Tampere University

MARKUS MATTILA

Vitamin C, Iron, Nitrate, and Nitrite in the Development of Islet Autoimmunity and Type 1 Diabetes

Two Prospective Birth Cohort Studies of Individuals at Increased Genetic Risk of Type 1 Diabetes

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ACADEMIC DISSERTATION
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Tampere, March 2023
Markus Mattila

ABSTRACT

Type 1 diabetes is the result of an autoimmune reaction against insulin-producing beta cells in the pancreas, leading to the eventual termination of insulin production. Since the body can no longer control blood glucose levels, lifetime insulin therapy is required. The causes and mechanisms are still relatively unknown, but the emergence of specific autoantibodies in blood circulation signifies islet autoimmunity development. Genetics and environmental triggers, such as diet, might affect inflammation markers, which either induce or suppress autoimmune reactions and further lead to development type 1 diabetes.

The aim of this thesis was to explore whether plasma vitamin C status in childhood is associated with the risk of type 1 diabetes development and whether vitamin C metabolism-related genotypes modify the association. Furthermore, the aim was to explore whether maternal intake of vitamin C, iron, nitrate, and nitrite during pregnancy is associated with the risk of developing type 1 diabetes.

This thesis was a part of multinational The Environmental Determinants of Diabetes in the Young (TEDDY) Study (n = 8,677) and Finnish Type 1 Diabetes Prediction and Prevention (DIPP) Study (n = 7,782). The children studied carry a high or moderate genetic risk for type 1 diabetes. Children were monitored for the emergence of autoantibodies associated with type 1 diabetes and the development of clinical type 1 diabetes between 3- and 12-month intervals up to 15 years of age (DIPP) or up to the diagnosis of type 1 diabetes (TEDDY). Childhood plasma vitamin C status was measured up to 6 years of age, and the genotypes related to vitamin C metabolism were assessed in the TEDDY Study. Maternal diet was assessed using a semiquantitative validated food frequency questionnaire (FFQ) covering total diet during 8th month of pregnancy in the DIPP Study. The statistical methods used were the Cox proportional-hazards model and condition logistic regression.

Childhood plasma vitamin C status was associated with a decreased risk of islet autoimmunity. Vitamin C metabolism-related genotypes did not modify this association. Maternal intake of vitamin C, iron, nitrate, or nitrite during pregnancy was not associated with the risk of islet autoimmunity or type 1 diabetes in offspring.

The results suggest that a high plasma vitamin C status in the early childhood might protect against islet autoimmunity. Maternal intake of vitamin C, iron, nitrate, or nitrite during pregnancy was not associated with childhood type 1 diabetes development. This may result from tightly regulated nutrient transportation in the placenta.

Keywords: vitamin C, children, pregnancy, islet autoimmunity, type 1 diabetes, autoantibodies, epidemiology, nutrition

TIIVISTELMÄ

Tyypin 1 diabetes on autoimmuunisairaus, jossa elimistön puolustusjärjestelmä tuhoaa haiman insuliinia tuottavia saarekesoluja johtaen lopulta insuliinin tuotannon loppumiseen. Kun elimistö ei kykene enää säätelemään veren sokeripitoisuutta, tarvitaan hoidoksi eliniän kestävää insuliinin annostelua. Toistaiseksi ei tarkasti tiedetä sairastumisen syitä eikä mekanismeja, mutta tiettyjen autovasta-aineiden ilmaantuminen verenkiertoon kuvastaa beetasoluauto-immuniteetin kehittymistä. Perimä ja ympäristötekijät kuten ravinto voivat vaikuttaa elimistön tulehdustekijöihin, jotka joko edistävät tai ehkäisevät autoimmuunireaktion ja edelleen kliiniseen tyypin 1 diabeteksen kehittymistä.

Väitöskirjatutkimuksessani selvitettiin lapsuusiän veren C-vitamiinipitoisuuden yhteyttä tyypin 1 diabeteksen kehittymiseen sekä muokkaavatko C-vitamiinin aineenvaihduntaa säätelevät genotyypit tätä yhteyttä. Lisäksi selvitettiin äidin raskaudenaikaisen ravinnon C-vitamiinin, raudan, nitraatin ja nitriitin saannin yhteyttä tyypin 1 diabeteksen kehittymiseen.

Aineistoni kerättiin kansainvälisestä The Environmental Determinants of Diabetes in the Young (TEDDY) (n=8,677) sekä suomalaisesta Type 1 Diabetes Prediction and Prevention (DIPP) (n=7,782) seurantatutkimuksista. Tutkimuksessa olevilla lapsilla on geneettisesti suurentunut sairastumisalttius tyypin 1 diabetekseen. Lapsilta seurattiin tyypin 1 diabetekseen liitettyjen autovasta-aineiden ilmaantumista sekä kliinisen tyypin 1 diabeteksen kehittymistä 3–12 kuukauden välein 15-vuotiaaksi asti (DIPP) tai kunnes tyypin 1 diabetes diagnosoitiin (TEDDY). TEDDY-tutkimuksessa veren C-vitamiinipitoisuus mitattiin vuosittain 6-vuotiaaksi asti ja lapsilta kerättiin tiedot C-vitamiinin aineenvaihduntaa säätelevistä geeneistä. DIPP-tutkimuksessa äitien ruoankäyttötiedot mitattiin validoidulla ruoankäytön kyselylomakkeella kahdeksannen raskauskuukauden aikana, mikä kuvailee koko raskaudenaikaisetta ruoankäyttöä. Tilastollisina analyyseinä käytettiin Cox regressioanalyysia sekä logistista regressioanalyysia.

Veren korkea C-vitamiininpitoisuus lapsuusiässä oli suojaavassa yhteydessä beetasolu-autoimmuniteetin riskiin. C-vitamiinin aineenvaihduntaa säätelevät genotyypit eivät muokanneet tätä yhteyttä. Äidin raskaudenaikainen C-vitamiinin,

raudan, nitraatin tai nitriitin saanti ei näyttäisi olevan yhteydessä lapsen beetasolu-autoimmuniteetin tai kliinisen tyypin 1 diabeteksen riskiin.

Tulokset viittaavat siihen, että korkea veren C-vitamiininpitoisuus varhaislapsuudessa saattaa suojata beetasolu-autoimmuniteetin kehittymiseltä. Äidin raskaudenaikaisen C-vitamiinin, raudan, nitraatin tai nitriitin saanti ei ole yhteydessä lapsen riskiin sairastua tyypin 1 diabetekseen. Ravintoaineiden tarkkaan säädelty kuljetus istukassa voi olla mahdollinen selittävä tekijä.

Avainsanat: C-vitamiini, lapset, raskaus, beetasolu-autoimmuniteetti, tyypin 1 diabetes, autovasta-aineet, epidemiologia, ravitsemus

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ABBREVIATIONS

DHA	Dehydroascorbic acid
DIPP	Type 1 Diabetes Prediction and Prevention Study
FFQ	Food frequency questionnaire
FDR	First degree relative
GADA	Glutamic acid decarboxylase antibody
HLA	Human leukocyte antigen
HR	Hazard ratio
IA-2A	Insulinoma antigen-2 antibody
IAA	Insulin autoantibody
ICA	Islet cell autoantibody
OR	Odds ratio
PCA	Principal component analysis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SLC23A1	Solute Carrier Family 23 Member 1
SLC23A2	Solute Carrier Family 23 Member 2
SLC2A/GLUT	Solute Carrier Family 2A/Glucose transporter
SNP	Single nucleotide polymorphism
SVCT-1	Sodium-dependent vitamin C transporter 1
SVCT-2	Sodium-dependent vitamin C transporter 2
TEDDY	The Environmental Determinants of Diabetes in the Young Study

ORIGINAL PUBLICATIONS

I. Mattila M, Erlund I, Lee H-S, Niinistö S, Uusitalo U, Aronsson CA, Hummel S, Parikh H, Rich SS, Hagopian W, Toppari J, Lernmark Å, Ziegler A, Rewers M, Krischer J, Norris JM, Virtanen SM for the TEDDY Study Group. Plasma ascorbic acid and the risk of islet autoimmunity and type 1 diabetes mellitus: The Environmental Determinants of Diabetes in the Young (TEDDY) Study. Diabetologia. 2020 Feb;63(2):278-286. doi: 10.1007/s00125-019-05028-z.

II. Mattila M, Niinistö S, Takkinen HM, Tapanainen H, Reinivuo H, Åkerlund M, Suomi J, Ahonen S, Ilonen J, Toppari J, Knip M, Veijola R, Virtanen SM. Maternal nitrate and nitrite intakes during pregnancy and risk of islet autoimmunity and type 1 diabetes – the DIPP cohort study. J Nutr. 2020 Nov 19;150(11):2969-2976. doi: 10.1093/jn/nxaa250.

III. Mattila M, Hakola L, Niinistö S, Takkinen HM, Tapanainen H, Ahonen S, Ilonen J, Toppari J, Veijola R, Knip M, Virtanen SM. Maternal vitamin C and iron intake during pregnancy and the risk of islet autoimmunity and type 1 diabetes in children: a birth cohort study. Nutrients. 2021 Mar 13;13(3):928. doi: 10.3390/nu13030928.

1 INTRODUCTION

Type 1 diabetes is a chronic autoimmune disease, and its incidence is increasing at 3–5% per year (Atkinson et al., 2014; Forlenza & Rewers, 2011). Finland has the highest incidence of type 1 diabetes (Harjutsalo, et al., 2013; Patterson et al., 2019). A steady increase continued from 1950 to 2006, after which the worldwide incidence rate became steadier (Patterson et al., 2019). Genetic susceptibility plays a substantial role in disease development, particularly genes in the human leukocyte antigen (HLA) region (Barrett et al., 2009; Noble & Erlich, 2012). Studies have observed different disease outcomes between identical twins, incidence variations between populations, and seasonal differences in disease development (Barrett et al., 2009; Knip & Simell, 2012). This strongly suggests that environmental exposure, in conjunction with genes, influences disease development (Knip & Simell, 2012; Moltchanova e al., 2009; Nisticò et al., 2012). Furthermore, the incidence in Finland began to decrease from 2003 to 2018, suggesting changes in environmental factors (Parviainen et al., 2020).

