression, interaction, diversity and disease. *J Hum Genet*. 2009 Jan;54(1):15-39.

Sidney J, Peters B, Frahm N, Brander C, Sette A. HLA class I supertypes: a revised and updated classification. *BMC Immunol*. 2008 Jan 22;9:1.

Sigmundsdottir H, Pan J, Debes GF, Alt C, Habtezion A, Soler D, Butcher EC. DCs metabolize sunlight-induced vitamin D<sub>3</sub> to 'program' T cell attraction to the epidermal chemokine CCL27. *Nat Immunol*. 2007 Mar;8(3):285-93.

Siljander HT, Simell S, Hekkala A Lähde J, Simell T, Vähäsalo P, Veijola R, Ilonen J, Simell O, Knip M. Predictive characteristics of diabetes-associated autoantibodies among children with HLA-conferred disease susceptibility in the general population. *Diabetes*. 2009 Dec; 58(12): 2835–2842.

Simpson M, Brady H, Yin X, Seifert J, Barriga K, Hoffman M, Bugawan T, Barón AE, Sokol RJ, Eisenbarth G, Erlich H, Rewers M, Norris JM. No association of vitamin D intake or 25-hydroxyvitamin D levels in childhood with risk of islet autoimmunity and type 1 diabetes: the Diabetes Autoimmunity Study in the Young (DAISY). *Diabetologia*. 2011;54(11):2779–2788.

Skaaby T, Husemoen LL, Thuesen BH, Pisinger C, Hannemann A, Jørgensen T, Linneberg A. Longitudinal associations between lifestyle and vitamin D: A general population study with repeated vitamin D measurements. *Endocrine*. 2016 Feb;51(2):342-50.

Sloka S, Grant M, Newhook LA. Time series analysis of ultraviolet B radiation and type 1 diabetes in Newfoundland. *Pediatr Diabetes*. 2008 Apr;9(2):81-6.

Snellman G, Melhus H, Gedeberg R, Byberg L, Berglund L, Wernroth L, Michaëlsson K. Determining vitamin D status: a comparison between commercially available assays. *PLoS One*. 2010 Jul 13;5(7):e11555.

Somersalo O. Studies of childhood diabetes. I. Incidence in Finland. *Ann Paediatr Fenn* 1954-1955;1(3):239-49.

Steck AK, Armstrong TK, Babu SR, Eisenbarth GS; Type 1 Diabetes Genetics Consortium. Stepwise or linear decrease in penetrance of type 1 diabetes with lower-risk HLA genotypes over the past 40 years. *Diabetes*. 2011 Mar;60(3):1045-9.

Steck AK, Vehik K, Bonifacio E, Lernmark A, Ziegler AG, Hagopian WA, She J, Simell O, Akolkar B, Krischer J, Schatz D, Rewers MJ; TEDDY Study Group. Predictors of Progression From the Appearance of Islet Autoantibodies to Early Childhood Diabetes: The Environmental

Determinants of Diabetes in the Young (TEDDY). *Diabetes Care*. 2015 May;38(5):808-13.

Stene LC, Joner G; Norwegian Childhood Diabetes Study Group. Use of cod liver oil during the first year of life is associated with lower risk of childhood-onset type 1 diabetes: a large, population-based, case-control study. *Am J Clin Nutr*. 2003 Dec;78(6):1128-34.

Stene LC, Ulriksen J, Magnus P and Joner G. Use of cod-liver oil during pregnancy associated with lower risk of Type 1 diabetes in the offspring. *Diabetologia*. 2000 Sep;43(9):1093-8.

Strom TB, Roy-Chaudury R, Manfro R, Zheng XX, Nickerson PW, Wood K, Bushell A. The Th1/Th2 paradigm and allograft response. *Curr Opin Immunol*. 1996;8:688-93.

Söderström U, Aman J, Hjern A. Being born in Sweden increases the risk for type 1 diabetes—a study of migration of children to Sweden as a natural experiment. *Acta Paediatr*. 2012 Jan;101(1):73-7

Sørensen IM, Joner G, Jennum PA, Eskild A, Torjesen PA, Stene LC. Maternal serum levels of 25-hydroxy-vitamin D during pregnancy and risk of type 1 diabetes in the offspring. *Diabetes*. 2012 Jan;61(1):175-8.

