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To my family

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ABSTRACT

The aims of this study were to get an overall view of 25-hydroxyvitamin D (25[OH]D) concentrations in healthy Finnish children, to find the major factors influencing the levels, and to find if there are differences in 25(OH) concentrations already at birth or later in childhood between children who develop islet autoimmunity (IA) or type 1 diabetes (T1D) and autoantibody negative children.

The study population comprised of children born in 1994-2004 participating in Type 1 Diabetes Prediction and Prevention study (DIPP) clinics in Turku, Oulu and Tampere University Hospitals, Finland.

In this study we showed there was a marked increase in the 25(OH)D concentrations in healthy children, who were over the age of two years, in year 2003. Seasonal variation was significant, and it remained the same after year 2003. A large-scale program for vitamin D fortification of dairy products and other foodstuffs was begun in Finland in 2003.

The median 25(OH)D concentrations were lowest at birth and highest at the age of six months, and they were not associated with the development of IA or T1D during childhood in follow-up lasting up to 14 years. The concentrations were associated with geographical location, sampling year and month, age of the child and for the samples taken at birth also with maternal age and maternal intake of vitamin D during pregnancy.

KEYWORDS: type 1 diabetes, serum, 25-hydroxyvitamin D, vitamin D, islet autoimmunity, pregnancy, neonate, infant, child, Finland

TURUN YLIOPISTO
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Lastentautioppi
Lasten ja nuorten klinikka
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TIIVISTELMÄ

Tutkimuksen tavoitteina oli luoda yleiskatsaus terveiden suomalaislasten seerumin 25-hydroksi-D-vitamiinin (25[OH]D) pitoisuuksista, pitoisuuksiin vaikuttavien tekijöiden kartoittaminen, ja sen selvittäminen eroavatko 25(OH)D pitoisuudet syntymähetkellä tai myöhemmin lapsuudessa niillä lapsilla, joille kehittyy saarekesoluvasta-aineita (IA) tai jotka sairastuvat tyypin 1 diabetekseen (T1D), kun heitä verrataan vasta-ainenegatiivisiin lapsiin.

Työssä tutkittiin vuosina 1994 - 2004 syntyneitä lapsia, jotka osallistuivat tyypin 1 diabeteksen ehkäisemiseen ja ennustamiseen tähtäävään DIPP-tutkimukseen Turun, Oulun ja Tampereen yliopistosairaaloissa.

Terveillä yli kaksivuotiailla lapsilla seerumin 25(OH)D pitoisuudet olivat merkittävästi korkeammat vuodesta 2003 alkaen kuin sitä ennen. Vuoden-aikavaihtelu oli huomattavaa ja pysyi samanlaisena vuoden 2003 jälkeen. Vuonna 2003 Suomessa aloitettiin maitotuotteiden ja muutamien muiden elintarvikkeiden laajamittainen D-vitaminointiohjelma.

Tutkimuksessa havaittiin, että seerumin 25(OH)D-mediaanipitoisuudet ovat alimmillaan syntymähetkellä ja korkeammillaan puolivuotiailla lapsilla, mutta pitoisuudet eivät assosioituneet IA:n tai T1D:n kehittymiseen lapsilla. Pitoisuuksiin vaikuttivat maantieteellinen sijainti, näytteenottovuosi ja –kuukausi, lapsen ikä sekä syntymäpitoisuuteen myös äidin ikä ja raskaudenaikainen D-vitamiininsaanti.

AVAINSANAT: tyypin 1 diabetes, seerumi, 25-hydroksi-D-vitamiini, D-vitamiini, raskaus, vastasyntynyt, lapsi, lastentaudit, Suomi

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Abbreviations

$1,25(OH)_2D$	1,25-dihydroxyvitamin D, calcitriol
7-DHC	7-dehydrocholesterol
25(OH)D	25-hydroxyvitamin D, calcidiol
BMI	body mass index
CI	confidence interval
CV	coefficient of variability
DASP	Diabetes Autoantibody Standardization Program
DBP	vitamin D binding protein
DEQAS	The External Vitamin D Assessment Scheme
DIPP	Type 1 Diabetes Prediction and Prevention study
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
GADA	antibodies to 65 kilodalton isoform of glutamic acid decarboxylase
HLA	human leukocyte antigen
IA	islet autoimmunity
IA-2A	antibodies to the protein tyrosine phosphatase related IA-2 protein
IAA	insulin autoantibodies
ICA	islet cell autoantibodies
IQR	inter-quartile range
IU	international unit
IVGTT	intravenous glucose tolerance test
JDFU	Juvenile Diabetes Foundation units
PCR	polymerase chain reaction
PTH	parathyroid hormone
RU	relative units
SD	standard deviation
SNP	single nucleotide polymorphism
T1D	type 1 diabetes
TEDDY	The Environmental Determinants of Diabetes in the Young study
UL	upper limit for daily intake
UV	ultraviolet

VDR	vitamin D receptor
ZnT8A	antibody to pancreas-specific zinc transporter 8

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Mäkinen Marjaana, Simell Ville, Mykkänen Juha, Ilonen Jorma, Veijola Riitta, Knip Mikael, Simell Olli, Toppari Jorma, and Hermann Robert. An increase in serum 25-hydroxyvitamin D concentrations preceded a plateau in type 1 diabetes incidence in Finnish children. *Journal of Clinical Endocrinology & Metabolism*, 2014; 99(11): E2353-2356.

II Mäkinen Marjaana, Mykkänen Juha, Koskinen Maarit, Simell Ville, Veijola Riitta, Hyöty Heikki, Ilonen Jorma, Knip Mikael, Simell Olli, and Toppari Jorma. Serum 25-hydroxyvitamin D concentrations in children with HLA-conferred disease susceptibility progressing to autoimmunity and clinical type 1 diabetes. *Journal of Clinical Endocrinology & Metabolism*, 2016; 101(2):723-729.

III Mäkinen Marjaana, Löyttyniemi Eliisa, Koskinen Maarit, Vähä-Mäkilä Mari, Siljander Heli, Nurmio Mirja, Mykkänen Juha, Virtanen Suvi, Simell Olli, Hyöty Heikki, Ilonen Jorma, Knip Mikael, Veijola Riitta, and Toppari Jorma. Serum 25-hydroxyvitamin D concentrations at birth in children screened for HLA-DQB1 risk for type 1 diabetes. *Journal of Clinical Endocrinology & Metabolism*, 2019; 104(6): 2277-2285

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1 Introduction

Type 1 diabetes (T1D) is a chronic disease caused by inadequate endogenous production of insulin and characterized by impaired glucose metabolism. This severe, life-threatening disease occurs all around the world, but the highest incidence is in Finland. The reason for the high incidence is unknown as is the aetiology of the disease.

Vitamin D is known to have important roles in human health. Serum level of the storage form of vitamin D, 25-hydroxyvitamin D (25[OH]D), is the best indicator to assess vitamin D deficiency, insufficiency, hypovitaminosis, adequacy, and toxicity, since it reflects both the intake of vitamin D from food and supplements, and its synthesis from precursors in the skin after exposure to sun or other sources of ultraviolet light. However, due to the angle of the sun, no vitamin D is produced subcutaneously from October to March to the north from 37 ºN. Finland is located far up in the north, between 60 ºN and 70 ºN, and several studies have shown low serum 25(OH)D levels in the Finnish population, especially during winter.

The aim of this study was to assess the possible connection between serum 25(OH)D concentration and the risk of T1D in children.

2 Review of the Literature

2.1 Epidemiology of type 1 diabetes in children

Type 1 diabetes (T1D) is an incurable autoimmune disease caused by selective destruction of the insulin-producing β-cells in the islets of Langerhans in pancreas. It is of particular interest in Finland, where the overall incidence of T1D in children is the highest in the world and has risen to 60.9 per 100 000 people per year (Patterson et al., 2019).

The incidence of T1D has been increasing world-wide since the 1950s but the trend is finally abating in some countries with high incidences, such as Finland, where the increase in incidence levelled off in 2005 and Norway, where this happened in 2007 (Patterson et al., 2019). The incidence rate in Finland is slightly different in different age groups (Harjutsalo et. al., 2013), but the trend can be seen in all of them, as seen in Fig.1.

In Finland T1D is slightly more common in males than in females, unlike in many other countries (Diabetes Epidemiology Research International Group, 1988), but rate of increase in incidence is now higher in boys than in girls globally in 10- to 14-year age group (Patterson et al., 2019).

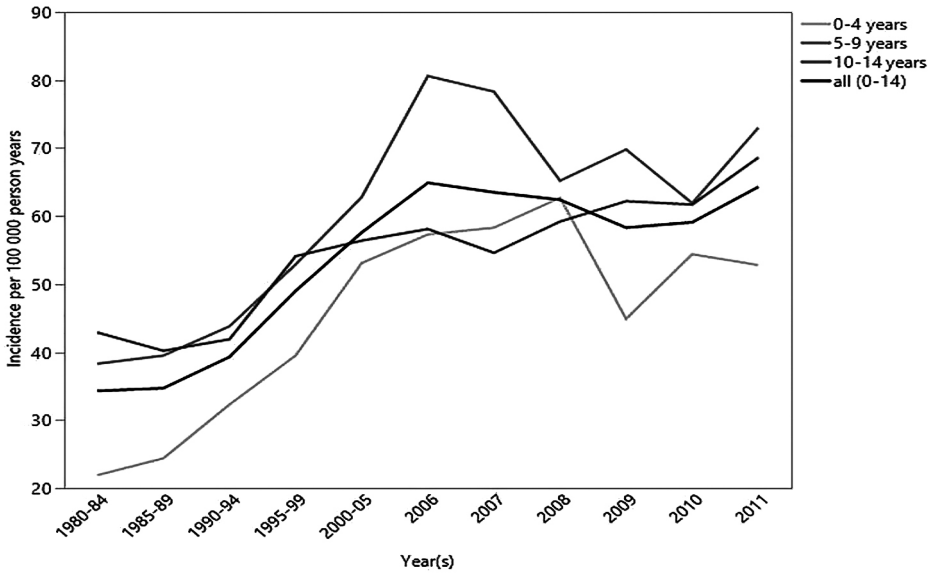

Figure 1. T1D incidence per 100 000 per year in Finnish children during 1980-2011 in different age groups (Harjutsalo et al., 2013; Harjutsalo et al., 2008): Incidence for 0-4 year-old children in light grey, for 5-9 year-olds in grey, for 10-14 year-olds in dark grey and for all age groups, 0-14 years, in black.