Several environmental factors have been suggested as causes for this decrease, such as diet, environmental toxins, reduced or changed exposure to environmental microbes (improved hygiene), viral infections, alteration in gut microbiota, obesity, and increased growth (Virtanen, 2016; Forlenza & Rewers, 2011; Craig et al., 2019; Knip & Simell, 2012; Lindberg et al., 1999; Moltchanova et al., 2009). Nutritional factors might influence the development of type 1 diabetes through several mechanisms, such as increased oxidative stress, decreased insulin response, increased inflammation, beta cell apoptosis, or impaired immune function (Bodin et al., 2015).

Nitrate and nitrite are naturally occurring compounds in foods and drinking water, but they are also used as food additives. A high intake of nitrate and nitrite might lead to the generation of peroxynitrite, reactive nitrogen intermediates, and N-nitroso compounds, which are suggested to be toxic to beta cells (de la Monte et al., 2009; Longnecker & Daniels, 2001; Wilson et al., 1983). However, previous studies on humans have been mostly ecological surveys and case-control studies which have turned out inconsistent results (Dahlquist et al., 1990; Helgason & Jonasson, 1981; Kostraba et al., 1992; Muntoni et al., 2013; Parslow et al., 1997; Virtanen et al., 1994).

Beta cells have low activity of free-radical detoxifying and redox-regulating enzymes; thus, dietary antioxidants might play an important role in protecting against oxidative

damage (Lazo-de-la-Vega-Monroy & Fernández-Mejía, 2013; Lei & Vatamaniuk, 2011). Vitamin C, as a dietary antioxidant, might protect against type 1 diabetes (al-Zuhair & Mohamed, 1998; Kaneto et al., 1999). However, the available results from retrospective case-control studies are inconsistent and limited (Benson et al., 2010; Dahlquist et al., 1990; Glatthaar et al., 1988). It is also suggested that in addition to assessment of dietary intake, plasma vitamin C status might also be required since it more accurately represents vitamin C availability in the body (Dehghan et al., 2007). Furthermore, plasma and tissue vitamin C availability differs between individuals due to genetic variations in vitamin C metabolism-related genes (Michels et al., 2013); thus, certain genotypes might contribute to the risk of type 1 diabetes.

Iron is an essential trace mineral required for oxygen transport in circulation and as a component of cellular enzymes. Maintaining iron balance is crucial, as iron has the ability to donate and accept electrons; thus, it functions as a catalyst for reactive oxygen species (ROS) (Conrad & Umbreit, 2000). It has been postulated that the generation of iron-induced ROS in beta cells might lead to beta cell dedifferentiation or cell death via apoptosis or ferroptosis, which could increase the risk of developing type 1 diabetes (Hansen et al., 2014).

The development of type 1 diabetes may be influenced as early as the fetal period (Lindberg et al., 1999; Oresic et al., 2013). Only one previous study explored the association between maternal intake of vitamin C during pregnancy and the risk of islet autoimmunity, and they found no association (Uusitalo et al., 2008). One previous cohort study suggested that maternal use of iron supplements during pregnancy might increase the risk of type 1 diabetes in offspring (Stordal et al., 2018). Furthermore, a nationwide case-control study suggested that a high maternal intake of nitrate and nitrite during pregnancy might increase the risk of type 1 diabetes in offspring (Virtanen et al., 1994).

Prospective studies are limited, and the results are inconsistent, which highlights the fact that more studies exploring the association between vitamin C, iron, nitrate, and nitrite intake and the development of type 1 diabetes are needed. The first aim of this dissertation is to study whether childhood high plasma vitamin C status protects against the development of islet autoimmunity or type 1 diabetes. Whether vitamin C metabolism-related genetic variations influence the outcome was the second objective of the study. The third main objective was to evaluate whether the maternal intake of nitrate, nitrite, vitamin C, or iron during pregnancy is associated with the development of islet autoimmunity or type 1 diabetes in childhood.

2 REVIEW OF LITERATURE

2.1 Pathogenesis of type 1 diabetes

Type 1 diabetes results from an autoimmune reaction in which the immune system attacks and destroys beta cells in pancreatic islets. Beta cells produce the insulin hormone that regulates the uptake of glucose in tissues; thus, beta-cell death leads to a progressive decrease in insulin. This further results in impaired glucose tolerance and ultimately hyperglycemia and loss of insulin production (Atkinson et al., 2014). Clinical symptoms of type 1 diabetes, such as fatigue, thirst, and polyuria, appear at the very end of disease development, which has most likely continued several months from the initiation of autoimmune reaction (Gan et al., 2012). The disease can occur at any age but is commonly triggered during childhood, and the incidence increases with age, peaking at around 10–14 years of age. The incidence in children under 15 years old can range from 1 to 3 per 100,000 people per year in Asian and South American countries and up to 30–60 per 100,000 people in Scandinavia (DIAMOND Project Group, 2006; Patterson et al., 2019). The global incidence rate has increased since the 1960s (Figure 1).

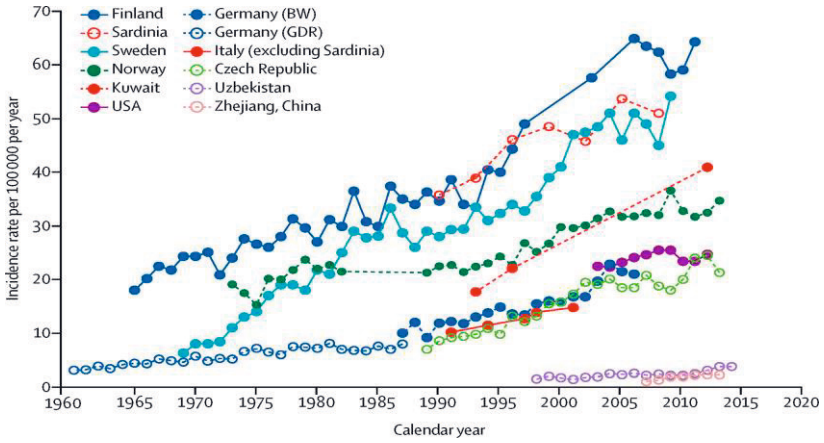

Figure 1. Incidence rates of type 1 diabetes from 1960–2015. Abbreviations: GDR, German Democratic Republic; BW, Baden-Württemberg (Data from Norris, et al., 2020)

The preclinical phase, called islet autoimmunity, is determined by the emergence of specific autoantibodies in the blood and the gradual loss of beta cells before the symptoms occur. Five disease-related autoantibodies have been found to predict type 1 diabetes: islet cell (ICA), insulin (IAA), glutamic acid decarboxylase (GADA), protein tyrosine phosphatase-related islet antigen 2 (IA-2A), and zinc transporter 8 (ZnT8A) autoantibodies (Knip, 2002; Knip & Siljander, 2008). Islet autoimmunity is staged according to the number of detectable autoantibodies (Mrena et al., 1999). Positivity for a single autoantibody is a marker of early islet autoimmunity, whereas two antibodies are a marker of advanced islet autoimmunity, and three to four antibodies suggest late progressive islet autoimmunity. ICA was discovered over 40 years ago and later confirmed to comprise a heterogenous group of autoantibodies: GADA, IA-2A, and ZnT8A (Knip et al., 2016). Islet autoimmunity observed in the first to second years of life is usually initiated by IAA (Ilonen et al., 2013). GADA is often the first-appearing antibody at 3–5 years of age or later (Ilonen et al., 2013), suggesting that positivity to GADA might precede a type 1 diabetes diagnosis in later childhood or adulthood. IA-2A and ZnT8A primarily appear in the later stage of autoimmunity development and suggest advanced beta-cell destruction rather than early autoimmunity development (Siljander & Knip, 2011; Williams & Long, 2019). Autoantibodies signal that disease development is ongoing, but they are not the cause of the disease. What triggers autoimmune reactions is still unknown.

Genetic variations play a substantial role in transnational differences in the incidence of type 1 diabetes. Distributions of specific DQ genotypes relate to a high risk for type 1 diabetes in the general population (Ronningen et al., 2001). Genes encoding class II HLA molecules suggest genetic risk; more closely, alleles DQB1*0302/0302 are associated with an increased risk for type 1 diabetes, and DQB1*0301, 0602, and 0603 are associated with a decreased risk in the Finnish population (Ilonen et al., 1996). As much as over 60% of Finnish subjects with type 1 diabetes carry high (DQB1 *0201/*0302) or moderate (DQB1*0302 without protecting alleles) risk compared to the general population, with a 14% prevalence of these genes.

Although genetic factors predict the risk of developing type 1 diabetes, the prevalence of high-risk HLA haplotypes has not increased over time. This suggests that environmental factors play a role in the development of type 1 diabetes (Gillespie et al., 2012; Norris et al., 2020). Environmental factors contribute to disease progression by either initiating the destruction of beta cells or modifying the progression of the pathogenetic process. Suggested early-life triggers might target children in utero, perinatally, or in the early years of life. It has been observed that autoantibody appearance has specific patterns depending on age; therefore, the environmental factors that trigger autoimmune reactions might also vary at different age points (Ilonen et al., 2013; Krischer et al., 2015). Proposed triggers

include virus infections, bacteria, diet, and toxins, which are suggested to influence gut development, gut permeability, or inflammation, either inducing or suppressing autoimmunity reaction (Bodin et al., 2015; Norris et al., 2020; Vaarala, 2011). Diet is also a subject of ongoing studies since it is a frequent daily exposure. Nutrients are required for normal development and well-being, but excessive amounts of certain nutrients might increase the risk of type 1 diabetes development by, for example, accelerating oxidative stress and apoptosis in beta cells (Bodin et al., 2015). Conversely, certain nutrients function as antioxidants, which might protect against developing type 1 diabetes (al-Zuhair & Mohamed, 1998).

2.2 Epidemiologic evidence on dietary factors in the development of type 1 diabetes

2.2.1 Vitamin C and the development of type 1 diabetes

A study performed on male albino rats showed that vitamin C suppressed the activity of interferon alpha, which is suggested to be cytotoxic to pancreatic islets (al-Zuhair & Mohamed, 1998). However, evidence in humans is limited. Studies exploring the association between maternal vitamin C intake during pregnancy and the development of type 1 diabetes in offspring are also scarce. One previous study within the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) Nutrition Study found no association between maternal intake of vitamin C and the risk of islet autoimmunity in offspring (Uusitalo et al., 2008) (Table 1). However, the association between vitamin C supplements exclusively and the risk of islet autoimmunity was not studied. In addition, type 1 diabetes as an outcome was not assessed in the study, and the follow-up time was short (median 4.4 y) (range: 0.2–8.8 y).