Taylor AE, Morris RW, Fluharty ME, Bjorngaard JH, Åsvold BO, Gabrielsen ME, Campbell A, Marioni R, Kumari M, Hällfors J, Männistö S, Marques-Vidal P, Kaakinen M, Cavadino A, Postmus I, Husemoen LL, Skaaby T, Ahluwalia TS, Treur JL, Willemsen G, Dale C, Wannamethee SG, Lahti J, Palotie A, Rääkkönen K, Kisiailiou A, McConnachie A, Padmanabhan S, Wong A, Dalgård C, Paternoster L, Ben-Shlomo Y, Tyrrell J, Horwood J, Fergusson DM, Kennedy MA, Frayling T, Nohr EA, Christiansen L, Ohm Kyvik K, Kuh D, Watt G, Eriksson J, Whincup PH, Vink JM, Boomsma DI, Davey Smith G, Lawlor D, Linneberg A, Ford I, Jukema JW, Power C, Hyppönen E, Jarvelin MR, Preisig M, Borodulin K, Kaprio J, Kivimäki M, Smith BH, Hayward C, Romundstad PR, Sørensen TI, Munafò MR, Sattar N. Stratification by smoking status reveals an association of CHRNA5-A3-B4 genotype with body mass index in never smokers. *PLoS Genet* 2014; 10: e1004799.

Thraikill KM, Fowlkes JL. The role of vitamin D in the metabolic homeostasis of diabetic bone. *Clin Rev Bone Miner Metab*. 2013 Mar 1;11(1):28-37.

Tizaoui K, Kaabachi W, Hamzaoui A, Hamzaoui K. Contribution of VDR polymorphisms to type 1 diabetes susceptibility: Systematic review of case-control studies and meta-analysis. *J Steroid Biochem Mol Biol*. 2014 Sep;143:240-9.

Toriola AT, Surcel HM, Agborsangaya C, Grankvist K, Tuohimaa P, Toniolo P, Lukanova A, Pukkala E, Lehtinen M. Serum 25-hydroxyvitamin D and the risk of ovarian cancer. *Eur J Cancer*. 2010 Jan;46(2):364-9.

Tsai S, Santamaria P. MHC Class II Polymorphisms, Autoreactive T-Cells, and Autoimmunity. *Front Immunol*. 2013 Oct 10;4:321.

Tuomilehto J, Karvonen M, Pitkaniemi J, Virtala E, Kohtamäki K, Toivanen L, Tuomilehto-Wolf E. Record-high incidence of Type I (insulin-dependent) diabetes mellitus in Finnish children. The Finnish Childhood Type I Diabetes Registry Group. *Diabetologia*. 1999 Jun;42(6):655-60.

Tuomilehto J. The emerging global epidemic of type 1 diabetes. *Curr Diab Rep*. 2013 Dec;13(6):795-804.

Uitterlinden AG, Fang Y, Van Meurs JB, Pols HA, Van Leeuwen JP. Genetics and biology of vitamin D receptor polymorphisms. *Gene*. 2004 Sep 1;338(2):143-56.

Uusitalo U, Liu X, Yang J, Aronsson CA, Hummel S, Butterworth M, Lernmark Å, Rewers M, Hagopian W, She JX, Simell O, Toppari J, Ziegler AG, Akolkar B, Krischer J, Norris JM, Virtanen SM; TEDDY Study Group.

Association of Early Exposure of Probiotics and Islet Autoimmunity in the TEDDY Study. *JAMA Pediatr.* 2016 Jan;170(1):20-8.

Valle T ja työryhmä. Diabeetikkojen hoitotasapaino Suomessa vuosina 2009-2010. *DEHKO-raportti 2010*;5 (in Finnish).