2.2 Natural course of T1D in children

The classical model of the natural course of type 1 diabetes presented over 30 years ago (Eisenbarth, 1986) is still essentially valid (Greenbaum et al., 2018) with its six stages: 1) genetic susceptibility, 2) triggering of autoimmunity, 3) active immunity, 4) progressive loss of glucose-stimulated insulin release, 5) overt T1D, 6) complete β-cell destruction.

2.2.1 Genetic susceptibility

The process leading to T1D in children is thought to commence with a genetic susceptibility (Davies et al., 1994). Increased genetic risk consists mainly of human leukocyte antigen (HLA) factors, so that HLA-DR/DQ region is the major determinant of the genetic risk for T1D, despite over 50 risk affecting loci have been found in other regions (Ilonen et al., 2016). A simplified classification of HLA-DQB1 genotypes used in risk assessment was to divide them into high, moderate, low and decreased risk (Veijola et al., 1996). Increased genetic risk is not an absolute requirement, as even the highest risk is found only in 22 % of the patients and 2 % of healthy controls (Ilonen et al., 2016), and some children with so called protective HLA alleles may develop T1D as well (Huopio et al., 2016). Nevertheless, having a

first-degree relative with T1D makes it 10 times more likely to develop the disease (Hippich et al., 2019).

2.2.2 Triggering of autoimmunity

Several environmental factors have been linked with the progression towards the second state, islet autoimmunity (IA). The studies are somewhat controversial, but the most prominentfactors are enteroviruses (Dotta et al., 2007), such as coxsackievirus (Sioofy-Khojine et al., 2018), and their close relatives, parechoviruses (Nilsson et al., 2013). Some early dietary factors, such as omega-3-fatty acids (Norris et al., 2007), have been associated with lower risk of IA, while others, such as cow milk components (Vaarala et al., 2012) and the age when a child was first exposed to cereals (Norris et al., 2014), or gluten (Uusitalo et al., 2018) have been implicated as potential triggers. Despite many efforts, there have been no successful interventions in preventing the development of IA (Couper et al., 2014). In some children endogenous events may be enough to initiate autoimmunity without any environmental triggers (Todd, 2010). The process begins many months or even years before any symptoms can be detected (Ziegler et al., 2013).

2.2.3 Active immunity

Islet autoimmunity can be detected in serum samples with autoantibody measurement. The first autoantibody found to have a connection with T1D was discovered already in 1970's, and it was an IgG class non-specific islet cell autoantibody (ICA) (Bottazzo et al., 1974). Biochemical islet autoantibodies were discovered later, these include insulin autoantibody (IAA) (Atkinson et al., 1986), antibody to the protein tyrosine phosphatase related IA-2 protein (IA-2A) (Bonifacio et al,. 1995), antibody to 65 kilodalton isoform of glutamic acid decarboxylase (GADA) (Baekkeskov et al., 1990) and antibody to pancreas-specific zinc transporter 8 (ZnT8A) (Wenzlau et al., 2007). Autoantibodies are produced by B cells with the help of follicular T helper cells (Serr et al., 2016). Young age at seroconversion and positivity for multiple autoantibodies are associated with high risk for disease progression (H. T. A. Siljander et al., 2009). IA is a dynamic process as both positive and inverse seroconversions may occur during follow-up (Knip et al., 2010), but risk for developing T1D remains elevated after seroconversion even if individual autoantibodies revert (Vehik et al., 2016).

2.2.4 Progressive loss of glucose-stimulated insulin release

First signs of insulitis development and advanced β-cell destruction is reduced early insulin response, detected via intravenous glucose tolerance test (IVGTT) (Koskinen et al., 2016). The velocity of the process and the severity of insulitis at disease onset are dependent on the age of the child (Rodriguez-Calvo et al., 2018).

2.2.5 Overt T1D

T1D is diagnosed when fasting blood glucose is 7.0 mmol/l or above, a random blood glucose is 11.0 mmol/l or above with symptoms of hyperglycemia, or two-hour plasma glucose level is 11.1 mmol/l or above in standardized oral glucose tolerance test (American Diabetes Association, 2018). T1D is generally characterized by insulin deficiency leading to hyperglycemia. Polydipsia and polyuria are often the first tangible symptoms of T1D, and they may begin months before marked weight-loss and debilitation, which in turn are then followed by ketoacidosis (Cahill Jr. & McDevitt, 1981) if left untreated. At the onset of T1D, there is a marked reduction of β-cells (Conget et al., 1993), but often even in long-lasting T1D some β-cells remain functional and this can be detected by measuring C-peptide (Steck et al., 2017). The higher the C-peptide level is, the more proinsulin secretion increases with meal stimulation (Sims et al., 2019).

2.2.6 Complete β-cell destruction

The β-cell loss is not linear as some β-cells are regenerated, but eventually the process will come to its final stage, and no C-peptide can be measured (Akirav et al. 2008). Some proinsulin secretion remains even after C-peptide is undetectable and the last sign of complete β-cell destruction is that proinsulin stimulation by meals is blunted (Sims et al., 2019).

2.3 Vitamin D

Vitamin D is a collective term for a family of closely related seco-steroids. As illustrated in Fig.2, 7-dehydrocholesterol (7-DHC), also known as pro-vitamin D3, undergoes photolytic conversion in the skin to pre-vitamin D_3, which then enters the bloodstream as vitamin D_3, also known as cholecalciferol. Vitamin D_3 can also be indigested from a variety of animal-based food. Other nutritional source of vitamin D are certain fungi and sprouts, which contain vitamin D_2, also known as ergocalciferol. These compounds are biologically inactive, but they are hydroxylated in the liver to 25-hydroxyvitamin D (25[OH]D), which is the storage form of this vitamin. There is no significant storage of vitamin D in the liver of mammals and

instead the storage form is rapidly released by the liver into the blood, where it circulates with a biological half-life of approximately 12-19 days. Approximately 85 % of 25(OH)D is bound to vitamin D binding protein (DBP), 15 % to albumin and 0.03 % is in free form. A small proportion of 25(OH)D that is bound to DBP becomes further hydroxylated, mainly in the kidneys, to the highly potent calciotropic hormone 1,25-dihydroxyvitamin D (1,25[OH]$_2$D). The production of 1,25(OH)$_2$D is tightly regulated and its serum half-life is only 4-6 h (Wootton, 2005). Macrophages and other cells are also capable of hydroxylating 25(OH)D into 1,25(OH)$_2$D, so that it can be produced in higher concentrations locally in the tissues (Mathieu & Badenhoop, 2005).

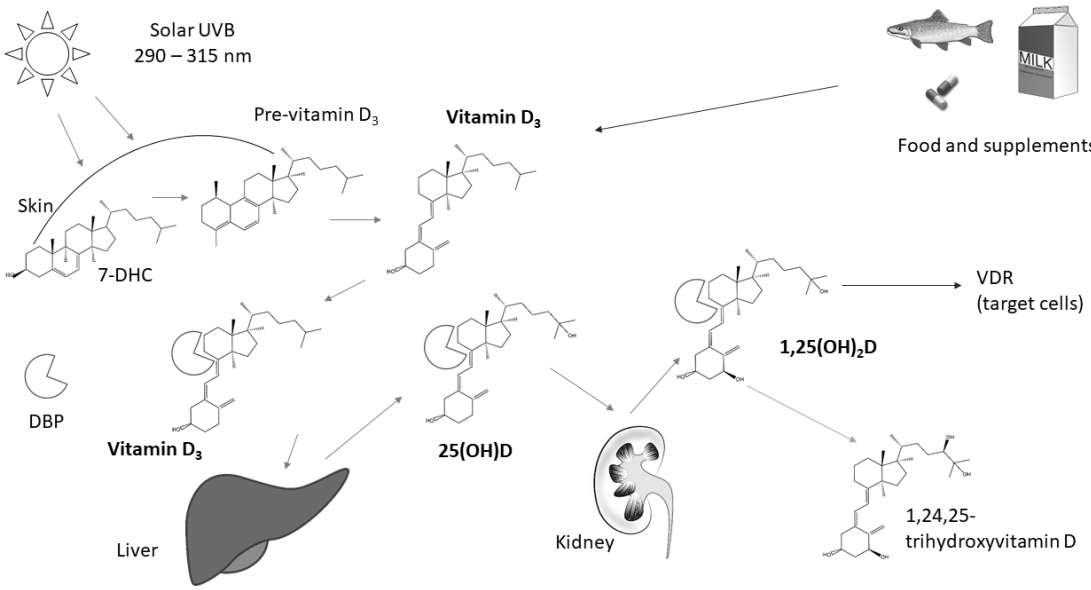

Figure 2. A simplified illustration of the vitamin D metabolism. Under ultraviolet B (UVB) radiation 7-dehydrocholesterol converts to previtamin D_3 in the skin and further to vitamin D, which can also be obtained from food and supplements. It enters the bloodstream, binds to vitamin D binding protein (DBP), is transported to the liver and converted to 25-hydroxyvitamin D (25[OH]D), which is the major circulating metabolite. Some of the 25(OH)D is transported to the kidney and hydroxylated into 1,25-dihydroxyvitamin D (1,25[OH]$_2$D, which is the active metabolite. The 1,25(OH)D then binds to vitamin D receptors (VDR) and the excess is transformed into a more soluble compound, 1,24,25-trihydroxyvitamin D (1,24,25{OH]$_3$D), which is then secreted into bile.

2.3.1 Determining 25-hydroxyvitamin D concentrations in serum samples

The knowledge related to vitamin D metabolism and its clinical importance has increased explosively during the last decades, mainly because of commercially available immunoassays. However, getting reliable 25(OH)D results has been troublesome.