Table 1. Epidemiologic evidence from study exploring the association between maternal intake of vitamin C during pregnancy and the risk of type 1 diabetes-related outcomes in offspring

Study design	No. of participants	Country / Population	Exposure	Outcome	Association HR (95% CI)	Reference
Cohort	165 with diagnosed IA 4,132 without	Finland / genetic risk for T1D	Vitamin C (mg / day)	IA	Adjusted HR	Uusitalo et al., 2008
			Diet		0.96 (0.76, 1.22)[1] 0.92 (0.72, 1.18)[2]	
			Diet + supplement		0.97 (0.80, 1.18)[1] 0.96 (0.78, 1.17)[2]	

Abbreviations: IA, islet autoimmunity; T1D, type 1 diabetes

[1] Adjusted for energy by the residual method, HLA genotype, and family history of diabetes.

[2] Adjusted for energy by the residual method, HLA genotype, and family history of diabetes, sex, gestational age, maternal age, maternal education, maternal parity, maternal smoking during pregnancy, degree of urbanization, and region of birth (Oulu vs. Tampere area).

Epidemiologic studies exploring the association between childhood vitamin C intake and the development of type 1 diabetes are summarized in Table 2. An Australian case-control study observed that the use of vitamin C supplements was less common in type 1 diabetes case children than in control children, but dietary intake was not measured (Glatthaar et al., 1988). A Swedish case-control study observed that a higher dietary intake of vitamin C was associated with an increased risk of type 1 diabetes, but the association was no longer significant when stratified for intake of nitrate and nitrite (Mantel-Haenszel risk estimate, > 75th centile vitamin C intake: odds ratio [OR] 1.04, $p = 0.86$) (Dahlquist et al., 1990). Furthermore, a Canadian case-control study assessing nutrients from both diet and drinking water found no association between the dietary intake of vitamin C and the risk of type 1 diabetes (Benson et al., 2010). However, none of the previous studies assessed plasma vitamin C status.

Table 2. Epidemiologic evidence from studies exploring the association between childhood intake of vitamin C and the risk of type 1 diabetes-related outcomes in offspring

Study design	No. of participants	Country / Population	Exposure	Outcome	Association OR (95% CI)	Reference
Case-control	194 cases 753 controls	Australia / GP	Use of vitamin C supplements more than one month yes vs. no	T1D	Unadjusted OR **0.46 (0.30, 0.70)**	Glatthaar et al., 1988
Case-control	339 cases 528 controls	Sweden / GP	Vitamin C from food, (frequency of intake) < 25th centile 25–50th centile > 75th centile	T1D	Unadjusted OR 1.00 0.79 (0.56, 1.13) **1.54 (1.06, 2.23)**	Dahlquist et al., 1990
Case-control	57 cases 105 controls	Canada / GP	Vitamin C from food (mg / day) < 81.2 81.2–130.9 130.9–203.9 ≥ 203.90	T1D	Adjusted OR[1] 1.00 0.73 (0.25, 2.17) 0.58 (0.17, 1.97) 0.61 (0.16, 2.34)	Benson et al., 2010

Abbreviations: GP, general population; T1D, type 1 diabetes

[1] Adjusted for a third-generation family history of T1D, number of infections during the first 2 years of life, residential area, father's education level, child's age, sex, and energy intake.

2.2.2 Nitrate and nitrite and the development of type 1 diabetes

Mice fed with smoked cured mutton containing N-nitroso compounds, such as nitrosamines and nitrosamides, developed insulin-dependent diabetes (Helgason et al., 1982), suggesting that dietary intake of these compounds might play a role in disease development. Ecological surveys have suggested that a high intake of nitrate and nitrite from drinking water could increase the risk of type 1 diabetes (Kostraba et al., 1992; Parslow et al., 1997; van Maanen et al., 2000), but others have found no association (Moltchanova et al., 2009; Muntoni et al., 2006). Observational studies in humans exploring nitrate and nitrite intake on the development of type 1 diabetes have been inconsistent. One study has assessed maternal intake of nitrate and nitrite and the childhood risk of type 1 diabetes (Table 3). In a Finnish case-control study, the highest quartile of maternal nitrite intake was associated with an increased risk of type 1 diabetes in offspring when adjusted for mother's education, age, smoking, and place of residence (Virtanen et al., 1994). Although only the highest quartile was statistically significant, the risk estimate increased as nitrite intake increased (Table 3). Furthermore, the dietary intake of nitrite was higher in the case children's mothers than in the control children's mothers (0.9 mg/d vs. 0.8 mg/d). Studies in general are few; no prospective studies have been conducted so far, and no

studies have explored the association between nitrate and nitrite intake and the risk of islet autoimmunity.

Table 3. Epidemiologic evidence from study exploring the association between maternal intake of nitrate and nitrite and the risk of type 1 diabetes-related outcomes in offspring

Study design	No. of participants	Country / Population	Exposure	Outcome	Association OR (95% CI)	Reference
Case-control	684 cases 595 controls	Finland / GP	Maternal diet (mg / day)	T1D	Adjusted OR[1]	Virtanen et al., 1994
			Nitrate			
			1st quartile		1.00	
			2nd quartile		**0.62 (0.42, 0.91)**	
			3rd quartile		0.87 (0.61, 1.26)	
			4th quartile		0.80 (0.56, 1.16)	
			Nitrite			
			1st quartile		1.00	
			2nd quartile		1.15 (0.76, 1.74)	
			3rd quartile		1.29 (0.87, 1.91)	
			4th quartile		**1.98 (1.35, 2.90)**	

Abbreviations: GP, general population; T1D, type 1 diabetes

[1] Adjusted for maternal education, smoking, child's age, and place of residence.

Studies assessing the intake of nitrate and nitrite during childhood and the risk of type 1 diabetes are summarized in Table 4. A Swedish study observed that a high intake of nitrate and nitrite from food in childhood could increase the risk of type 1 diabetes (Dahlquist et al., 1990). The associations remained similar when standardized for the child's age, sex, family history of insulin-dependent diabetes, maternal education, and maternal age. Similarly, a Finnish study found a positive association between a high dietary intake of nitrite in childhood and an increased risk of type 1 diabetes (Virtanen et al., 1994). Conversely, an Australian case-control study found no association between childhood nitrosamine (a form of N-nitroso compound) intake from diet and the risk of type 1 diabetes (Verge et al., 1994). Furthermore, a Canadian case-control study found no association between nitrite and nitrosamine intake from diet and the risk of type 1 diabetes in childhood (Benson et al., 2010). Other studies assessed nitrate and nitrite intake from drinking water but not from diet (Table 4). Nitrite, which is used as a preservative in processed meat products along with animal protein, might accelerate N-nitroso compound formation. In addition to animal study (Helgason et al., 1982), high consumption of red meat and especially processed meat products in childhood were found to increase the risk of type 1 diabetes in a Sardinian case-control study (Muntoni et al., 2013). Childhood intake of nitrosamines was associated with an increased risk of type 1 diabetes in a Swedish case-control study (Dahlquist et al., 1990). When stratified by the intake of protein, an increased risk of type 1 diabetes was observed in children with the highest intake of both N-nitroso compounds and protein (OR 2.12, 95% Cl 1.11, 4.04).

Table 4. Epidemiologic evidence from studies exploring the association between intake of nitrate, nitrite, and nitrosamines during childhood and type 1 diabetes-related outcomes

Study design	No. of participants	Country / Population	Exposure	Outcome	Association OR, HR, or SIR (95% CI)	Reference
Case-control	339 cases 528 controls	Sweden / GP	Diet (% of intake)	T1D	Unadjusted OR	Dahlquist et al., 1990
			Nitrate or nitrite			
			< 25%		1.00	
			25–75%		0.84 (0.59, 1.19)	
			> 75%		**2.41 (1.64, 3.54)**	
			Nitrosamines			
			< 25%		1.00	
			25–75%		**1.73 (1.23, 2.44)**	
			> 75%		**2.56 (1.83, 3.59)**	
Case-control	684 cases 595 controls	Finland / GP	Diet (mg / day)	T1D	Adjusted OR[1]	Virtanen et al., 1994
			Nitrate			
			1st quartile		1.00	
			2nd quartile		0.82 (0.59, 1.14)	
			3rd quartile		0.99 (0.72, 1.36)	
			4th quartile		0.94 (0.68, 1.29)	
			Nitrite			
			1st quartile		1.00	
			2nd quartile		1.16 (0.82, 1.65)	
			3rd quartile		**1.49 (1.06, 2.10)**	
			4th quartile		**2.32 (1.67, 3.24)**	
Case-control	217 cases 258 controls	Australia / GP	Nitrosamines from diet	T1D	Adjusted OR[2]	Verge et al., 1994
			lowest tertile		1.00	
			middle tertile		0.71 (0.44, 1.14)	
			highest tertile		1.07 (0.66, 1.74)	
Retrospective cohort	517 with T1D	UK / GP	Nitrate from tap water (mg / L)	T1D	SIR **111.8 (96, 129)**	Zhao et al., 2001
Nested case-control	95 cases 139 controls	Germany / family history of T1D	Nitrate from tap water (mg / L)	T1D	Adjusted OR[3] 0.6 (0.4, 1.0)	Winkler et al., 2008

Abbreviations: GP, general population; SIR, standardized incidence ratio; T1D, type 1 diabetes

[1] Adjusted for maternal education, age, smoking, and place of residence.

[2] Adjusted for intake of fluids, cow's milk protein, and/or cereal protein.

[3] Adjusted for HLA-DR 3/4 genotype, HLA-DR 4/4 genotype, and maternal type 1 diabetes.