Vaiserman AM, Carstensen B, Voitenko VP, Tronko MD, Kravchenko VI, Khalangot MD, Mechova LV. Seasonality of birth in children and young adults (0-29 years) with type 1 diabetes in Ukraine. *Diabetologia.* 2007 Jan;50(1):32-5.

van der Knaap NJ, El Marroun H, Klumpers F, Mous SE, Jaddoe VW, Hofman A, Homberg JR, White T, Tiemeier H, Fernández G. Beyond classical inheritance: the influence of maternal genotype upon child's brain morphology and behavior. *J Neurosci.* 2014 Jul 16;34(29):9516-21.

Vehik K, Hamman RF, Lezotte D, Norris JM, Klingensmith G, Bloch C, Rewers M, Dabelea D. Increasing incidence of type 1 diabetes in 0- to 17-year-old Colorado youth. *Diabetes Care.* 2007 Mar;30(3):503-9.

Vehik K, Lynch KF, Schatz DA, Akolkar B, Hagopian W, Rewers M, She JX, Simell O, Toppari J, Ziegler AG, Lernmark Å, Bonifacio E, Krischer JP; TEDDY Study Group. Reversion of  $\beta$ -Cell Autoimmunity Changes Risk of Type 1 Diabetes: TEDDY Study. *Diabetes Care.* 2016 Sep;39(9):1535-42.

Viljakainen HT, Saarnio E, Hytinantti T, Miettinen M, Surcel H, Mäkitie O, Andersson S, Laitinen K, Lamberg-Allardt C. Maternal vitamin D status determines bone variables in the newborn. *J Clin Endocrinol Metab.* 2010 Apr;95(4):1749-57.

Virtanen JK, Nurmi T, Voutilainen S, Mursu J, Tuomainen TP. Association of serum 25-hydroxyvitamin D with the risk of death in a general older population in Finland. *Eur J Nutr.* 2011 Aug;50(5):305-12.

Viskari H, Ludvigsson J, Uibo R, Salur L, Marciulionyte D, Hermann R, Soltész G, Fuchtenbusch M, Ziegler AG, Kondrashova A, Romanov A, Kaplan B, Laron Z, Koskela P, Vesikari T, Huhtala H, Knip M, Hyöty H. Relationship between the incidence of type 1 diabetes and maternal enterovirus antibodies: time trends and geographical variation. *Diabetologia.* 2005 Jul;48(7):1280-7.

Voipio AJ, Pahkala KA, Viikari JS, Mikkilä V, Magnussen CG, Hutri-Kähönen N, Kähönen M, Lehtimäki T, Männistö S, Loo BM, Jula A, Marniemi J, Juonala M, Raitakari OT. Determinants of serum 25(OH)D concentration in young and middle-aged adults. The Cardiovascular Risk in Young Finns Study. *Ann Med.* 2015 May;47(3):253-62.

Vukusic S, Hutchinson M, Hours M, Moreau T, Cortinovis-Tourniaire P, Adeleine P, Confavreux C; Pregnancy In Multiple Sclerosis Group. Pregnancy and multiple sclerosis (the PRIMS study): clinical predictors of post-partum relapse. *Brain.* 2004 Jun;127(Pt 6):1353-60.

Wang Y, He D, Ni C, Zhou H, Wu S, Xue Z, Zhou Z. Vitamin D induces autophagy of pancreatic  $\beta$ -cells and enhances insulin secretion. *Mol Med Rep.* 2016 Sep;14(3):2644-50.

Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, Kiel DP, Streeten EA, Ohlsson C, Köller DL, Peltonen L, Cooper JD, O'Reilly PF, Houston DK, Glazer NL, Vandenput L, Peacock M, Shi J, Rivadeneira F, McCarthy MI, Anneli P, de Boer IH, Mangino M, Kato B, Smyth DJ, Booth SL, Jacques PF, Burke GL, Goodarzi M, Cheung CL, Wolf M, Rice K, Goltzman D, Hidiroglou N, Ladouceur M, Wareham NJ, Hocking LJ, Hart D, Arden NK, Cooper C, Malik S, Fraser WD, Hartikainen AL, Zhai G, Macdonald HM, Forouh NG, Loos RJ, Reid DM, Hakim A, Dennison E, Liu Y, Power C, Stevens HE, Jaana L, Vasani RS, Soranzo N, Bojunga J, Psaty BM, Lorentzon M, Forouf T, Harris TB, Hofman A, Jansson JO, Cauley JA, Uitterlinden AG, Gibson Q, Järvelin MR, Karasik D, Siscovick DS, Econs MJ, Kritchevsky SB, Florez JC, Todd JA, Dupuis J, Hyppönen E, Spector TD.

Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet*. 2010 Jul 17;376(9736):180-8.

Weber DR, Haynes K, Leonard MB, Willi SM, Denburg MR. Type 1 diabetes is associated with an increased risk of fracture across the life span: a population-based cohort study using The Health Improvement Network (THIN). *Diabetes Care*. 2015 Oct;38(10):1913-20.

Weber DR, Schwartz G. Epidemiology of Skeletal Health in Type 1 Diabetes. *Curr Osteoporos Rep*. 2016 Dec;14(6):327-336.

Whittemore R, Jaser S, Chao A, Jang M, Grey M. Psychological experience of parents of children with type 1 diabetes: a systematic mixed-studies review. *Diabetes Educ*. 2012 Jul-Aug;38(4):562-79

Winkler TW, Justice AE, Graff M, Barata L, Feitosa MF, Chu S, Czajkowski J, Esko T, Fall T, Kilpeläinen TO, Lu Y, Mägi R, Mihailov E, Pers TH, Rieger S, Teumer A, Ehret GB, Ferreira T, Heard-Costa NL, Karjalainen J, Lagou V, Mahajan A, Neinast MD, Prokopenko I, Simino J, Teslovich TM, Jansen R, Westra HJ, White CC, Absher D, Ahluwalia TS, Ahmad S, Albrecht E, Alves AC, Bragg-Gresham JL, de Craen AJ, Bis JC, Bonnefond A, Boucher G, Cadby G, Cheng YC, Chiang CW, Delgado G, Demirkan A, Dueker N, Eklund N, Eiriksdottir G, Eriksson J, Feenstra B, Fischer K, Frau F, Galesloot TE, Geller F, Goel A, Gorski M, Grammer TB, Gustafsson S, Haitjema S, Hottenga JJ, Huffman JE, Jackson AU, Jacobs KB, Johansson Å, Kaakinen M, Kleber ME, Lahti J, Mateo Leach I, Lehne B, Liu Y, Lo KS, Lorentzon M, Luan J, Madden PA, Mangino M, McKnight B, Medina-Gomez C, Monda KL, Montasser ME, Müller G, Müller-Nurasyid M, Nolte IM, Panoutsopoulou K, Pascoe L, Paternoster L, Rayner NW, Renström F, Rizzi F, Rose LM, Ryan KA, Salo P, Sanna S, Scharnagl H, Shi J, Smith AV, Southam L, Stancáková A, Steinthorsdottir V, Strawbridge RJ, Sung YJ, Tachmazidou I, Tanaka T, Thorleifsson G, Trompet S, Pervjakova N, Tyrer JP, Vandenput L, van der Laan SW, van der Velde N, van Setten J, van Vliet-Ostaptchouk JV, Verweij N, Vlachopoulou E, Waite LL, Wang SR, Wang Z, Wild SH, Willenborg C, Wilson JF, Wong A, Yang J, Yengo L, Yerges-Armstrong LM, Yu L, Zhang W, Zhao JH, Andersson EA, Bakker SJ, Baldassarre D, Banasik K, Barcella M, Barlassina C, Bellis C, Benaglio P, Blangero J, Blüher M, Bonnet F, Bonnycastle LL, Boyd HA, Bruinenberg M, Buchman AS, Campbell H, Chen YD, Chines PS, Claudi-Boehm S, Cole J, Collins FS, de Geus EJ, de Groot LC, Dimitriou M, Duan J, Enroth S, Eury E, Farmaki AE, Forouhi NG, Friedrich N, Gejman PV, Gigante B, Glorioso N, Go AS, Gottesman O, Gräßler J, Grallert H, Grarup N, Gu YM, Broer L, Ham AC, Hansen T, Harris TB, Hartman CA, Hassinen M, Hastie N, Hattersley AT, Heath AC, Henders AK, Hernandez D, Hillege H, Holmen O, Hovingh KG, Hui J, Husemoen LL, Hutri-Kähönen N, Hysi PG, Illig T, De Jager PL, Jalilzadeh S, Jørgensen T, Jukema JW, Juonala M, Kanoni S, Karaleftheri M, Khaw KT, Kinnunen L, Kittner SJ, Koenig W, Kolcic I, Kovacs P, Krarup NT, Kratzer W, Krüger J, Kuh D, Kumari M, Kyriakou T, Langenberg C, Lannfelt L, Lanzani C, Lotay V, Launer LJ, Leander K, Lindström J, Linneberg A, Liu YP, Lobbens S, Luben R, Lyssenko V, Männistö S, Magnusson PK, McArdle WL, Menni C, Merger S, Milani L, Montgomery GW, Morris AP, Narisu N, Nelis M, Ong KK, Palotie A, Pérusse L, Pichler I, Pilia MG, Pouta A, Rheinberger M, Ribel-Madsen R, Richards M, Rice KM, Rice TK, Rivolta C, Salomaa V, Sanders AR, Sarzynski MA, Scholtens S, Scott RA, Scott WR, Sebert S, Sengupta S, Sennblad B, Seufferlein T, Silveira A, Slagboom PE, Smit JH, Sparsø TH, Stirrups K, Stolk RP, Stringham HM, Swertz MA, Swift AJ, Syvänen AC, Tan ST, Thorand B, Tönjes A, Tremblay A, Tsafantakis E, van der Most PJ, Völker U, Vohl MC, Vonk JM, Waldenberger M, Walker RW, Wennauer R, Widén E, Willemssen G, Wilsgaard T, Wright AF, Zillikens MC, van Dijk SC, van Schoor NM, Asselbergs FW, de Bakker PI, Beckmann JS, Beilby J, Bennett DA, Bergman