Immunoassays can always be challenging (Hölzel, 1991), but the major problem in measuring 25(OH)D is attributable to the molecule itself. It is hydrophobic, exists in two forms, $25(OH)D_2$ and $25(OH)D_3$, and it is especially vulnerable to matrix effects. The matrix factors affect the ability of the binding agent, antibody, or DBP to associate with 25(OH)D in the sample or standard in equal fashion. DBP is more susceptible to these matrix effects than antibodies. (Hollis, 2004)

Marked variability was observed in serum 25(OH)D measurements between laboratories early on (Binkley et al., 2004). No central authority has determined which cut-off points to use, so that a single individual may be deemed deficient or sufficient depending on the laboratory and many laboratories use much higher cut-off points than the Institute of Medicine committee suggests (IOM, 2010). The international Vitamin D External Quality Assessment Scheme (DEQAS) was set to help verify the results obtained from different laboratories and it has been monitoring the performance of 25(OH)D assays since 1989. It has grown to comprise over 1000 registered participants in 56 countries (Binkley & Carter, 2017). Each participant receives five serum samples four times a year, measures the 25(OH)D concentrations, sends back the results and reports what method was used. The All-Laboratory Trimmed Mean (ALTM) is then calculated from all the results received and it has proven to be a good surrogate for the "true" total 25(OH)D target value produced by gas chromatography-mass spectrometry (Carter et al. 2004a). The laboratory then receives a certificate if it meets the performance target, which was initially to get 80 % of the results within ± 33 % of the ALTM (Carter et al., 2004b) and later 80 % within ± 30 % of the ALTM (Carter et al., 2010). Even though the performance target was quite wide, only 52 % of the participants met the criteria in 2000, and 59 % in 2003 (Carter et al. 2004a).

2.3.2 Sources of vitamin D

Sun is a major source of vitamin D due to ultraviolet (UV) wavelengths of 20-315 nm causing skin to produce a biologically inert precursor of vitamin D, 7-DHC, which is converted into provitamin D3 by body heat (Holick, 2009). Sun alone is not always enough, as vitamin D deficiency and rickets is not uncommon even in the sunniest of countries, such as Kenya (Jones et al., 2018). Natural sources of vitamin D include fish, meat, dairy products, eggs and certain mushrooms (Kiely &

Cashman, 2018). Not all fish, for example tuna and saithe, contain vitamin D, but many popular fish in Finland, such as pikeperch, rainbow trout, salmon, perch, baltic herring and whitefish, contain substantial amounts of it, 8-22 µg/100g, while other sources contain significantly less, as eggs for example contain 2.2 µg/100 g (Valtion ravitsemusneuvottelulautakunta, 2010).

2.3.2.1 Vitamin D recommendations in Finland

As was stated already in 1960's, it has long been known that children need vitamin D for healthy growth, but at the same time there has been concerns over the negative effects of an overdose. The recommendation was to give 2000 IU/day in supplements from the age of two weeks until the age of two years. This was considered safe and sufficient, but if fish liver oil was used, 1600 IU/day was sufficient and it could be given to children older than two years of age if needed. (Hallman et al. 1964)

In the 1987 Finnish Nutrition Committee report, less vitamin D supplements were recommended than in the 1960's; 10 µg (400 IU) per day throughout the first year of life and for growing children during the "dark period of the year", which was not defined in detail. The daily recommended intake for children was 10 µg and 5 µg (200 IU) for all adults, which had to include pregnant and breastfeeding women although they were not mentioned, and it was stated that intake should be adjusted to sunlight exposure. (Valtion ravitsemusneuvottelulautakunta, 1987)

In the 1998 Finnish Nutrition Recommendations 10 µg/day was the appropriate vitamin D intake for children from two weeks until three years of age and for pregnant and lactating women, and for children between the ages four and ten years, 5 µg/day was regarded sufficient. (National Nutrition Council, 1999). In the longer version of the report in Finnish it was explained that vitamin D is needed to prevent rickets and for that reason 10 µg/day was recommended for children with dark skin type during winter. Tolerable upper limit for average daily intake of vitamin D was set at 50 µg (2000 IU). (Valtion ravitsemusneuvottelulautakunta, 1998)

Importantly, in 2003 it was noted that people in all age groups were getting inadequatly vitamin D. The groups needing the same special attention small children had been receiving were pregnant and lactating women and the elderly (> 61 years), and they were all recommended a dose of 10 µg/day, i.e. twice the amount that young adults were recommended to receive. The recommendation was thus the same as it had been five years earlier, but it was admitted that vitamin D supplements were needed during winter to achieve the recommendations. (Valtion ravitsemus-neuvottelulautakunta, 2003)

After 2003 there have been only small changes over the years, as in the latest general Finnish nutritional recommendations suitable vitamin D intake was10

µg/day from two weeks to two years of age, 7.5 µg/day (300 IU) for children between the ages of 2 an 18 years of age, 10 µg/day for pregnant and lactating mothers and 20 µg/day for the elderly. Tolerable upper daily limit was set at 100 µg (4000 IU) for adults and 50 µg for children. (Valtion ravitsemusneuvottelulautakunta, 2014)

According to EU regulation 2016/127 baby formulas, as well as baby porridges and gruels, are fortified with significant amounts of vitamin D (The European Comission, 2016) and thus use of vitamin D supplementation should be adjusted according to the amount of formula used (Valtion ravitsemusneuvottelulautakunta, 2018). Tolerable upper daily limit is set at 25 µg (1000 IU) for babies between the ages of 0 to 6 months and 35 (1400IU) µg for babies between 6 and twelve months (Valtion ravitsemusneuvottelulautakunta, 2018).

It should be stated that The Finnish Nutrition Recommendations are directed by definition for groups of healthy, fairly active people and may not always be ideal for every individual (National Nutrition Council, 1999).

2.3.2.2 Vitamin D fortification of foodstuffs in Finland

Systematic vitamin D fortification began in 2003 when it was noted that although 5 µg per day was estimated to be enough, many people did not get that much from food during winter, as they ate les fish than was recommended. The fortification was 0.5 µg/100 ml for liquid milk products and it was agreed upon in collaboration with the major dairy companies. (Valtion ravitsemusneuvottelulautakunta, 2003) In 2010 the fortification was increased to1 µg/100 ml for liquid milk products and 20 µg/100 ml for fat spreads (Valtion ravitsemusneuvottelulautakunta, 2010).

2.3.3 Factors associated with serum 25(OH)D concentrations

There are some differences of opinion about the optimal 25(OH)D concentration, although some views remain the same. Vitamin D deficiency is generally linked with osteomalacia, vitamin D depletion with osteoporosis, secondary hyperparathyroidism and calcium malabsorption, vitamin D sufficiency with skeletal health and eucalcemia, and vitamin D toxicity with hypercalcemia (Morris, 2005). Circulating 25(OH)D levels of 20-29 nmol/l are required in preventing rickets (Whiting & Calvo, 2005), 50-80 nmol/l can be considered satisfactory (Lamberg-Allardt, 2006) and levels closer to 75-80 nmol/l may be needed to fully support both endocrine and autocrine functions (Whiting & Calvo, 2005). This means that the canonical function of vitamin D, i.e. optimizing intestinal calcium absorption, is fully expressed, elevated parathyroid activity is minimized and osteoporotic fractures are reduced when serum levels are approximately 80 nmol/l (Heaney, 2005). Skin type

determines the amount of UVB radiation required for sufficient 25(OH)D production, and it is in the range of 20-80 % of the personal sunburn threshold dose (Shih et al., 2018). The same amount of UV radiation causes a more significant increase in 25(OH)D concentration in people with light skin than in people with darker skin, even when they have a higher baseline concentration (Felton et al., 2016). Using sunscreens is an easy way to block the cutaneous absorption of UVB radiation and vitamin D synthesis for all skin types (Matsuoka et al., 1987).

2.3.3.1 Seasons

As UVB radiation is a major source of vitamin D, seasons and sunlight exposure are major factors influencing circulating 25(OH)D concentrations, especially near the polar regions. General clinical observation in the past was that rickets was common in spring but rare in autumn (Maxwell, 1994). In Finland the mean 25(OH)D concentrations dropped from 82.5 nmol/l in the summer to 36.3 nmol/l in the winter (Savolainen et al. 1980) and in Norway the concentrations were roughly 50% higher in late summer compared to late winter (Moan et al., 2005). In Finland the summer values were somewhat lower in the elderly (Savolainen et al., 1980) and also in Norway it was discovered that the 25(OH)D concentrations were lower and the seasonal variation was greater in the young than in people over 30 years of age (Porojnicu et al. 2007). Seasonal variation is seen even in higher 25(OH)D concentrations, for example in a British study, where the mean concentration was already quite high, 72.5 nmol/l (Berry et al. 2011). 25(OH)D concentrations were significantly higher in Italians compared to Estonians both in winter (December-February) and in summer (July-August) (Cutolo et al., 2006).

2.3.3.2 Body mass

It has long been known that in general, the higher the body mass index (BMI) of a person, the lower the serum 25(OH)D concentration (Szternel et al. 2018), whereas the $1,25(OH)_2D$ does not differ significantly between obese children and controls (Reinehr et al., 2007). Weight loss leads to a significant increase in 25(OH)D levels, but less than would be expected if all 25(OH)D would be mobilised from the lost fat tissue into circulation, suggesting that some of it is converted into inactive metabolites and secreted (Pannu et al. 2016). The low levels have been speculated to be attributed to decreased exposure to sunlight in obese subjects due to limited mobility, their clothing habits, or the excessive deposition of vitamin D in adipose tissue, with little evidence. In some studies lifestyle factors have had a more significant effect on 25(OH)D concentrations in children than their body composition (McVey et al., 2019). Recently, it has been shown in mice, that the

expression of the enzyme CYP2R1 responsible for 25(OH)D hydroxylation is reduced in obesity leading to decreased circulating 25(OH)D concentrations (Roizen et al., 2019). The interaction between body mass and vitamin D may also be inverse, as low maternal 25(OH)D concentrations were associated with increased body weight and thus increased BMI in children at the ages of one and three years (Neelon et al., 2018).