Table 4. Continue

Study design	No. of participants	Country / Population	Exposure	Outcome	Association OR, HR, or SIR (95% CI)	Reference
Case-control	57 cases 105 controls	Canada / GP	Diet	T1D	Adjusted OR[4]	Benson et al., 2010
			Nitrate (mg / day)			
			< 5.66		1.00	
			5.66–7.27		1.01 (0.28, 3.61)	
			7.28–9.00		1.19 (0.31, 4.52)	
			≥ 9.01		2.25 (0.45, 11.14)	
			Nitrite (mg / day)			
			< 1.83		1.00	
			1.83–3.26		0.19 (0.28, 3.18)	
			3.27–4.81		1.24 (0.35, 4.47)	
			≥ 4.82		1.30 (0.30, 5.59)	
			Nitrosamine (µg / day)			
			< 0.01		1.00	
			0.01–0.029		0.57 (0.21, 1.57)	
			0.03–0.039		0.66 (0.18, 2.45)	
			≥ 0.04		0.62 (0.19, 2.00)	
			Diet and drinking water			
			Nitrate (mg / day)			
			< 7.20		1.00	
			7.20–9.86		3.22 (0.93, 11.17)	
			9.87–11.88		1.02 (0.23, 4.46)	
			≥ 11.89		2.81 (0.60, 13.23)	
			Nitrate and nitrite (mg / day)			
			< 9.56		1.00	
			9.56–13.20		2.70 (0.77, 9.43)	
			13.21–16.72		1.66 (0.42, 6.58)	
			≥ 16.73		2.39 (0.46, 12.37)	
Case-control	130 cases 323 controls	Finland / high and low risk popluation[1]	Nitrate from tap water (mg / L)	T1D	Adjusted OR[5]	Samuelsson et al., 2011
			Nitrate		**1.32 (1.06, 1.64)**	
			Nitrite		0.36 (0.06, 2.03)	

Abbreviations: GP, general population; SIR, standardized incidence ratio; T1D, type 1 diabetes

[4] Adjusted for a third-generation family history of T1D, number of infections during the first 2 years of life, residential area, father's education level, child's age, sex, and energy intake.

[5] Adjusted for municipality of residents. A total of seven municipalities with high annual incidence of type 1 diabetes and six municipalities with lowest incidence.

2.2.3 Iron and the development of type 1 diabetes

Studies have suggested that prenatal exposure to iron might increase the risk of type 1 diabetes in offspring. In a Danish case-control study, a high cord blood iron status was associated with an increased risk of type 1 diabetes in offspring (Kyvsgaard et al., 2017). Studies assessing maternal iron intake on the risk of type 1 diabetes are summarized in Table 5. The Norwegian Mother and Child Cohort study found no association between cord plasma iron biomarkers and the risk of type 1 diabetes, but maternal use of iron supplements was associated with an increased risk of type 1 diabetes in offspring (Stordal et al., 2018). A Danish National Birth Cohort study found no association between maternal iron supplement use and type 1 diabetes risk in offspring (Thorsen et al., 2019). The association between maternal iron intake and the childhood risk of islet autoimmunity was not assessed in these studies.

Table 5. Epidemiologic evidence from studies exploring the association between maternal intake of iron during pregnancy and the risk of type 1 diabetes-related outcomes in offspring

Study design	No. of participants	Country / Population	Exposure	Outcome	Association OR HR (95% CI)	Reference
Cohort	373 with T1D 93,872 without	Norway / GP	Use of iron supplement Cord plasma ferritin (+50mg/l) Cord plasma transferrin (0,5 mg/l)	T1D	Adjusted HR or OR[1] **HR 1.33 (1.06, 1.67)** OR 1.05 (0.99, 1.13) OR 0.91 (0.81, 1.01)	Størdal et al., 2018
Cohort	238 with T1D 63,693 without	Denmark / GP	Use of pure iron supplements Yes vs. no	T1D	Adjusted HR[2] 1.05 (0.76, 1.45)	Thorsen et al., 2019

Abbreviations: GP, general population; T1D, type 1 diabetes

[1] Adjusted for maternal age and education, smoking, parity, birth weight and prematurity, pre-pregnancy body mass index (BMI), mode of delivery, diagnosed maternal anemia, maternal type 1 diabetes, and maternal celiac disease.

[2] Adjusted for socioeconomic status, mode of delivery, pre-pregnancy BMI, age, smoking during pregnancy, parity, gestational age, maternal age, and breastfeeding.

The epidemiologic studies exploring association between childhood iron intake and the development of type 1 diabetes are summarized in Table 6. Primarily, studies have assessed iron intake only from drinking water or iron supplements. The results of these studies have been inconsistent. In a US case-control study, dietary iron intake during the first 4 months of age was associated with an increased risk of type 1 diabetes (Ashraf et al., 2010). In contrast, a Canadian case-control study observed no association between dietary intake of iron and the risk of type 1 diabetes (Benson et al., 2010). Furthermore, a Norwegian cohort study found no association between children's iron supplement use up to 18 months of age and the risk of type 1 diabetes (Stordal et al., 2018). However, a Danish cohort study observed an association between iron droplet use at the age of 18 months and a decreased risk of type 1 diabetes (Thorsen et al., 2019). Childhood dietary intake was not assessed in these studies.

Table 6. Epidemiologic evidence from studies exploring the association between childhood intake of iron and type 1 diabetes-related outcomes

Study design	No. of participants	Country / Population	Exposure	Outcome	Association OR, HR, or IRR (95% CI)	Reference
Retrospective cohort[1]	517 with T1D	UK / GP	Iron from tap water (mg / L)	T1D	IRR 98.2 (84, 115)	Zhao et al., 2001
Nested case-control[1]	95 cases 139 controls	Germany / family history of T1D	Iron from tap water (mg / L)	T1D	Adjusted OR[2] 1.0 (0.4, 2.3)	Winkler et al., 2008
Case-control[1]	128 cases 67 controls	US. / GP and high risk poplulation[4]	Iron from diet (mg / day)	T1D	Adjusted OR[5] **2.01 (1.18, 3.41)**	Ashraf et al., 2010
Case-control	57 cases 105 controls	Canada / GP	Iron from diet (mg / day) <10.00 10.00–13.27 13.28–16.11 ≥16.12	T1D	Adjusted OR[7] 1.00 1.19 (0.34, 4.20) 0.87 (0.20, 3.75) 1.22 (0.22, 6.85)	Benson et al., 2010
Case-control[1]	130 cases 323 controls	Finland / high and low risk population	Iron from tap water (mg / L)	T1D	Adjusted OR[3] 1.56 (0.99, 2.44)	Samuelsson et al., 2011
Cohort	373 with T1D 93,872 without	Norway / GP	Iron supplement (yes vs. no) < 6 mo. of age < 18 mo. of age Any use by 0–18 mo.	T1D	Adjusted HR[8] 1.43 (0.80, 2.56) 1.20 (0.49, 2.91) 1.36 (0.82, 2.25)	Størdal et al., 2018
Cohort	191 with T1D 51,668 without	Denmark / GP	Iron droplets at 18 mo. of age Yes vs. no	T1D	Adjusted HR[9] **0.74 (0.55, 1.00)**	Thorsen et al., 2019

Abbreviations: IRR, incidence rate ratio; GP, general population; T1D, type 1 diabetes

[1] Meta-analysis of three case-control and one retrospective cohort study indicating inconsistent results between childhood iron intake and type 1 diabetes (Sogaard et al., 2017).

[2] Adjusted for HLA-DR 3/4 genotype, HLA-DR 4/4 genotype, and maternal type 1 diabetes.

[3] Adjusted for municipality of residents. A total of seven municipalities with high annual incidence of type 1 diabetes and six municipalities with lowest incidence.

[4] Cases and controls were selected based on whether or not the sibling had type 1 diabetes.

[5] Adjusted for birth weight, birth order, and age at the time of the survey.

[6] Adjusted for age, sex, and energy intake.

[7] Adjusted for a third-generation family history of T1D, number of infections during the first 2 years of life, residential area, father's education level, child's age, sex, and energy intake.

[8] Adjusted for parity, smoking, birth weight, prematurity, pre-pregnancy BMI, mode of delivery, maternal anemia, maternal age, maternal education, maternal type 1 diabetes, and maternal celiac disease.

[9] Adjusted for socioeconomic status, mode of delivery, pre-pregnancy BMI, age, smoking during pregnancy, parity, gestational age, maternal age, breastfeeding.

2.3 Characteristics of vitamin C and possible mechanisms of action in the development of type 1 diabetes

Water-soluble vitamin C is an essential nutrient for humans because the human body cannot synthesize it endogenously (Frei et al., 2012). It was first shown to prevent scurvy, but several other functions were later identified Table 7. Vitamin C occurs in three forms l-ascorbic acid, intermediate ascorbate radical, and l-dehydroascorbic acid (DHA) which are interchangeable enzymatically. The function of vitamin C is based on ascorbate recycling, which is the ability to circulate between ascorbic acid and DHA.

Table 7. Vitamin C functions, formulated from (Padayatty & Levine, 2016)

Vitamin C functions
Cofactor of enzymes (mammals)
Biosynthesis
• Norepinephrine
• Carnitine
Hydroxylation
• Collagen
• Hypoxia-inducible factor (HIF)
Metabolism
• Tyrosine
Amidation
• Peptide hormones
Demethylation
• Histone
Reducing agent
• Enhanced iron absorption in the small intestine
Antioxidant
• Gene expression and mRNA translation regulation
• Oxidant damage prevention to DNA and intracellular proteins
• Prevention of N-nitroso compounds formation in the stomach
Pro-oxidant
• Damage to DNA
• Damage to cancer cells via hydrogen peroxide formation

2.3.1 Dietary sources of vitamin C

Vitamin C is found in many fruits, berries, and vegetables (Table 8). Vitamin C is sensitive to decline during food preparation. Transportation, storage conditions and time, peeling, cutting, and cooking can reduce the vitamin C content in vegetables and fruits via ascorbate oxidase enzyme (World Health Organization [WHO], 2004). Blanching vegetables and acidity, such as pickling, prevent enzyme activity.

In general, the Finnish adult population reaches the recommended 75 mg/day of vitamin C, but one out of five men does not due to low consumption of vegetables, according to the National FinDiet 2017 Survey (Valsta et al., 2018). During pregnancy, the requirement for vitamin C increases moderately, and an approximately 10 mg/day increase in vitamin C intake is suggested to supply the need for growing fetuses in the last trimester (WHO, 2004).