RN, Bergmann S, Böger CA, Boehm BO, Boerwinkle E, Boomsma DI, Bornstein SR, Bottinger EP, Bouchard C, Chambers JC, Chanock SJ, Chasman DI, Cucca F, Cusi D, Dedoussis G, Erdmann J, Eriksson JG, Evans DA, de Faire U, Farrall M, Ferrucci L, Ford I, Franke L, Franks PW, Froguel P, Gansevoort RT, Gieger C, Grönberg H, Gudnason V, Gyllensten U, Hall P, Hamsten A, van der Harst P, Hayward C, Heliövaara M, Hengstenberg C, Hicks AA, Hingorani A, Hofman A, Hu F, Huikuri HV, Hveem K, James AL, Jordan JM, Julia A, Kähönen M, Kajantie E, Kathiresan S, Kiemeny LA, Kivimäki M, Knekt PB, Koistinen HA, Kooner JS, Koskinen S, Kuusisto J, Maerz W, Martin NG, Laakso M, Lakka TA, Lehtimäki T, Lettre G, Levinson DF, Lind L, Lokki ML, Mäntyselkä P, Melbye M, Metspalu A, Mitchell BD, Moll FL, Murray JC, Musk AW, Nieminen MS, Njølstad I, Ohlsson C, Oldehinkel AJ, Oostra BA, Palmer LJ, Pankow JS, Pasterkamp G, Pedersen NL, Pedersen O, Penninx BW, Perola M, Peters A, Polašek O, Pramstaller PP, Psaty BM, Qi L, Quertermous T, Raitakari OT, Rankinen T, Rauramaa R, Ridker PM, Rioux JD, Rivadeneira F, Rotter JI, Rudan I, den Ruijter HM, Saltevo J, Sattar N, Schunkert H, Schwarz PE, Shuldiner AR, Sinisalo J, Snieder H, Sørensen TI, Spector TD, Staessen JA, Stefania B, Thorsteinsdóttir U, Stumvoll M, Tardif JC, Tremoli E, Tuomilehto J, Uitterlinden AG, Uusitupa M, Verbeek AL, Vermeulen SH, Viikari JS, Vitart V, Völzke H, Vollenweider P, Waeber G, Walker M, Wallaschofski H, Wareham NJ, Watkins H, Zeggini E; CHARGE Consortium.; DIAGRAM Consortium.; GLGC Consortium.; Global-BPGen Consortium.; ICBP Consortium.; MAGIC Consortium., Chakravarti A, Clegg DJ, Cupples LA, Gordon-Larsen P, Jaquish CE, Rao DC, Abecasis GR, Assimes TL, Barroso I, Berndt SI, Boehnke M, Deloukas P, Fox CS, Groop LC, Hunter DJ, Ingelsson E, Kaplan RC, McCarthy MI, Mohlke KL, O'Connell JR, Schlessinger D, Strachan DP, Stefansson K, van Duijn CM, Hirschhorn JN, Lindgren CM, Heid IM, North KE, Borecki IB, Kutalik Z, Loos RJ. The Influence of Age and Sex on Genetic Associations with Adult Body Size and Shape: A Large-Scale Genome-Wide Interaction Study. *PloS Genet* 2015; 11: e1005378.