2.3.3.3 Individual response to vitamin D intake

The 25(OH)D increase following oral vitamin D_3 supplementation varies greatly between people. In white adolescents, vitamin D intakes of 10 µg/day resulted in 6.7 ± 9.9 nmol/l difference in serum 25(OH)D concentrations from the baseline of roughly 50 nmol/l (49.2 ± 12 nmol/l)which means that in some individuals 10 µg was not enough in maintaining the previously obtained level, while in others the same amount caused the concentration to rise, and it took 20 µg/day to get a rise in 25(OH)D concentrations in all study subjects (Smith et al., 2016). Similar results were obtained in Finnish infants, who received either 10, 30 or 40 µg of vitamin D daily, and 10 µg was not enough for all, but was for some (Holmlund-Suila et al., 2012). Some of the variation can be explained through differences in basal 25(OH)D levels, BMI and hormonal factors (Mazahery & von Hurst, 2015), but some of it is genetic. Especially genes involved with the hydroxylation of 25(OH)D, DBP (Soininen et al., 2018) and VDR (Barry et al., 2014) have an effect on 25(OH)D concentrations, but there are several other genes involved as well, widely distributed in the genome (Jiang et al., 2018). In general, genetic determinants cause less variation in 25(OH)D concentrations than environmental factors (D. Berry & Hyppönen, 2011), but in some populations the difference in 25(OH)D concentrations has been approximately 20 nmol/l between people with different genotypes (Ahn et al., 2010). Many VDR genes of peripheral blood mononuclear cells can be used as transcriptomic biomarkers for classifying individuals who possible benefit from additional vitamin D supplementation (Wilfinger et al., 2014).

2.3.4 Hypervitaminosis D

Very high, 200,000-300,000 IU/day doses of vitamin D cause hypervitaminosis D and hypercalcemia within a few weeks (Hallman et al., 1964). Accidental overfortification of milk in the U.S. in 1987- 1991 caused an outbreak of hypervitaminosis D with a median 25(OH)D concentration of 560 nmol/l (range: 140-1740 nmol/l) and lead to death of two people (Blank et al., 1995). It is noteworthy that some people were symptomatic already with rather low levels of 25(OH)D, i.e. 140 nmol/l. at As some individuals are very sensitive to vitamin D, in

rare cases vitamin D intoxication is possible with recommended vitamin D intake (Waheed et al., 2018). With vitamin D fortification of foodstuffs and better compliance of recommendations regarding vitamin D supplementation, the 25(OH)D concentrations have become very high in some individuals, up to 280 nmol/l, in the traditionally low umbilical cord serum samples in Finland (Hauta-Alus et al., 2019).

2.4 Vitamin D and type 1 diabetes in children

When receptors for 1,25-hydroxyvitamin D were discovered in human leukocytes in the early 1980's (Provvedini et al. 1983), it became clear that vitamin D had a role in human immunology. First the effects were seen in monocytes changing toward macrophage phenotype in the absence of $1,25(OH)_2D$ (Provvedini et al., 1986) and when $1,25(OH)_2D$ was found to regulate differentiation of T cell subsets CD4+ and CD8+ (Willheim et al., 1999) previously found important in the development of T1D (Hänninen et al., 1992), a direct link between T1D and vitamin D could be suggesteded.

An indirect link could also be suggested, as low 25(OH)D concentrations have been associated with more frequent respiratory infections (D. J. Berry et al., 2011) and viral infections have been suspected to trigger T1D (Dotta et al., 2007).

Moreover, children diagnosed with type 1 diabetes have been found vitamin D deficient (Svoren et al. 2009). In some studies vitamin D deficiency has been much more common in children with T1D than in controls (Bener et al., 2009), while in others there have been no differences (Garcia et al., 2007). Vitamin D deficiency has been more prevalent in diabetic subjects with albuminuria, as urinary DPB excretion is simultaneously increased (Thrailkill et al., 2011).

2.4.1 25(OH)D concentrations and type 1 diabetes

2.4.1.1 During pregnancy or at birth

Low 25(OH)D concentrations during pregnancy are quite common all around the world (Bischoff-Ferrari, 2011; Cavalier et al., 2008; Clifton-Bligh et al., 2008; Eggemoen et al., 2016), and vitamin D deficiency is especially common if the mother has gestational diabetes (Eggemoen et al., 2018) or has dark skin type (Bodnar et al., 2007). Recently it has been shown that maternal 25(OH)D concentrations have a U-shaped association with cord serum insulin and C-peptide, which in turn may have an impact on glucose regulation and growth (Switkowski et al., 2019).

As the 25(OH)D concentrations are somewhat higher during pregnancy in women originating in Western European countries with high paediatric T1D

incidence than in women living in the same area but who originate in developing countries and whose offspring has similar or lower incidence (Agrawal et al., 2019; Eggemoen et al., 2016; The DIAMOND Project Group, 2006), it seems unlikely that low maternal 25(OH)D concentrations alone would increase the risk for T1D in the offspring. In a Norwegian cohort, with samples taken 1992-1994, a trend toward a higher risk for T1D was found with lower maternal levels of vitamin D during pregnancy (Sørensen et al., 2012), but the difference was no longer statistically significant after adjusting for DBP concentration, even though neither was the effect of DBP alone (Sørensen et al., 2016). In another cohort, with samples taken 1999-2005, the same group found that low DBP concentration at birth, but not during pregnancy, was associated with increased risk of T1D and that increased 25(OH)D levels may lower the risk in certain *VDR* genotype (Tapia et al., 2019). By analysing neonatal blood spots, a small Italian study found no association between 25(OH)D levels and risk of T1D, except in an even smaller subgroup of migrant babies of mainly African origin, where the difference was small but statistically significant between cases (N=23) and controls (N=57) (Cadario et al., 2015). A Danish research group showed with a very large cohort (study population 331,623 individuals, of which 886 cases) that neonatal 25(OH)D status was not associated with a later risk of T1D (Jacobsen et al., 2016). The result was confirmed by a large Danish and Norwegian study, which found that maternal 25(OH)D measured during different trimesters or at birth were not associated with later risk of T1D in the offspring (Thorsen et al., 2018). In a Norwegian study it was shown that $1,25(OH)_2D$ concentration is strongly correlated with 25(OH)D concentration

In Finnish mothers, with samples taken during first trimester 1986-1989 and matched to the exact day they were taken, no difference was found in 25(OH)D concentrations between those whose children later developed T1D (mean 47.9 nmol/l) and those whose did not (mean 47.7 nmol/l), but 70 % were vitamin D deficient or insufficient (Miettinen et al., 2012).

2.4.1.2 Before onset of diabetes

Alike pregnant mothers, children have low 25(OH)D levels all around the world; in countries with plenty of sunshine and low T1D incidence, such as Iran (Ardestani et al., 2010), China (Li et al., 2018), United Arab Emirates (Haq et al., 2018), Mexico (Acosta-Bendek et al., 2017), and also in Finland (Savilahti et al., 2016).

It has been speculated that the low 25(OH)D levels are the reason why T1D incidence in children is globally rising, and for example in Poland the 25(OH)D levels used to be higher in children, but decreased over the past decades and were low in the beginning of this decade (Wójcik et al. 2018). However, the findings regarding an association between low 25(OH)D levels and risk of IA or T1D have

been contradictory. In many studies no association has been found between 25(OH)D concentrations and risk of IA (Reinert-Hartwall et al., 2014) or T1D or both (Simpson et al., 2011), but there were some weaknesses in these studies, as the first study was small, with only 35 cases and 115 children in total (Reinert-Hartwall et al., 2014), and, although larger with 90 children developing T1D, 211 IA children and 2,686 controls, the latter defined IA already when just one autoantibody was present, and as only one sample was drawn each year after the child had turned two years of age, no seasonal variation could be taken into account (Simpson et al., 2011). In a study with 376 IA children and 1041 controls high plasma 25(OH)D concentration was associated with lower risk of IA (Norris et al., 2018) and in somewhat smaller study with 108 IA, 244 T1D and 406 control children levels were lower in children with IA than in controls, with the largest difference in the summer levels and no samples from the cases before seroconversion (Raab et al., 2014). A study conducted in the U.S. military with 1000 adults who later developed T1D and 1000 matched controls reported a 3.5-fold lower risk of T1D with a serum 25(OH)D concentrations of 60 nmol/l or higher, where only single sample per subject was drawn 1 month to up to 10 years before diagnosis (Gorham et al., 2012), which can be considered as a clear limitation. Additionally, in a study with 284 otherwise healthy children 25(OH)D concentrations ≤ 50 nmol/l were associated with impaired fasting glucose tolerance (Szternel et al., 2018). In a reasent international study of 15 countries the 25(OH)D concentrations were lower in the case children (for IA mean 57.7 nmol/l, N=84, and for T1D mean 58,0 nmol/l, N=65) 18 months before seroconversion than in controls (mean 64.8 nmol/l for IA controls and 65.0 nmol/l vfor T1D controls), and for T1D children also at the age of 12 months (mean 70.1 nmol/l vs. 75.9 nmol/l), but not at other time points (Miettinen et al., 2020).

2.4.1.3 After diagnosis

It seems to be a global phenomenon that the 25(OH)D concentrations are lower in paediatric patients with T1D than in controls. In a study of Australian children, 25(OH)D concentrations were lower in the 56 T1D children (78.7 nmol/l) than in the 46 unmatched controls (91.4 nmol/l) (Greer et al., 2013). In Egyptian children, 25(OH)D concentrations were lower in the 120 T1D patients (35.5 nmol/l) than in their 120 controls matched for age, sex and ethnic origin (46.6 nmol/l), and even though the difference in the mean values may partly be due to the higher BMI of control children, the difference remained significant after adjustment with BMI (Abd-Allah et al., 2014). In Qatar vitamin D concentration was lower in the 170 T1D children (median 39.4 nmol/l) than in their matched 170 controls (42.6 nmol/l), but there were some differences in BMI and skin type between cases and controls (Bener et al., 2009). The mean serum 25(OH)D was lower in 296 Chinese children with

T1D (48.7 nmol/l) than in 295 controls matched for age and sex (57.9 nmol/l) and there was no statistically significant difference in 25(OH)D levels in 190 children with established T1D compared with 106 children with newly diagnosed T1D, but there was no data on BMI of these children (Liu et al., 2018).