Table 8. Vitamin C content of selected foods (Finnish Institute for Health and Welfare, 2019b)

Food source	Vitamin C mg/100g
Rose hip	601
Red sweet pepper	185
Blackcurrant	128
Orange	51
Strawberry	46
Swede / rutabaga, cooked	30
Tomato	14
Lingonberry	11
Bilberry	7
Potato, boiled	6

2.3.2 Metabolism of vitamin C

Vitamin C is absorbed in the intestine as ascorbic acid or DHA via specific membrane transporters in the apical brush border membrane (Lykkesfeldt & Tveden-Nyborg, 2019). Ascorbic acid is transported actively through a sodium L-ascorbic acid transporter (SVCT), which has two isoforms: hSVCT1 and hSVCT2. In the basolateral membrane, ascorbic acid is transported to plasma by diffusion but possibly also by facilitated diffusion or active transporter protein. DHA is transported to the epithelium by facilitating diffusion via glucose transporters (GLUT): GLUT1 or GLUT3. It is then either converted to ascorbic acid or transported to the bloodstream via GLUT1 and GLUT2 transporters in the basolateral membrane.

SVCT1 maintains systemic ascorbic acid homeostasis, while SVCT2 accounts for ascorbic acid locally demands (Eck et al., 2013). High capacity/low affinity SVCT1 is an absorptive transporter regulating the uptake and reuptake of ascorbic acid; thus, these are found in the intestinal membrane, renal tubules, and liver (Padayatty & Levine, 2016). Transport of ascorbic acid from the bloodstream to various tissues is primarily regulated by low capacity/high affinity SVCT2. DHA is transported to various tissues by facilitated diffusion via GLUTs (Shaghaghi et al., 2016). A total of 5 transporters have been identified: SLC2A1 (GLUT1), SLC2A2 (GLUT 2), SLC2A3 (GLUT3), SLC2A4 (GLUT4), and SLC2A8 (GLUT8), from which GLUT1, GLUT3, and GLUT4 are the most important DHA transporters in humans (Wilson, 2005). Inside the cell, DHA is immediately reduced to ascorbic acid, as DHA has a half-life of only a few minutes. As ascorbic acid is oxidized intracellularly, the resulting DHA is transported out of the cell or reduced back to ascorbic acid (Lykkesfeldt & Tveden-Nyborg, 2019). DHA transport is important, especially during inflammation, during which the antioxidant reaction of ascorbic acid results in increased oxidation to DHA (Schorah, 1992). DHA competes with glucose in transportation; thus, DHA diffusion is inhibited in some cells by excessive glucose in the plasma (Rumsey et al., 2000, 1997). However, clinical significance in humans requires further evidence.

In healthy individuals, the plasma steady-state vitamin C status, primarily in the form of ascorbic acid, reaches a maximum of about 70–80 μM when the intake is increasing (Padayatty & Levine, 2016). It has been suggested that a daily intake of 200–400 mg of vitamin C is needed to keep the plasma status saturated (Frei et al., 2012). Vitamin C is absorbed completely when intake is under 30 mg/day but when the intake is between 30–180 mg/day, the absorption decreases to 80–90% (Mutanen et al., 2021) (Table 9).

Although diet is the only source of vitamin C in humans, the relationship between dietary intake and plasma vitamin C status may be more complex. Based on observational studies, several factors influence the diet–plasma relationship, such as smoking, pregnancy, infection, stress, body size, and intake of some other nutrients (Dehghan et al., 2007). Furthermore, diet-recording methods and food databases cannot reliably take into account the declining vitamin C content during the processing of vegetables, fruits, and berries. As a water-soluble vitamin, vitamin C is prone to decline due to long storage times, peeling, cutting, cooking, and other types of processing (Frei et al., 2012). Thus, it has been proposed that plasma vitamin C can reflect vitamin C function more accurately than intake (Dehghan et al., 2007).

Table 9. The absorption efficiency of ascorbic acid (formulated from Mutanen et al., 2021)

Ingested ascorbic acid (mg)	Absorption efficiency (%)	Absorbed amount (mg)
180	90	160
1 000	75	750
3 000	40	1 200
5 000	24	1 200
12 000	16	1 920

2.3.3 Vitamin C transport across the placenta

The nutrients from maternal to fetal circulation are transferred via the syncytiotrophoblast, which is the primary barrier between maternal and fetal circulation (Prasad et al., 1998). Fetus rely solely on maternal vitamin C, most likely by transport via SVCT2 (Lykkesfeldt & Tveden-Nyborg, 2019). Maternal vitamin C status declines gradually from the first to the third trimester due to the increased volume of distribution and selective accumulation across the placenta (Juhl et al., 2016). Vitamin C is shown to be important for the development of brain and cognition in child (Lykkesfeldt & Tveden-Nyborg, 2019), and deficiency is observed to increase the risk of pre-eclampsia via oxidative stress. However, supplementation trials have not been shown to be beneficial (Conde-Agudelo et al., 2011).

2.3.4 Plasma vitamin C status and the impact of genetic variation

It has been suggested that plasma and tissue vitamin C availability differ between individuals due to genetic variation-induced alterations in proteins regulating vitamin C transport (Michels et al., 2013). The two SVCT proteins regulating ascorbic acid transport are encoded by the genes *SLC23A1* (Solute Carrier Family 23 Member 1) and *SLC23A2* (Solute Carrier Family 23 Member 2). Single nucleotide polymorphisms (SNPs) in these genes occur worldwide, some of which are ineffective, while others have been found to affect plasma ascorbic acid status. The most prominent is rs33972313, a low-frequency missense variant in *SLC23A1*, which has been consistently associated with a lower circulating ascorbic acid status (Timpson et al., 2010). Two intronic SNPs, rs6596473 and rs4257763, and one promoter SNP, rs10063949 in *SLC23A1*, have also been associated with ascorbic acid status, although not uniformly (Amir Shaghaghi et al., 2014; Cahill & El-Sohemy, 2009; Skibola et al., 2008; Timpson et al., 2010). *SLC23A2* intronic SNP rs6053005 has been associated with increased

plasma ascorbic acid status (Skibola et al., 2008), and rs1279683 has been associated with decreased plasma ascorbic acid status (Zanon-Moreno et al., 2011).

GLUTs managing the facilitated diffusion of DHA are encoded by members of the *SLC2A* solute carriers' gene family. The importance of *SLC2A* polymorphisms in vitamin C status homeostasis remains unknown (Michels et al., 2013). Genetic variants of GLUTs have been associated with diabetes complications and cancer (Shaghaghi et al., 2016). Whether these associations are the result of impaired vitamin C function is inconclusive, as plasma vitamin C status might not reflect tissue-specific vitamin C function (Banhegyi et al., 2014). However, since the interplay between ascorbic acid and DHA plays a substantial role in vitamin C function, it can be hypothesized that alterations in GLUTs could influence vitamin C status in the body.

Studies have suggested an association between vitamin C status–related genotypes and vitamin C status or oxidative stress–related diseases (Amir Shaghaghi et al., 2014; Michels et al., 2013). However, no studies have explored whether these genotypes are associated with the development of type 1 diabetes.

2.3.5 Oxidative stress in pancreatic beta cells and antioxidant properties of vitamin C

Mitochondria in cells produce energy by respiration and oxidative phosphorylation (Lei & Vatamaniuk, 2011). However, this also generates byproducts which are oxygen, nitrogen, or sulfur-based molecules with unpaired electrons known as free radicals. These molecules are also generated via external sources such as environmental toxins and infections. These active unstable molecules are prone to chemical reactions with other molecules and attack nearby cell molecules, causing damage. Oxygen-centered free radicals are called ROS, and nitrogen-centered ones are called reactive nitrogen species (RNS). Accumulation of these molecules would be detrimental for tissues and thus antioxidant mechanisms are required to regulate oxidative damage. The imbalance of the free radical accumulation and antioxidant defense causes oxidative stress.

Most tissues have intracellular free radical detoxifying and redox-regulating enzymes, such as catalase, glutathione peroxidase, and superoxide dismutase (Lei & Vatamaniuk, 2011). The intracellular formation of ROS in pancreatic beta cells is partly accountable for the release of pro-inflammatory cytokines from immune cells and further beta-cell death (Donath et al., 2008; Lenzen et al., 1996). Pancreatic beta cells are vulnerable to ROS accumulation because the activity of antioxidant enzymes is low. In comparison to the liver, islet cells contain only 1% catalase, 2% glutathione peroxidase, and 29%

superoxide dismutase activities (Lenzen et al., 1996; Tiedge et al., 1997). Thus, dietary antioxidants might be vital for beta-cell protection (Lei & Vatamaniuk, 2011; Miki et al., 2018).

Vitamin C is a strong antioxidant, as ascorbic acid scavenges oxygen and nitrogen radicals, during which it donates electrons and oxidizes to acerbate radicals further to DHA (Carr & Frei, 1999; Frei et al., 2012; Padayatty & Levine, 2016). In the endoplasmic reticulum, acerbate radical and DHA are reduced back to ascorbic acid by glutathione and other thiols. DHA is reduced within minutes; thus, very low levels can be detected in plasma in comparison to ascorbic acid. Studies in both animals and humans have suggested that vitamin C could be essential for the management of beta cells from oxidative stress and the further development of type 1 diabetes (al-Zuhair & Mohamed, 1998; Davison et al., 2008; Kaneto et al., 1999; Lei & Vatamaniuk, 2011; Miki et al., 2018). As hyperglycemia itself induces oxidative stress, vitamin C may also prevent progression from islet autoimmunity to type 1 diabetes (Acharya & Ghaskadbi, 2010). However, vitamin C can also reduce metals, such as iron and copper, which leads to the formation of superoxide and hydrogen peroxide (Padayatty & Levine, 2016; Valko et al., 2005). Nevertheless, vitamin C has not been shown to function as a pro-oxidant (Valko et al., 2005).

2.4 Characteristics of nitrate and nitrite and possible mechanisms of action in the development of type 1 diabetes

Nitrate (NO_3^-) and nitrite (NO_2^-) are essential inorganic nutrient compounds and primary sources of nitric oxide, which is required for the regulation of vasodilation and neurotransmitters (Kobayashi, 2018). Nitrate occurs in the soil after lightning or when soil microbes convert nitrogen to nitrate. Naturally occurring nitrite is much lower and is mostly found in drinking water. A prominent exposure to nitrite is from food additives, sodium, or potassium nitrite used as a preservative (European Food Safety Authority [EFSA], 2008; Hord et al., 2009).

2.4.1 Dietary sources of nitrate and nitrite

The sources of nitrate in the selected foods are shown in Table 10. The main source of nitrate is vegetables with the highest amounts in beets, radishes, and green leafy vegetables, whereas tubers, fruit vegetables, and seeds contain lower amounts (Hord et al., 2009). Drinking water can be a substantial source of nitrate and nitrite, but the contents vary depending on the location or source (Hord et al., 2009). Therefore, the WHO has determined acceptable concentrations of < 50 mg/L of nitrate and < 3 mg/L nitrite in drinking water (WHO, 2003).