Xiang F, Lucas R, de Gruijl F, Norval M. A systematic review of the influence of skin pigmentation on changes in the concentrations of vitamin D and 25-hydroxyvitamin D in plasma/serum following experimental UV irradiation. *Photochem Photobiol Sci.* 2015 Dec;14(12):2138-46.

Ylppö A. Lastentautien erikoisuuksia Suomessa, etupäässä ilmaston vaikutuksia silmälläpitäen. *Duodecim* 1925;11: 653-65 (in Finnish).

Yoon JW, Jun HS. Viruses cause type 1 diabetes in animals. *Ann N Y Acad Sci.* 2006 Oct;1079:138-46.

Zajicková K, Krepelová A, Zofková I. A single nucleotide polymorphism under the reverse primer binding site may lead to BsmI mis-genotyping in the vitamin D receptor gene. *J Bone Miner Res.* 2003 Oct;18(10):1754-7.

Zella JB, McCary LC, DeLuca HF. Oral administration of 1,25-dihydroxyvitamin D(3) completely protects NOD mice from insulin-dependent diabetes mellitus. *Arch Biochem Biophys* 2003; 417: 77-80.

Zerwekh JE. The measurement of vitamin D: analytical aspects. *Ann Clin Biochem.* 2004 Jul;41(Pt 4):272-81

Zhang J, Li W, Liu J, Wu W, Ouyang H, Zhang Q, Wang Y, Liu L, Yang R, Liu X, Meng Q, Lu J. Polymorphisms in the vitamin D receptor gene and type 1 diabetes mellitus risk: an update by meta-analysis. *Mol Cell Endocrinol.* 2012 May 15;355(1):135-42.

Zhang H, Wu H, Liu L, Li H, Shih DQ, Zhang X. 1,25-dihydroxyvitamin D3 regulates the development of chronic colitis by modulating both T helper (Th)1 and Th17 activation. *APMIS.* 2015 Jun;123(6):490-501.



Ziegler AG, Bonifacio E, Powers AC, Todd JA, Harrison LC, Atkinson MA. Type 1 Diabetes Prevention: A Goal Dependent on Accepting a Diagnosis of an Asymptomatic Disease. *Diabetes*. 2016 Nov;65(11):3233-3239.

Ziegler AG, Rewers M, Simell O, Simell T, Lempainen J, Steck A, Winkler C, Ilonen J, Veijola R, Knip M, Bonifacio E, Eisenbarth GS. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. *JAMA*. 2013 Jun 19;309(23):2473-9.

Zivkovic AM, German JB, Lebrilla CB, Mills DA. Human milk glycobiome and its impact on the infant gastrointestinal microbiota. *Proc Natl Acad Sci U S A*. 2011 Mar 15;108 Suppl 1:4653-8.

*references*