Also in Korean children and youth between six and twenty years of age, mean serum 25(OH)D was lower in 85 T1D patients (53.9 nmol/l) than in 518 controls (69.9 nmol/l), the deficiency was independent of sex, age and BMI, and the levels were not associated with duration of the disease, insulin dose, C-peptide or glycosylated hemoglobin (Bae et al. 2018). In a large study with 1,426 children and youth diagnosed with T1D, lower 25(OH)D concentrations were associated with insulin resistance (The et al., 2013). In children glycemic control, duration of T1D, and age have been associated with vitamin D inadequacy in bivariate analysis of 128 children with a mean age of 10.8 years (range 1.6-17.5), however, only age remained a significant predictor in multivariate analysis (Svoren et al., 2009). At the onset of T1D the 25(OH)D concentrations (mean 46.2 nmol/l, N=142) were inversely related with the severity of diabetic ketoacidosis (Savastio et al., 2016). Diabetic children with 25(OH)D concentrations ≤ 25 nmol/l showed higher insulin requirement and HbA1c than others (Savastio et al., 2016). In 129 Swiss children with T1D the majority was vitamin D deficient, especially during the winter, but neither age, calcium intake, duration of diabetes, HbA1c nor serum magnesium concentration associated with the 25(OH)D concentrations (Janner et al., 2010).

Only two peripheral immune mediators, chemokine ligand 8 and leptin, were associated with 25(OH)D concentrations in newly diagnosed paediatric T1D patients (N=460), while 16 other peripheral immune mediators studied were not, and the 25(OH)D concentrations were not statistically different from the controls, which were siblings to the patients (N=453) (Thorsen et al., 2017).

25(OH)D concentrations were lower in newly diagnosed T1D patients (N=244) than in unmatched autoantibody-negative children (N=406) throughout the year and 51% of children with T1D were vitamin D deficient (Raab et al., 2014).

2.4.2 Vitamin D intake or supplementation and T1D

2.4.2.1 During pregnancy

Maternal vitamin D intake from food, but not from supplements, during pregnancy was associated with decreased risk of IA in the offspring (Fronczak et al., 2003). In a small Norwegian pilot study of 85 subjects and 1071 controls, a strong negative association between mothers taking cod liver oil during pregnancy and the risk of T1D in their children was found, and it was independent of multivitamin use (Stene et al. 2000), but in a larger Norwegian study with 545 cases and 1668 controls, the

use of cod liver oil or other vitamin D supplements during pregnancy were not associated with T1D (Stene & Joner, 2003). In a large Danish birth cohort study of children born in 1983-1988 found no evidence that vitamin D fortification of margarine would have an effect on the risk of T1D in the offspring (Jacobsen et al., 2015). Vitamin D intake was not associated with the risk of advanced beta cell autoimmunity/T1D in offspring in the Finnish Diabetes Prediction and Prevention study (DIPP), where the maternal mean daily intake of vitamin D was 5.1 µg from food and 1.3 µg from supplements when10 µg was recommended (Marjamäki et al., 2010). In the recently published international The Environmental Determinants of Diabetes in the Young (TEDDY) study, vitamin D supplementation during pregnancy was not associated with the risk of IA (Silvis et al., 2019).

2.4.2.2 During childhood before the onset of T1D

In a European study, vitamin D supplement use in early childhood was associated with a decreased risk of T1D (EURODIAB Substudy 2 Study Group, 1999). In a large Finnish birth-cohort study of children born in 1966 low vitamin D supplementation during the first year of life was associated with increased risk of T1D later in life (Hyppönen et al., 2001). In an American study no association was found between vitamin D intake and risk of IA or T1D (Norris et al., 2007; Simpson et al., 2011). In a meta-analysis the risk of T1D was significantly reduced in infants who were supplemented with vitamin D compared to those who were not, and infants using higher amounts were at a lower risk, when also cod liver oil was considered a vitamin D supplement (Zipitis & Akobeng, 2008). However, cod liver oil is also a source of fatty acids and dietary intake of omega-3-fatty acids has been associated with a reduced risk of IA (Norris et al., 2007), and also in the aforementioned large Norwegian study the use of cod liver oil, but not the use of other vitamin D supplements, during the first year of life showed association with lower risk of T1D (Stene & Joner, 2003).

2.4.2.3 After T1D diagnosis

In a British study, paediatric T1D patients who were vitamin D deficient (≤ 30 nmol/l) and vitamin D insufficient (≤ 50 nmol/l) were supplemented with 150 µg (6000 IU) or 10 µg (400 IU), respectively, daily for 3 months, and this improved the HbA1c levels (Giri et al., 2017). Vitamin D supplementation (25 µg = 1000 IU/day) improved glycaemic control in children with T1D (Savastio et al., 2016). These promising effects may be temporary of nature, as in another study where T1D were treated with 25 µg/day (1000 IU) of vitamin D, no differences in C-peptide, HbA1c, or insulin requirement were present after one year of follow-up (Napoli et al., 2013).

As some promising results were obtained with vitamin D, it was thought that maybe administering the active form of vitamin D, 1,25(OH)D_2 directly would be even more effective, but this does not seem to be the case. When young adults with recently diagnosed T1D received 0.25 µg/day of 1,25(OH)D_2 for 9 months, the treatment was found to be safe but ineffective, as it did not reduce loss of β-cell function or change the titres of autoantibodies (Walter et al., 2010). Similar results were obtained when Italian paediatric and adolescent recently diagnosed T1D patients were treated with 0.2 µg/day of 1,25(OH)D_2, and followed-up for two years; no differences were found in levels of A1C or insulin requirements and C-peptide levels dropped similarly in the untreated control group (Bizzarri et al., 2010).

2.4.3 Vitamin D linked genetic polymorphism and T1D

Already over a decade ago a common inherited variation of the vitamin D metabolism CYP27B1 gene encoding for the enzyme 1-α-hydroxylase was associated with increased risk of T1D in a British study of nearly 8000 patients and over 8000 controls (R. Bailey et al., 2007). The other studies have been smaller. In a meta-analysis of the early small studies it was concluded that VDR polymorphism *Bsm*I has some association with increased risk of T1D, especially in Asian populations (Zhang et al., 2012). A large study with families from the U.K., Finland, Norway, Romania and the U.S. no association was detected between common sequence variation in the VDR gene and T1D (Nejentsev et al., 2004).

In Kuwaiti Arab T1D children the frequency of variant alleles of VDR gene polymorphisms *Fok*I and *Taq*I was significantly higher than in controls (Rasoul et al. 2019) and in Egyptian children VDR *Bsm*I and *Fox*I polymorphisms were associated with T1D (Abd-Allah et al., 2014), while no difference in VDR polymorphism distribution in Australian children with or without T1D could be observed (Greer et al., 2013).

In the TEDDY study where high plasma 25(OH)D concentration was associated with lower risk of IA, it was found that this association is further stratified by VDR polymorphism (Norris et al., 2018). VDR polymorphism alone was not associated with risk of IA in the DAISY study, but it was associated with the rate of disease development in IA positive children (Frederiksen et al., 2013) and the strength of association between VDR polymorphism and 25(OH)D concentration was found stronger in mothers of T1D children during pregnancy than in control mothers, emphasising the importance of *in utero* environment for the development of T1D in the child (Miettinen et al., 2017a).

3 The Aims of the Present Study

The aims of this study were to get an overall view of serum 25-hydroxyvitamin D (25[OH]D) concentrations in Finnish children, and to investigate the possible connection of the concentrations with T1D pathogenesis.

The specific aims of this study were:

* To analyse 25(OH)D concentrations in healthy Finnish children in consecutive serum samples from the age of 3 months in 3- to 12-month intervals during different calendar years
* To analyse 25(OH)D concentrations in the follow-up samples of children who develop T1D, and in their carefully matched autoantibody-negative counterparts
* To find if there are differences in 25(OH) concentrations already at birth between children who develop islet autoimmunity (IA) or type 1 diabetes (T1D) and autoantibody negative children

4 Subjects and Methods

4.1 Subjects and overview of the study cohorts and design

All the subjects were born in 1994-2004 and participated in the Type 1 Diabetes Prediction and Prevention (DIPP) study, where new-born infants with HLA-conferred susceptibility to T1D are recruited from the University Hospitals of Turku (60 ºN), Tampere (61 ºN) and Oulu (65 ºN), Finland.

4.1.1 Healthy children (Study I)

The sampling period of this study was limited to 1998-2006 and to the Turku area in order to avoid overrepresentation of young children and to limit variation due to location. The first serum samples were collected at the age of 3 months and at 3- to 6-month intervals thereafter. In total, 5334 serum samples from 387 children (233 boys, 154 girls) were measured for 25(OH)D.

4.1.2 Children developing T1D and their controls (Study II)

There were 3702 serum samples from 252 children in this study: 126 cases who developed T1D by the end of 2012, and 126 control children, matched pairwise for age (birth within one month), sex, study site, and HLA-DQB1. In the Turku cohort, the first serum samples were obtained at the age of 3 months followed by sample collection at 3-month intervals during the first 2 years after which autoantibody-negative children were monitored at 6-month intervals. In the Tampere and Oulu cohorts the serum samples were drawn at the age of 3, 6, 12, 18, and 24 months and yearly thereafter, if they were autoantibody negative. If the participant seroconverted, the follow-up was changed to 3-month intervals in all the centres and the follow-up continued to the diagnosis of T1D. As the number of samples per child was different in the two groups due to the study protocol a sub-cohort from the full data set with a matching number of samples from each pair of children was established by selecting the samples closest to each other based on sampling dates to

enable further analysis. This restricted data set included 3060 samples from the same 252 children.

4.1.3 25(OH)D levels at birth (Study III)

In the birth study there were 764 umbilical cord serum samples from 764 DIPP study participants: 133 case-control pairs in the Turku cohort, 137 in the Oulu cohort and 112 in the Tampere cohort. Case children comprised two groups; 250 children, who were diagnosed with T1D by the end of July 2016 (T1D+) and 132 children, who were IA positive for multiple (≥2) autoantibodies, but did not progress to overt T1D by the end of the study period (IA+). The cases were included based on the availability of samples and one control subject was selected for each case child. All control subjects (T1D- and IA-) remained autoantibody-negative and non-diabetic throughout the follow-up, and they were pairwise matched for age (birth within 30 days), sex and study centre.