Table 10. Mean nitrate content of selected foods (Suomi et al., 2013)

Food source	Nitrate mg/100g
Leaf lettuce (Finnish)	280
Beetroot	152
Spinach (Finnish)	122
Cabbage	29
Carrot	19
Potato (import)	18
Potato (Finnish)	6
Strawberry	6
Tomato	4
Orange	1

Nitrite contents in foods are shown in Table 11. The highest amount of nitrite is observed in processed meat products, where it is added as a preservative to prevent botulism (EFSA, 2008; Hord et al., 2009). There is no recommendation for nitrate and nitrite intake, but acceptable daily intake values are set as 3.7 mg/kg body weight for nitrate and 0.07 mg/kg body weight for nitrite (EFSA Panel on Food Additives and Nutrient Sources added to Food [ANS] et al., 2017). The acceptable amounts of nitrite added to meat products are regulated and monitored. However, high consumption of these products can exceed the acceptable daily intake of nitrite, particularly in children due to their smaller body size (Suomi et al., 2013, 2016). This can lead to methemoglobinemia, where increased methemoglobin causes poor capacity for oxygen transport and anemia.

Table 11. Mean nitrite content of selected foods (EFSA Panel on ANS et al., 2017)

Food source	Nitrite mg/100g
Bacon	2.2
Cooked smoked sausage	2.0
Ham	1.1
Meat, poultry	1.1
Fruit vegetables	1.1
Meat, pork	1.0
Meat, beef	0.9
Leaf vegetables	0.9
Grains and cereal products	0.08
Milk	0.02

2.4.2 Metabolism of nitrate and nitrite and endogenous formation of N-nitroso compounds

Exogenous nitrite from food accounts for approximately 60–80% of the total nitrate intake (Archer, 2002). Exogenous nitrite accounts for only under 10% of the total intake, as 80–85% is obtained from endogenous conversion from nitrate (Mensinga et al., 2003). Nitrite from the diet is almost completely absorbed in the duodenum and jejunum (Hunault et al., 2009). After ingestion of nitrate, plasma levels of nitrate increase to its peak around 15–30 minutes. Ingested nitrite is converted to nitrate in the blood.

Nitrite is formed endogenously in the stomach from reduction of saliva nitrate (EFSA, 2008). Nitrite is partially reduced to nitric oxide, and the remaining nitrate and nitrite are absorbed in the small intestine into the bloodstream and kidneys for excretion (Suomi et al., 2013). Nitrite, both derived from diet or reduction of salivary nitrate, is converted in the acidic stomach to nitroso compounds, such as S-nitroso, N-nitroso, O-nitroso compounds, and nitric oxide (Kobayashi, 2018). Low pH in the stomach (Caulfield et al., 1996), ascorbic acid in the gastric juice (Sobala et al., 1989), and intake of vegetables and fruits high in antioxidants, polyphenols, vitamin C, and vitamin E (Helser et al., 1992; Tannenbaum et al., 1991)—favor the formation of S-nitroso compounds and nitric oxide. S-nitroso compounds, which favor stable nitric oxide activity, have been suggested to have a beneficial influence on cardiovascular health and the prevention of cancer (Kobayashi et al., 2015), which could address the benefits of consuming vegetables rich in nitrate. On the contrary, increased gastric pH due to helicobacter pylori infection, intake of proton pump inhibitors, and low ascorbic acid status in the stomach favors N-nitroso compound formation in the stomach (Leach et al., 1987; Sobala et al., 1989), which has been associated with increased risk of gastric cancer (Ruddell et al., 1976).

Nitrate and nitrite are primarily circulated and catalyzed into nitroso-related compounds in the stomach and small intestine, but these compounds are also formed in the large intestine. High consumption of dietary nitrate, nitrite, and heme protein enchases the nitrosylation of heme, while high consumption of meat provides undigested protein residues, which are converted to amines by microbial fermentation. As a nitrosating agent, nitrosyl-heme reacts with nitrosatable amines, which could lead to the formation of N-nitroso compounds (Lakshmi et al., 2005; Mirvish et al., 2008). Furthermore, the composition of the diet could influence intestinal microbe composition, favoring N-nitroso compound formation. An animal-based diet has been suggested to increase *Bacteroides*, while plant-based diets favor *Firmicutes*, but the bacteria that increase the formation of N-nitroso compounds have yet to be elucidated *in vivo* (Kobayashi, 2018). Although more studies are warranted, the formation of N-nitroso compounds, especially in the large intestine, is suggested to be influenced by the high consumption of red meat. N-nitroso compounds per se are also ingested from the diet (e.g., from processed meat and beer, in which the compounds are formed during food processing) (Lijinsky, 1999).

2.4.3 Nitrate and nitrite transport across the placenta

Nitrate is transplacentally transferred through an active transport system. Nitric oxide is required in several stages of placental development, such as implantation, embryo development, and placental vascular tone (Krause et al., 2011). Nitric oxide is produced endogenously, and it is unclear how large a portion of nitric oxide is as a result of dietary nitrate and nitrite intake. Nitrite content is similar in maternal and fetal blood before birth (Jones et al., 2015).

2.4.4 N-nitroso compound formation and its potential toxicity to beta cells

Animal studies have demonstrated that streptozotocin, a glucosamine-nitrosourea compound, is toxic to pancreatic beta cells and induces diabetes (Wilander & Gunnarsson, 1975). An *in vitro* study using animal cultures observed that 1-methyl-1-nitrosourea, bis-chloroethylnitrosourea, streptozotocin, and its analogue chlorozotocin damaged beta cells (Wilson et al., 1983). However, the formation of these specific compounds in humans *in vivo* has not been demonstrated. Exposure to N-nitroso compounds has been suggested to induce DNA damage, oxidative stress, and lipid peroxidation (de la Monte et al., 2009).

Observational studies in humans have presented inconsistent results on the association between N-nitroso compounds and the development of type 1 diabetes. A previous ecological survey suggested that foods containing high amounts of N-nitroso compounds in childhood might increase the risk of type 1 diabetes (Helgason & Jonasson, 1981). Furthermore, a case-control study observed that a high intake of nitrosamines from diet in childhood might increase the risk of type 1 diabetes, particularly in the presence of high consumption of protein (Dahlquist et al., 1990). However, two population-based case-control studies found no association between the intake of N-nitroso compounds or the consumption of foods high in N-nitroso compounds and the risk of type 1 diabetes (Benson et al., 2010; Verge et al., 1994).

2.5 Characteristics of iron and possible mechanisms of action in the development of type 1 diabetes

Iron is a trace metal with the ability to transfer electrons. Iron can transit between two oxidative states: ferrous (Fe2+) and ferric (Fe3+), from which it takes part in several biological processes (Duck & Connor, 2016). Iron is primarily found in hemoglobin and myoglobin, which are proteins that transport oxygen in blood and muscle tissues. Iron is also the metal nucleus in redox enzymes; thus, it is required to maintain a balance in cell signaling and homeostasis. To maintain adequate body iron status, iron is needed from the diet, where it is found in two forms: heme iron and non-heme iron. Iron, as an active metal, can also function as a catalyst in the production of ROS, which leads to oxidative stress in large amounts. Thus, the absorption and excretion of iron in the body must be tightly regulated.

2.5.1 Dietary sources of iron

Dietary sources of iron in the selected foods are shown in Table 12. Heme iron is found in animal flesh, organs, and blood, while non-heme iron occurs in plant-based foods, such as vegetables, legumes, whole grains, nuts, and seeds. According to the National FinDiet 2017 Survey, the mean iron intake of adult Finnish women was 10 mg/day, which is under the recommended 15 mg/day (Valsta et al., 2018). Conversely, the iron intake of men was 11 mg/day, which exceeded the recommended 9 mg/day for men. The main source of iron was cereal products, followed by meat and egg-based dishes. During pregnancy, iron requirement is increased and approximately 500 mg iron stores

are required for iron balance in early pregnancy, based on Finnish recommendation (Finnish Institute for Health and Welfare, 2019a). Iron supplements may be needed after the first trimester due to the increased need for iron.

Table 12. Iron content of selected foods (Finnish Institute for Health and Welfare, 2019b)

Food source	Iron mg/100g
Blood pancake	22
Liver, cooked	21
Wheat bran	20
Liver sausage	10
Oat bran	8
Blood sausage	7
Rye crisp bread	4–5
Liver casserole	4
Rye bread	2–3
Pasta, whole wheat, cooked	1

2.5.2 Metabolism and distribution of iron

The mean absorption efficiency of iron in women is 13% for ingestion and 6% for men (Mutanen et al., 2021). The absorption efficiency is dependent on the iron status. Iron is absorbed as heme or free ferrous (non-heme) iron in the duodenal intestine (Hansen et al., 2014). Non-heme iron is less readily absorbed in the gastrointestinal track than heme iron. Other plant-based compounds, such as fiber, phytates, and tannins, and ingestion of other micronutrients, such as zinc, copper, and calcium, inhibit absorption. Ingestion of heme iron or vitamin C along with non-heme iron improves the absorption of non-heme iron. Ferrous iron is transported into erythrocytes by divalent metal transporter, while heme form is transported via heme carrier protein 1 (Andrews & Schmidt, 2007; Evstatiev & Gasche, 2012). Iron is then transported from erythrocytes' basolateral membrane into the bloodstream by ferroportin, where it is bound to plasma protein transferrin for transportation into circulation (Andrews & Schmidt, 2007; Donovan et al., 2005). Iron is stored in the liver as ferritin and hemosiderin and is transferred to other tissues via transferrin.

Excess iron is toxic; therefore, absorption is tightly controlled. Iron absorption is regulated by the hepatic peptide hormone hepcidin to maintain iron homeostasis (Qiao et al., 2012; Sangkhae & Nemeth, 2017). Absorption is decreased during sufficient iron status to prevent iron overload in circulation. Hepcidin expression is regulated by plasma iron status, body iron stores, infection, and inflammation (Sangkhae & Nemeth, 2017). In addition to absorption, hepcidin also regulates cellular iron transport and thus plasma iron status. Impaired hepcidin regulation can induce iron overload, such as in

hereditary hemochromatosis, while increased expression can result in iron-restricted anemia. Although absorption is regulated, excretion of excess iron is not possible; thus, iron accumulation is possible (Woodman et al., 2017).

2.5.3 Iron transport across the placenta

During pregnancy, maternal red cell mass expansion, fetal development, and placental development and function require approximately 1 g of iron throughout the pregnancy (Fisher & Nemeth, 2017). A fetal requirement of around 270 mg of iron is maintained through the placenta, which itself requires approximately 90 mg of iron (Bothwell, 2000).

The main transport of nutrients through the placenta occurs via the syncytiotrophoblast. Iron from maternal circulation is delivered to the placenta in the form of ferritin and heme, but the main transportation is suggested to occur in the form of transferrin (Sangkhae & Nemeth, 2019). Transferrin receptor 1 is highly expressed in the syncytiotrophoblast apical side facing maternal circulation (Bastin et al., 2006; Seligman et al., 1979; Wada et al., 1979). Transferrin is dissociated in the acidic endosome, where ferric iron is reduced to soluble ferrous iron by ferrireductase (Sangkhae & Nemeth, 2019). It is then exported into the cytoplasm via endosomal iron transporter and stored as ferritin or transferred to ferroportin in the basal membrane toward fetal circulation. Iron may also be transferred from maternal circulation to the placenta in the form of ferritin or heme. However, the exact mechanisms of placental iron transport remain unknown.

Placental iron transport is tightly regulated, and neonatal iron stores depend on maternal iron status (Radlowski & Johnson, 2013). Maternal hepcidin regulates the bioavailability of iron by suppressing the iron flow in the circulation (Nemeth et al., 2004). Pregnancy affects hepcidin expression (Sangkhae & Nemeth, 2017), and hepcidin levels decrease substantially during the second and third trimesters of pregnancy, most likely to ensure iron bioavailability to fetal development (Koenig et al., 2014).

Iron supplementation is often used during pregnancy to maintain adequate iron stores. However, if the mother is iron-replete per se, supplement use during pregnancy can lead to excess iron overload.

2.5.4 Iron in beta-cell function and excess iron-induced oxidative stress

In pancreatic beta cells, iron is required for insulin secretion and glucose metabolism, but in large amounts, iron might increase the generation of ROS (Hansen et al., 2012; Sampaio et al., 2014). The generation of ROS might lead to dedifferentiation of beta cells, activation of apoptosis or ferroptosis, and further beta-cell death (Hansen et al., 2014). Markers of oxidative stress have been found in placental tissues after iron supplementation during pregnancy (Devrim et al., 2006). Elevated ferritin levels in mothers have been shown to increase the risk of pre-eclampsia (Scholl, 2005), which in turn could increase the risk of type 1 diabetes in offspring, but evidence so far is very limited (Henry et al., 2011). Whether a high maternal intake of iron during pregnancy influences a baby's pancreatic beta cells has not been studied.

2.6 Summary and basis of the current study

Figure 2 presents the suggestive mechanisms of dietary nitrate, nitrite, vitamin C, and iron intake in the development of islet autoimmunity and type 1 diabetes. There is suggestive case-control evidence that maternal high consumption of nitrite during pregnancy might increase the risk of type 1 diabetes. The use of iron supplements during pregnancy has been suggested to increase the risk of type 1 diabetes in offspring, but studies are few. Vitamin C is a dietary antioxidant, which might be beneficial for the protection of beta cells from oxidative damage since they lack antioxidant enzymes. However, studies on humans are 1) limited, 2) include case-control studies that have produced inconsistent results, and 3) have not assessed plasma ascorbic acid status. Prospective studies with comprehensive dietary assessments of maternal intake of these dietary components and their association with islet autoimmunity and type 1 diabetes are limited.

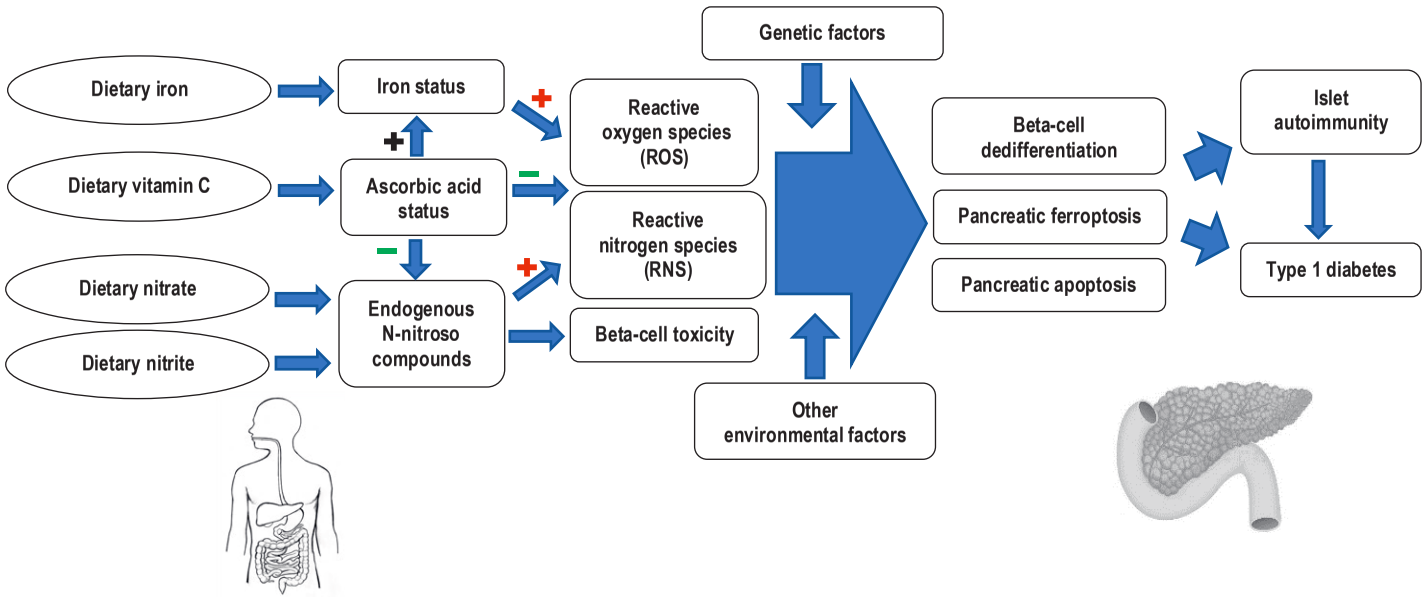

Figure 2. Suggested hypothesis of the mechanisms between dietary intake of vitamin C, iron, nitrate and nitrite and type 1 diabetes development. High consumption of iron increases body iron status, which could lead to the generation of ROS. The high consumption of nitrate and nitrite might lead to the increased endogenous formation of N-nitroso compounds, which might be toxic to beta cells, or they might lead to the generation of RNS. Vitamin C increases plasma ascorbic acid status, which reduces the formation of N-nitroso compounds. It also enhances iron absorption, but this mechanism might not increase the generation of ROS. In conjunction with genetic and other environmental factors, ROS- and RNS-induced oxidative and nitrosative stress might lead to pancreatic apoptosis. Furthermore, iron-generated ROS might lead to iron-dependent pancreatic apoptosis (ferroptosis) or loss of functional beta-cell mass due to altered genetic regulation (dedifferentiation). Impaired beta-cell function might trigger autoimmune reactions and the development of type 1 diabetes.

3 AIMS OF THE STUDY

The purpose of this study was to assess the association between plasma status of ascorbic acid during early life and the development of islet autoimmunity and type 1 diabetes. The second aim was to assess the association between maternal intake of vitamin C, iron, nitrate, and nitrite during pregnancy and islet autoimmunity and the development of type 1 diabetes in offspring. The specific research questions were as follows:

- Is high plasma ascorbic acid status in childhood associated with a decreased risk of islet autoimmunity, islet autoimmunity starting with IAA or GADA, and type 1 diabetes, and do polymorphisms in the vitamin C metabolism-related genetic variation affect the associations (study I)?

- Is maternal high vitamin C intake during pregnancy associated with a decreased risk of islet autoimmunity and type 1 diabetes in offspring (study III)?

- Is maternal high intake of nitrate and nitrite during pregnancy associated with an increased risk of islet autoimmunity and type 1 diabetes in offspring (study II)?

- Is maternal high iron intake during pregnancy associated with an increased risk of islet autoimmunity and type 1 diabetes in offspring (study III)?

4 STUDY POPULATION

The subjects for the current study were collected from two prospective cohort studies: one multinational and one Finnish. Since the risk of type 1 diabetes is small in the general population, subjects were collected from a population with an increased genetic risk of the disease.

4.1 The Environmental Determinants of Diabetes in the Young (TEDDY) Study (study I)

The Environmental Determinants of Diabetes in the Young (TEDDY) Study is a multinational observational study identifying environmental exposures associated with the development of islet autoimmunity and type 1 diabetes in genetically at-risk children based on the HLA genotype (The TEDDY Study Group, 2007, 2008). The study was conducted in six research centers in four countries: the United States (Colorado, Georgia/Florida, and Washington State), Finland, Sweden, and Germany. If families consented, a cord blood sample was collected to assess the genetic risk. The eligibility criteria for the first contact were one of the HLA class II genotypes: HLA-DR3/4, -DR4/4, -DR4/8, -DR3/3, and -DR4/4. Furthermore, HLA-DR genotypes—DR4/1, -DR4/13, -DR4/9, and -DR3/9—were included only if children had a first-degree relative (FDR)—mother, father, or sibling—with type 1 diabetes (Hagopian et al., 2011). Clinic visits were scheduled every 3 months until the child's age of 4 years and thereafter every 6 months up to 15 years of age or until being diagnosed with type 1 diabetes. Children who had type 1 diabetes-related autoantibodies were followed up every 3 months throughout the study. Children were excluded from the study if they had birth defects or illnesses that required long-term follow-up, or if they had had treatment that might alter the natural development of diabetes, such as the use of steroids or insulin.