4.1.4 T1D criteria and ethical aspects

The children were diagnosed with T1D according to the World Health Organisation criteria (World Health Organization, 1999). The three studies were conducted according to the guidelines of the Declaration of Helsinki. They were a part of the DIPP study that was approved by the Joint Commission on Ethics of the University of Turku and the Turku University Central Hospital (decision 1.2.1994 §228). Written informed consent for the DIPP study was obtained from all subjects and/or their guardians as explained in (Kupila et al., 2001).

4.2 Methods

4.2.1 Genetic screening

All children in the study were genetically screened for an increased risk of T1D using HLA-DQB1 alleles in the laboratory of Immunogenetics, University of Turku, Turku, Finland. For genetic screening droplets of umbilical cord blood were dried on filter paper, from which DNA was directly amplified by PCR, as previously described (Sjöroos et al. 1995). During 1994-2004 HLA-DQB1*02/0302 was considered high-risk genotype and the HLA DQB1*0302/x; *0301, *0602, *0603, and for male children in the Turku cohort also *02, were considered moderate-risk genotypes (Hermann et al., 2003; Ilonen et al., 1996; Nejentsev et al., 1999), and the children carrying them were then eligible for the prospective follow-up study.

4.2.2 Nutrition during pregnancy

Maternal dietary data of the eighth month of pregnancy was collected by a validated food frequency questionnaire after the birth of the child, as previously described (Marjamäki et al., 2010).

4.2.3 Blood and serum samples

Mixed arterial/venous umbilical cord blood was collected in the delivery room. For genetic screening, fresh blood was brought to the laboratory of Immunogenetics, where 4 blood drops from each child were dried on a filter paper (Schleicher & Schuell, Dassel, Germany). Disks of 3 mm in diameter of these blood spots were punched out directly onto 96-well PCR plates (Thermowell, Corning Costar, the Netherlands). DNA was amplified by PCR and hybridised with europium, terbium or samarium lanthanide chelates, which were then detected with time-resolved fluorometry (VICTOR fluorometer, Perkin-Elmer Finland, Turku, Finland) (Sjöroos et al., 1995). Analgesic lidocaine-prilocaine cream (EMLA, Astra Zeneca, Sweden) was used before venepuncture for serum samples. Serum was extracted, divided into 3 to 9 aliquots, and stored at -70 to -85 ºC.

4.2.4 Immunological methods

T1D-associated autoantibodies were analysed in the Research Laboratory, Department of Paediatrics, University of Oulu, Finland. Islet cell antibodies (ICA) were used as the primary screening tool for beta cell autoimmunity until the end of 2002. If a child was ICA positive or progressed to clinical type 1 diabetes during the follow-up, all their preceding and subsequent samples were also analysed for insulin autoantibodies (IAA), for the 65 kDa isoform of GAD autoantibodies (GADA) and for islet antigen 2 autoantibodies (IA-2A). Since 2003, all samples were analysed directly for ICA, IAA, GADA and IA-2A for study participants born in 2003 and later.

ICA were measured using a standardized indirect immunofluorescence staining method on frozen human pancreas sections from a blood group O donor, as described previously (Bottazzo et al., 1974). The cut-off limit for ICA positivity was 2.5 Juvenile Diabetes Foundation units (JDFU). The biochemical autoantibodies IAA, GADA and IA-2A were analysed with radiobinding assays on 96-well microplates, as previously described (Kulmala et al., 1998; Savola et al., 1998a; Savola, et al., 1998b; Williams et al., 1997). Titres of the biochemical autoantibodies were expressed in relative units (RU). RU were based on a standard curve prepared for each assay plate using MultiCalc software (PerkinElmer Life Sciences, Wallac, Turku, Finland). Cut-off values for positivity were defined as

the 99th percentile levels in 370–374 healthy Finnish children: IAA, 3.48 RU for the new method starting 2000 (Pöllänen et al., 2017) and 1.56 RU for the old method before 2000 (Hämäläinen et al., 2002); GADA, 5.36 RU; IA-2A, 0.43 RU. All the initially ICA-positive samples were retested to confirm positivity, as were the study samples with titres between the 97th and 99.5th percentiles of the reference population values.. The disease sensitivity of the old IAA microassay was 14%, and the specificity was 100% in the 2001 Diabetes Autoantibody Standardization Program (DASP) Workshop. Subsequently, the IAA assay was optimized, resulting in a disease sensitivity of 30%, the specificity remaining at 100%, based on a blinded reanalysis of the 100 DASP Workshop samples. The intra-assay coefficient of variation was 7%, and the interassay coefficient of variation was less than 9% in the IAA assay (Kimpimäki et al., 2002). Based on the 2005 DASP Workshop, the disease sensitivity values for the new IAA, GADA and IA-2A assays were 58%, 82%, and 72 %, respectively, while the corresponding disease specificity estimates were 98%, 96%, and 100 %. In the 2007 DASP IAA Affinity Workshop, the combination of the assay for the IAA level with the affinity assessment increased the disease sensitivity and specificity of the IAA assay to 90 % and 100 %, respectively (H. Siljander et al., 2009). The time point for seroconversion was defined as the date of the first autoantibody-positive sample. Confirmed autoantibody positivity was defined as positivity for at least one autoantibody in at least two consecutive samples.

4.2.5 Determination of serum 25(OH) D

25--hydroxy-vitamin D concentrations were measured in serum using commercial 25-Hydroxy Vitamin D EIA analysis kit (Immunodiagnostic Systems Limited, UK). As every drop of the DIPP serum samples is very precious and could be used for a number of other analysis, the protocol was optimized to require only 10 µl instead of the original 25 µl of serum, in collaboration with the kit manufacturer. The changes affected only the first few steps as the samples were diluted into 400 µl of sample buffer containing biotin-labelled 25(OH)D antibody, on a 1.2 ml polypropylene 96-well plate (ABgene, Epson, UK), instead of 1 ml in separate sample tubes. This made it possible to use a 12-channel pipette (BioHit, Finland) and to get the samples on the analysis plate almost simultaneously, thus minimizing the differences on incubation time and assay drift. Standards were added in duplicate but controls and samples in single wells, so that 80 samples were fitted per plate.

Standards with seven different concentrations, two controls and all the samples were diluted with biotin labelled 25(OH)D. The diluted samples were incubated in microtitre wells coated with a highly specific sheep 25(OH)D antibody for two hours

at room temperature before aspiration and washing. Horseradish peroxidise labelled avidin was added and following a further washing step, colour developed using a chromogenic substance (TMB). The reaction was stopped with hydrochloric acid and the absorbance of the reaction mixtures read at 450 nm using Victor (PerkinElmer Finland). The developed colour intensity is inversely proportional to the concentration of 25(OH)D and the working range was from 0 to 418 nmol/l. The concentrations were calculated from the absorbance values obtained, using Wallac MultiCalc™ (PerkinElmer Finland).

Since the only way for laboratories to demonstrate the accuracy of their results is to participate in an external quality assessment scheme (Carter, Carter, Jones, & Berry, 2004) also we participated in The International Vitamin D Quality Assessment Scheme (DEQAS) and the small participation fee was paid by the kit manufacturer (IDS Ltd, UK). Our results were in the acceptable range (using 50 samples, the R^2 was 0.72, R^2 adjusted 0.71, correlation of estimates 0.9265) from the All-Laboratory Trimmed Mean (ALTM) values, which has been considered a good surrogate for the "true" target values obtained by gas chromatography-mass spectrometry. (Tahsin-Swafiri et al., 2012). During our participation, the number of laboratories participating in 25(OH)D measurement in DEQAS increased from 121 to 1119 making the ALTM results more reliable towards the end.

All samples from each child, or case-control pair, were analysed with the same batch of kits whenever possible. This was challenging in work II, where the median number of samples per sample-matched pair was 20 (IQR 12-34), but even there the median number of batches was 1 (IQR 1-2) and the median number of different plates 3 (IQR 1-7.25). The results obtained in 2005-2007 with the first four batches of kits were re-calculated according to the instructions provided by the kit manufacturer and verified by the kit importer (Biofellows, Helsinki, Finland).

Both inter and intra assay variations were calculated using four different samples which were measured ten times. Two batches of kits were used in the inter-assay variation measurement, but only one for each analyte, lot #53336 for the first two samples and lot #56128 for the second two samples. In the intra assay analysis the mean concentration of 25(OH)D in the first sample was 24.1 nmol/l and the CV was 4.6 %. In the second sample the mean concentration was 52.3 nmol/l and CV 5.2 %, in the third 89.0 nmol/l and CV 7.1 %, and in the fourth 87.3 nmol/l and CV 7.4 %. In the inter assay analysis the mean 25(OH)D concentration was 42.3 nmol/l, CV 9.6 % for the first sample and 112.2 nmo/l and CV 7.4 % for the second sample of the first batch of kits. In the second batch the means were 21.9 nmol/l and 92.2 nmol/l and the CVs 10.3 % and 7.0 %, respectively. The sensitivity reported by the kit manufacturer was 5 nmol/l.

4.2.6 Collection of clinical data

During the early years of the DIPP study, the study nurses have recorded information on clinical well-being of the children by hand on paper files by each visit. These files have then been transformed in an organized manner to the electronic forms of the DIPP databases and the original files have been stored under lock and key. Additional information was obtained from Turku, Oulu, and Tampere University Hospital records and National Institute of Health and Welfare.

4.2.7 Statistical methods

In the first study (I) the statistical analyses were carried out using chi-square tests and mixed model analyses, where subject was used as a random effect. The effect of sample year was studied separately in two age groups (over/under 2 years) and together with sample month and sex were used as independent variables. The associations were further studied by adding the interaction between the sample year and sex as independent variables. Statistical analyses were performed using SAS for Windows version 9.3. *P*-values lower than 0.05 were considered statistically significant.

In the second study (II) the associations between 25(OH)D concentrations and case-control status were studied using conditional logistic regression. Associations between response variable, i.e. 25(OH)D concentrations and various predictor variables were studied using mixed models with the subject as a random variable. The results were adjusted for sex, age, sample month and year when appropriate. Numbers of samples in each group of the original data were compared using independent samples t-test. All statistical analyses were performed with SAS for Windows version 9.3. For all analyses, statistical significance was defined as $P \leq 0.05$.

For the third study (III) season of birth and study centre were used as adjusting factors in all analysis. Log-transformation was used when necessary in analyses requiring normal distribution. Possible confounding factors were controlled for by adding background variables to the statistical models. Statistical analyses were performed with JMP Pro 12.0.1 and SAS for Windows version 9.4 using multi-way analysis of variance and Fisher's exact test for categorical variables. The value of $P < 0.05$ (two-tailed) was taken to indicate statistical significance.

For additional analysis on intra-assays, DEQAS results, and on work III, SPSS version 25 for Windows, JMP Pro 12.0.1 and R version 3.5.1 were used.

5 Results

5.1 Serum vitamin D in healthy children

Mean serum 25(OH)D levels were markedly lower in healthy children during the first period (years 1998-2002) than during the second (years 2003-2006) (69.3±1.0 nmol/l vs. 84.9±1.3 nmol/l, respectively, $P<0.001$) in both genders (Publication I, Figure 1; Table 1). The mean difference between the periods was 15.7±1.3 nmol/l ($P<.001$). Seasons had a large effect on the 25(OH)D concentrations with peak values in late summer and the lowest values in late winter (Publication I: Figure 1; $P<0.001$) during both of the time periods. Young infants (<2 years) had higher 25(OH)D concentrations than older children (Publication I: Table 1). Importantly, the frequency of children with low serum 25(OH)D levels (< 50 nmol/l) was almost halved from 1998-2002 to 2003-2006 (37.3 % versus 69.9 %; $P<0.001$). Similarly, the frequency of severe vitamin D deficiency (<25 nmol/l) was significantly lower after 2002 than before (2.7% vs. 7.7%; $P=0.005$).

5.2 Serum vitamin D and development of type 1 diabetes

Distibution of the 25(OH)D concentrations at different age points was very similar in cases and controls as shown in the original publication II, Figure 1, and no statistically significant differences were observed. The median 25(OH)D concentrations were in the inadequate range of 50 to 75 nmol/l (Roizen et al. 2013) in both cases and controls. In the full data set the median 25(OH)D concentration was 66.6 nmol/l (range 14.0-262.8) in cases and 67.4 nmol/l (range 19.9-213.0) in controls ($P= 0.56$). Also in the restricted data set with matched samples from cases and controls they had very similar ($P= 0.78$) median concentrations (66.7 nmol/l [range 18.8-262.8] and 68.0 nmol/l [range 19.9-213.0], respectively). Month, year and the age of the child at sample draw had each a significant influence on 25(OH)D concentrations ($P<0.001$) both in the full and restricted data sets.

The median 25(OH)D concentration were highest in Turku, approximately 70 nmol/l, and lowest in Oulu, approximately 50 nmol/l. In Tampere the median

concentration were around 55 nmol/l in all groups. The differences between the centres were statistically significant in both the full and restricted data set (P <0.001).

In the full data set 10.3 % of children had at least one sample in the range of severe vitamin D deficiency with concentrations below 25 nmol/l (Kull et al. 2009), but the difference between cases (17 children) and controls (9 children) was not statistically significant (P=0.10) and the situation was very similar in the restricted data set, where the proportion was 9.5 %, 15 cases vs. 9 controls (P=0.19). None of the children had 25(OH)D concentration below 25 nmol/l in more than 50 % of the samples.

There was no correlation between 25(OH)D concentrations and the age of a case child at seroconversion for autoantibodies (P=0.79) or the age of disease manifestation (P=0.13). The 25(OH)D concentrations were similar before and after seroconversion, when adjusted for sample month, sample year, age of child at sample draw and sex (P=0.22) (Publication II: Figure 3) and when compared to controls before (P=0.35) or after seroconversion (P=0.23). There was neither any correlation of 25(OH)D concentrations with the number of autoantibodies present (Publication II: Suppl. Fig. 1), nor with the titres of the biochemical autoantibodies, GAD (P=0.40), IA2A (P=0.35), and IAA (P=0.46), when adjusted for sample month, sample year, age of child at sample draw and sex (Publication II: Suppl. Fig. 2).

Since body mass index (BMI) may affect circulating 25(OH)D concentrations, we analysed the BMI in all children (Publication II: Table 1). As BMI is dependent on age and sex of the child (Cole et al. 2000), it is difficult to take it into account in analysis when analysing different number of samples in growing children. We overcame this issue by first analysing the median age of the BMI data and it was similar between the groups (P=0.21). The median BMI was also the same in cases and controls (P=0.65), so it does not distort the results. There was an inverse association with BMI and the 25(OH)D concentrations; when BMI increased with 1 kg/m^2, median 25(OH)D concentration decreased with 1.3 nmol/l, similarly in cases and controls (P=0.57).

5.3 Serum vitamin D at birth

The median 25(OH)D concentrations in cord serum were essentially the same between the study groups (P=0.70). Most of the cord serum samples were below 50 nmol/l, so that 88 % of the study children had suboptimal (Lamberg-Allardt et al. 2013) vitamin D levels. As in older children, serum 25(OH)D concentrations were statistically significantly affected by year (P=0.001), month of birth (P=0.002) and study centre (P=0.03), so that the children of the Oulu centre (65°N) had significantly lower levels than the children in Turku (60°N) and Tampere (61°N) centres, but not by sex (P=0.64). The children were born evenly around the year: 210 children were

born in the winter, 182 in the spring, 187 in the summer, and 185 were born in the autumn.

The basic characteristics were similar between the study groups T1D+, T1D-, IA+ and IA-, except that as could be expected (Ziegler et al., 2013), the T1D+ children seroconverted to autoantibody positivity at an earlier age than IA+ children, and the follow-up time was shorter in the T1D+ group due to the design of the study. The median age at the diagnosis of T1D was 6.7 years and the median age at seroconversion to autoantibody positivity in the T1D+ group was 2.0 years and 4.0 years in the IA+ group (N=132). The 25(OH)D concentrations were not associated with the age at T1D diagnosis (P=0.53) nor with the age of seroconversion to autoantibody positivity (P=0.72).

There were no statistically significant differences in 25(OH)D concentrations between T1D+ and IA+ groups positive for different number of autoantibodies (P=0.38 for all autoantibodies, P=0.32 for biochemical autoantibodies).

No differences were found in 25(OH)D concentrations between the groups of cases and their matched 97 controls in a sub-cohort defined by the first appearing persistent biochemical autoantibody (P=0.61 for IAA and P=0.51 for GADA).

HLA-DQB1 classification (moderate risk, high risk) was not associated with 25(OH)D concentrations (P=0.57), and neither were the extended HLA-groups (non-increased risk, slightly increased risk, moderately increased risk, and highly increased risk in HLA-DR/DQ) (P=0.46).

The age of the mother at the time of birth was positively associated with 25(OH)D concentration (P=0.002) in cord serum, so that the concentration increased by approximately 0.05 nmol/l for each additional year. The median age of the mother was 29.8 (IQR 26.6-33.6) years. The ponderal index of the child, calculated as weight in kg per height^3, was inversely associated with 25(OH)D concentrations (P=0.031), but there was no statistically significant association with either weight (P=0.29) or length (P=0.75) alone to 25(OH)D. Furthermore, the ponderal index did not correlate with the age of the mother (P=0.16).

Nutrition during pregnancy

Nutritional information on the eighth month of pregnancy was available in almost half (48%) of the study population, and the general characteristics of this subpopulation are presented in Publication III: Table 3.

Intake of vitamin D during pregnancy showed a statistically significant association with 25(OH)D concentrations in all forms, from food (P<0.0001), from supplements (P<0.0001), and in total (P<0.0001). The energy intake was not associated with 25(OH)D concentration (P=0.27).

The age of the mother had a statistically significant association with 25(OH)D concentration also in this subpopulation with roughly the same impact (*P*=0.025), and the median age of mothers was similar in the subpopulation as in the entire cohort (29.3 years vs. 29.8 years). Maternal age did not correlate with energy intake (*P*=0.64), vitamin D intake from food (*P*=0.22), intake from supplements (*P*=0.51), nor with total vitamin D intake (*P*=0.22).

Interestingly, ponderal index of the baby was not associated with the 25(OH) concentration in this subpopulation (*P*=0.097) but its correlation with the age of the mother was close to significant (*P*=0.059) and it did not correlate with energy intake (*P*=0.34), vitamin D intake from food (*P*=0.30), from supplements (*P*=0.52), nor with total vitamin D intake (*P*=0.93).

The total vitamin D intake during pregnancy was quite low, which is not surprising as there were only 20 % of the mothers who reported the recommended total vitamin D intake during pregnancy ≥ 10 µg per day and only 9 % used vitamin D supplementation (≥ 5 µg vitamin D/day).

Additional analysis

Another way of analysing the cord serum 25(OH)D concentrations in matched case-control pairs was tested after the results were published. Conditional logistic regression analysis confirmed the results. Using the matched data set with the same number of samples from cases and controls unadjusted OR was 1.002 (95% CI 0.989-1.015). Adjustment with the age of the mother changed the results only slightly to the positive side and OR was 1.014 (95% CI 0.986-1.044). Adding ponderal index to the model had an equally slight reverse effect: OR 0.970 (95% CI 0.915-1.028).

6 Discussion

Vitamin D is crucial for human well-being and not surprisingly, evidence for positive evolutionary selection of genes linked with higher circulating 25(OH)D have been found in Northern populations (Kuan et al. 2013). With other type of genetic distribution the 25(OH)D concentrations may drop to dangerously low levels without vitamin D supplementation, and this is a problem especially with some immigrant populations. It seems that Northern populations have also evolved to survive on low serum 25(OH)D and our group found a difference in VDR genes within Finland, so that more efficient VDRA and VDRB genes, which increase receptor function and upregulate vitamin D-induced protein expression, had higher frequencies in the Oulu than in Turku region (Turpeinen et al., 2003). This might explain why the low 25(OH)D concentrations measured in this study in the Oulu subjects seem to have so little observed adverse effects.

Even though low 25(OH)D concentrations at birth (Publication III) or during childhood (Publication II) were not associated with the risk of T1D later in life, the low concentrations may have other adverse effects. Vitamin D deficiency during pregnancy increases risk of gestational diabetes independently of other factors (Wang et al., 2018). Low 25(OH)D levels near birth have been associated with several neurodevelopmental disorders later (García-Serna & Morales, 2019), with increased risk of multiple sclerosis (Munger et al., 2016), and high blood pressure in 3-year olds (Petersen et al., 2017). In obese children, low 25(OH)D concentrations have been associated with markers of cardiovascular disease independently of other factors (Censani et al., 2018).

There is some evidence that cord blood might not be predictive of postnatal immunity of the child and samples taken at three months of age would best represent the real set point where human immune system develops and is further modified by environmental factors (Olin et al., 2018). In this work the three-month samples were measured for 25(OH)D and analysed together with subsequent samples (II) and only umbilical cord serum samples were analysed separately (III). It seems unlikely that analysing 25(OH)D concentrations separately from the three-month samples would reveal something unique about the T1D progress. The cord serum samples were

analysed because they reflect the environment during the near past, i.e. during foetal development, and in that perspective, they are quite unique.

It is noteworthy that expecting mothers did not use vitamin D supplementation during the study period, despite recommendations, and thus their 25(OH)D levels were very low (publication III), but they gave vitamin D supplementation to their babies, who then had higher 25(OH)D concentrations in the bloodstream, without any seasonal variation (publication II). The study period proved to be rather interesting as it happened to overlap with the Finnish vitamin D fortification program, which somewhat changed the nutritional status of the Finnish population regarding vitamin D. The first fortification step in 2003 (Valtion ravitsemusneuvottelulautakunta, 2003) made a marked difference in 25(OH)D levels in children, as we showed in publication I. However, it was not enough for pregnant mothers to meet the daily intake recommendations of vitamin D (Prasad et al., 2010) and that could be seen in publication III as well. In 2010 the fortification was doubled and this seemed to be enough for adults, as the mean vitamin D intake in women increased from 3 µg/day in 2002 to 18 µg/day in 2012, and in men from 5 µg/day to 17 µg/day (Raulio et al., 2017). This, together with better compliance with the recommended vitamin D supplementation, reflected in the umbilical cord serum samples taken 2013-2014 so that mean concentration was 82.5 nmol/l (Hauta-Alus et al., 2019), some 50 nmol/l higher than we found during earlier years (1994-2004) in (Publication III). In children the difference was not all that prominent, as in 12-month samples taken in 2013-2015 the mean 25(OH)D concentration was 98.9 nmol/l (Hauta-Alus et al., 2019), roughly 25 nmol/l higher than the median for 1-year-olds in publication II.

The major strengths in this study lie within the design of the DIPP study; recruitment at birth over many years followed by the long closely monitored follow-up period with abundance of both serum samples and clinical data of a large paediatric population of both sexes. To date this study provides the largest number of serum 25(OH)D measured in consecutive follow-up samples, which permits for the first time the analysis of the seasonal changes in serum 25(OH)D during different calendar years before and after the beginning of national vitamin D fortification program. In children developing T1D there were samples quite uniquely at three-month intervals over many years before the onset of the disease, if the children were IA positive, and at three- to six-month intervals before the appearance of autoantibodies. It is also noteworthy that the children were born quite evenly around several calendar years, a feature which is not obtained in studies with short recruitment periods.

When different DIPP centres are compared, in the Turku data set the major strength was that the serum samples were collected in six-month intervals in all autoantibody-negative children disregard of age, and the major weakness that limited

nutritional data have been collected. The Tampere and Oulu data sets were quite alike, with annual sampling of autoantibody-negative children and a good coverage in nutritional information during the eighth month of pregnancy.

One of the strengths of this study was that the immunoassays were done in in well-designed batches in a single laboratory thus minimizing the variation (Snellman et al., 2010) using a method which is specific for total 25(OH)D concentration. The assay had some emphasis on 25 $(OH)D_3$ as only 75 % of $25(OH)D_2$ is measured according to the kit manual. However, it is important that it ignores other forms of vitamin D, such as 3-epi-25(OH)D, and thus simplifies interpretation of the results (Singh et al., 2006). The function of 3-epi-25(OH)D is still unknown, but interestingly it can be found in all age groups and it is most abundant in neonates, as reviewed in (D. Bailey et al., 2013). Free 25(OH)D, a form not bound to VBP, was not measured, even though it might be of some importance and perhaps should be measured at least in individuals with altered DBP levels (Bikle et al. 2017). If a patient with T1D suffers from diabetic nephropathy, DBP is excreted in the urine and this may cause vitamin D deficiency commonly seen in diabetic patients (Thrailkill et al., 2011), and measuring free 25(OH)D would probably be beneficiary. In the development of T1D the role of the free form is quite unclear and as the subjects in this study did not have overt T1D, it is unlikely that they would have suffered from diabetic complications requiring measurement of free 25(OH)D.

Much has been learned over the years, but the final conclusive environmental trigger or other factors behind diabetic autoimmunity and the development of T1D remain unknown. Families sometimes try to prevent T1D in children based on something they hear or read, and this seem to happen especially if the child has a first-degree relative with T1D (Baughcum et al., 2005). This in turn requires scrutiny from the follow-up studies, as it is necessary to know and to record what experimental preventive methods the family might be undertaking and take this into account as a variable in analysis or already in case-control matching. In the early years of the DIPP study there were some inconsistencies depending on study centres and even on individual study nurses on what questions were asked from the families and how the results were then marked on the papers, as no electronic records were used at the research clinics in the 1990's. For this reason for example reliable data on vitamin D supplementation of the children starting from birth was not available in all children, which is one of the weaknesses of this study.

The cohort in Publication II was large, but a larger cohort of children would have made it possible to divide it into different subgroups, for example those who developed T1D before/after the age of 2 or 4 years, those with vitamin D deficiency vs. those with vitamin D sufficiency at different age points. Now the power for these analyses was too limited. Another weakness of study II is the somewhat limited number of vitamin D deficient samples, so that there is not enough power to examine

the effects of long-term vitamin D deficiency in childhood. It may also be considered a weakness of all of these studies that the possible variables affecting 25(OH)D concentrations, such as skin type, clothing habits, nutritional factors and supplement use of the children, or single nucleotide polymorphisms (SNP) of genes linked to vitamin D metabolism, were not assessed. In an earlier study it had been shown that *VDR* SNPs were not associated with the risk of T1D in the DIPP cohort (Turpeinen et al., 2003). We did some preliminary analysis (data not shown) including 484 children with 6989 samples from the age of three months onwards, and there were 277 children with one SNP analysed (CC 23, CT 127, TT 127) and 211 children with another SNP (29 GG, 113 GT and 69 TT). It was not revealed what SNPs these were in order not to look for a specific result in the analysis. More SNPs were available in cases than in controls, but no association with 25(OH)D concentrations nor a statistically significant differences between cases and controls were found when sample month, age and sex of the child were included in the analysis, and so we decided not to proceed with this line of research. Some of the most interesting genetic variants are rare, and it would require a lot of children to get the groups large enough for detailed analysis. We decided to focus on the serum concentrations in consecutive samples over a long period of time. However, since then there have been some interesting findings. In a Finnish study maternal *VDR* variants were associated with the risk of T1D in the offspring (Miettinen et al., 2015) and in an international study, which included also Finnish children, increased childhood 25(OH)D was associated with decreased risk of IA, especially in some minor *VDR* alleles (Norris et al., 2018). Our aim was to study whether the concentrations are different in children who progress to IA or overt T1D compared to those who do not, irrespective of the possible mechanisms behind the differences.

The genetic screening method in the DIPP study during the study years1994-2004 relied on HLA-DQB1 and was later updated when more information on the genetic risk for T1D was available (Ilonen et al., 2016). This more detailed HLA-screening which was used later would have improved the case-control match in Publication II and having more data on HLA-sequences might have been beneficial as HLA-B polymorphism has been associated with 25(OH)D concentrations (Miettinen et al., 2017b).

There are many possible ways to analyse a nested case-control study like this and several methods were tested before selecting the ones we used in the publications. Autoantibody positivity and/or T1D were selected as end points and matched case-control pairs made the analysis more reliable in reducing the background noise. We tried area-under-the-curve –analysis, multiple of means –analysis, chi-square tests, the published analysis and finally conditional regression analysis, and they all gave similar results: there were no differences between cases and controls in this data set.

We found that children who progress to IA or T1D have similar circulating 25(OH)D concentrations as unaffected children, when they are carefully matched for age, geographic location in latitude of the living place, HLA-DQB1 risk, sex and time of blood draw. The results of this prospective study suggest that the development of T1D is not associated with marked changes in the vitamin D status.

7 Conclusions and Future Perspectives

Conclusions

* Serum 25(OH)D concentrations were very low at birth 1994-2004, and maternal vitamin D intake was below recommendations during pregnancy.
* From the age of three months to the age of two years, no seasonal variation can be seen in 25(OH)D concentrations, and most children in the study cohort had sufficient vitamin D levels (>50 nmol/l).
* Vitamin D fortification of foodstuffs, mainly dairy products, which was begun in 2003, increased serum 25(OH)D concentrations remarkably in Finnish children.
* Seasonal variation is prominent from the age of two years, and especially in 1998-2003, many children were vitamin D deficient in winter.
* Seasonal variation of serum 25(OH)D is remarkable in the Northern latitudes and several samples are needed in order to determine the vitamin D status of an individual, as the individual variation is great as well.
* In matched case-control pairs, serum 25(OH)D concentrations were not directly associated with the appearance of diabetic autoantibodies nor with the risk of T1D, not at birth nor later in the childhood.

Future perspectives

* The current vitamin D status of Finnish children should be studied.
* In future studies, adding data on genetic polymorphisms linked with vitamin D pathways on mothers, if not both parents, might deepen our understanding of vitamin D and the risk of T1D in the offspring.
* A detailed HLA-analysis providing more information on the genetic risk of T1D in children might further improve also our understanding on serum 25(OH)D concentrations.
* Large studies on serum concentrations of other vitamins and T1D, together with their genetic and environmental interactions, may shed more light on the complex interaction between vitamin D, different cell types of the immune system, and T1D.

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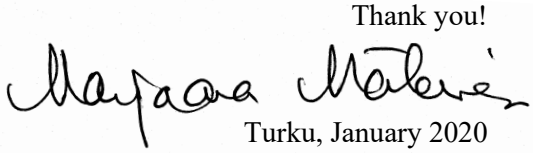

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