4.2 Type 1 Diabetes Prediction and Prevention (DIPP) Study (studies II & III)

The DIPP Study is a Finnish population-based birth cohort of children with an increased genetic risk of type 1 diabetes based on HLA-conferred susceptibility (Ilonen et al., 1996; Kupila et al., 2001). Cord blood samples from all newborn infants were screened for HLA-DQB1 alleles in the university hospitals of Turku, Oulu, and Tampere. Families of children with HLA-DQB1-conferred genetic susceptibility to type 1 diabetes (HLA-DQB1*02/0302 heterozygous and DQB1*0302/x-positive individuals, x standing for homozygosity or a neutral allele) were invited to participate in a prospective follow-up study. HLA-DQB1 (*02/*03:02) were determined as high-risk genotypes, and HLA-DQB1*03: 02/x (x ≠ *02, *03:01, *06:02) were determined to be moderate-risk genotypes for type 1 diabetes. The follow-up visits were scheduled at the child's ages of 3, 6, 12, 18, and 24 months and annually thereafter. Children were excluded from the study if they had severe systemic diseases or congenital anomalies. Furthermore, children whose parents were not Finnish origin or did not speak Finnish, Swedish, or English fluently were excluded.

4.3 Ethical aspects

The TEDDY Study was approved by local institutional review or ethics boards at each study site (University of Washington, Seattle; University of Colorado; Medical College of Georgia, Augusta; University of South Florida, Tampa; University of Turku, Finland; Technische Universität, Munich, Germany; and Lund University, Malmö, Sweden). The study is monitored by an external evaluation committee formed by the National Institutes of Health. Written informed consent for genetic screening and participation in prospective follow-up was obtained from the participating families.

The DIPP Study adheres to the Declaration of Helsinki, and the ethical committees of Oulu and Tampere University Hospitals approved the study protocol. Parents gave their written informed consent for genetic testing of their newborn infants from the cord blood sample and another one for participation in the follow-up. Since newborns were screened for genetic susceptibility to type 1 diabetes in the DIPP Study, the generalizability of the current results to the general population may be limited. However, children recruited for the study do benefit from the study due to constant medical and nutritional guidance and screening.

5 METHODS

5.1 Measurement of type 1 diabetes associated autoantibodies

The children in the TEDDY Study were monitored for the appearance of autoantibodies to IAA, GADA, or IA-2A, which were measured in two laboratories by radiobinding assays (The TEDDY Study Group, 2007, 2008). In the United States, sera samples were analyzed at the Barbara Davis Center for Childhood Diabetes at the University of Colorado Denver, while sera samples in Europe were analyzed at the University of Bristol, UK. Both laboratories have demonstrated high sensitivity, specificity, and concordance (Bonifacio et al., 2010). All positive islet autoimmunity and 5% of negative samples were retested in the other reference laboratory and considered confirmed if concordant.

In the DIPP Study, ICA was screened at 3- to 12-month intervals (Kupila et al., 2001). If a child was observed to be positive for ICA, all available samples from the child in question were analyzed for IAA, GADA, and IA-2A, and the follow-up interval was narrowed to 3 months. Autoantibodies were measured in the Research Laboratory, Department of Pediatrics, University of Oulu. The standard indirect immunofluorescence method was used for the quantification of ICA (Kimpimaki et al., 2000). A microassay was used for the IAA, and specific radiobinding assays were used for GADA and IA-2A quantification. If autoantibodies were present in the cord blood but disappeared during infancy, they were considered maternally transferred and excluded from the analyses (Hamalainen et al., 2000).

5.2 Definition of type 1 diabetes-related outcomes

The ethological analyses included two outcomes: 1) islet autoimmunity and 2) the diagnosis of type 1 diabetes. In the TEDDY Study, persistent confirmed islet autoimmunity was defined by the appearance of at least one of the type 1 diabetes-related autoantibodies, IAA, GADA, and IA-2A, confirmed at two subsequent clinical visits. Islet autoimmunity with persistent multiple autoantibodies was defined as the appearance of two or more autoantibodies at a single clinical visit. The current studies

included three secondary outcomes within islet autoimmunity outcomes: 1) islet autoimmunity with IAA as the first only observed autoantibody (IAA first), 2) islet autoimmunity with GADA as the first only observed autoantibody (GADA first), and 3) islet autoimmunity with multiple autoantibodies (multiple autoantibodies). The type 1 diabetes outcome definition was based on American Diabetes Association criteria (American Diabetes Association, 2014).

In the DIPP Study, islet autoimmunity was defined by repeated positivity for ICA and at least one other autoantibody (IAA, GADA, or IA-2A) or having a type 1 diabetes diagnosis. Type 1 diabetes is included in the islet autoimmunity outcome, as the majority of the children had one or more autoantibodies detected from a single sample before or at the time of diagnosis. Furthermore, blood samples were not available for some children. The type 1 diabetes outcome included only children with diagnosed type 1 diabetes obtained from the Finnish Pediatric Diabetes Register and University Hospitals (Parkkola et al., 2013), and diagnosis was defined according to World Health Organization criteria (Alberti & Zimmet, 1998).

5.3 Plasma ascorbic acid measurement

Plasma samples for ascorbic acid measurement were collected at the ages of 6 and 12 months and onwards annually up to 6 years of age or until seroconversion of the islet autoimmunity cases. For the type 1 diabetes cases, samples were collected up to 6 years of age or up to the visit just preceding the type 1 diabetes diagnosis (with corresponding time for matched controls). The case samples were paired with matched control samples.

Ascorbic acid measurements were performed at the Biochemistry Laboratory, Genomics and Biomarkers Unit, National Institute for Health and Welfare, Helsinki, Finland. Ascorbic acid was determined by an ion-paired, reversed-phase, high-performance liquid chromatographic method using electrochemical detection (Salminen & Alfthan, 2008). Isoascorbic acid was used as an internal standard for the quantitation of ascorbic acid.

5.4 Assessment of dietary intake

In the DIPP Study, maternal dietary intake during pregnancy was assessed using a validated semi-quantitative FFQ customized for the study (Erkkola et al., 2001). The FFQs were mailed to the mothers after delivery and checked at their children's 3-month follow-up visits. Mothers were asked retrospectively to describe their diet during the 8th month of pregnancy, the last month preceding maternity leave in Finland. The FFQ comprised a list of 181 food items and mixed dishes. Mothers were instructed to report the use of dietary supplements during the entire pregnancy, including brand names and manufacturers of the supplements, in addition to frequency of use. Each nutrient intake was summed from all supplements used. The nutrient intake calculation was made using the in-house software Finessi of the Finnish Institute for Health and Welfare, Finland, using Fineli as the source of food composition data (Reinivuo et al., 2010). FFQs with more than 10 missing items were excluded.

For study II, the special Fineli database was updated to contain recently measured nitrate and nitrite values in foods, which were based on analyses by the Finnish Customs Laboratory and Finnish Food Authority during 2008–2012 (Suomi et al., 2013, 2016). For the foods not included in these analyses, the nitrate and nitrite contents were determined from the scientific literature (EFSA Panel on ANS et al., 2017; Foedevaredirektoratet., 1999; Laitinen et al., 1993; Susin et al., 2006; Ysart et al., 1999). The highest priority was given for the latest scientific literature from the year 2000 onwards, followed by literature from 1980–2000. Preferably, European food items were chosen for analytical values, if possible. Values not found in the literature were derived from aggregation, recipe calculation, or imputation from similar foods.

5.5 Assessment of sociodemographic characteristics

For study I within TEDDY, the data on basic demographic characteristics and family history of type 1 diabetes were received from the infant screening form. The chosen characteristics were clinical center, sex, and family history of type 1 diabetes, which were used as matching variables in a nested case-control design (Lee et al., 2014). A family history of type 1 diabetes was defined as having an FDR (mother, father, or sibling) with type 1 diabetes. Otherwise, the child was categorized as the general population. Weight and height z-scores were received from the Centers for Disease Control and Prevention standardized growth charts.

In the DIPP Study, mothers were asked to fill out a questionnaire after delivery inquiring about maternal education, height and weight at antenatal visits, diabetes (type not specified), and family history of diabetes among FDRs. Birth registers of the hospitals provided information on offspring sex, maternal age, and smoking during pregnancy.

5.6 Genotyping of single nucleotide polymorphisms related to plasma vitamin C status

For study I, the SNPs were genotyped using Illumina Infinium ImmunoChip custom microarray, based upon robust genome-wide association analyses in 12 autoimmune diseases, including type 1 diabetes. The ImmunoChip array included 195,806 SNPs genotyped on TEDDY DNA samples, from which one SNP from ascorbic acid transport gene *SLC23A1* and three from DHA transport genes *SLC2A1* and *SLC2A2* were selected for the analyses Table 13. All the included SNPs passed the quality control metrics and were therefore selected for analysis. Principal component analysis (PCA) using EIGENSTRAT software was performed using each unrelated TEDDY participant to estimate ancestry. Two most significant principal components were used as covariates in the analytic models.

Table 13. Vitamin C metabolism-related SNPs genotyped with ImmunoChip microarray.

Gene	SNP (minor allele)
SLC23A1	rs33972313 (A)
SLC2A1	rs1105297 (A)
SLC2A1	rs3754223 (A)
SLC2A2	rs5400 (A)

Abbreviations: SNP, single nucleotide polymorphism

5.7 Study designs

5.7.1 Plasma ascorbic acid status in childhood and risk of islet autoimmunity and type 1 diabetes (study I)

The study flow chart for the TEDDY participants included in study I is presented in Figure 3. The primary outcomes were persistent and confirmed islet autoimmunity (defined as positivity for at least one of the type 1 diabetes-related autoantibodies) and

type 1 diabetes. The association between plasma ascorbic acid status and the outcomes was analyzed using a nested case-control design. Three matched controls were selected per case, and children were matched for family history of type 1 diabetes, clinical center, and sex. The islet autoimmunity data set included 350 cases with a median seroconversion age of 23 months (range 6–72 months). A control child was defined as a participant who had not developed persistent islet autoimmunity by the time the corresponding matched case plus 45 days. The type 1 diabetes dataset consisted of 102 cases with a median age of 31 months at diagnosis (range 8–75 months). A control child was defined as a participant who had not developed type 1 diabetes by the time the corresponding matched case plus 45 days. Secondary outcome analyses were performed within the islet autoimmunity outcome (Figure 3). The data set included 163 IAA first cases with a median seroconversion age of 18 months (range 6–72 months). From the islet autoimmunity cases, there were 120 GADA first cases with a median seroconversion age of 28 months (range 6–68 months).

The islet autoimmunity case-control set included 3,371 ascorbic acid samples. Samples from the controls were processed only when the matched case had an available sample at a corresponding visit. The mean childhood ascorbic acid status for each child was calculated from all available measurements.

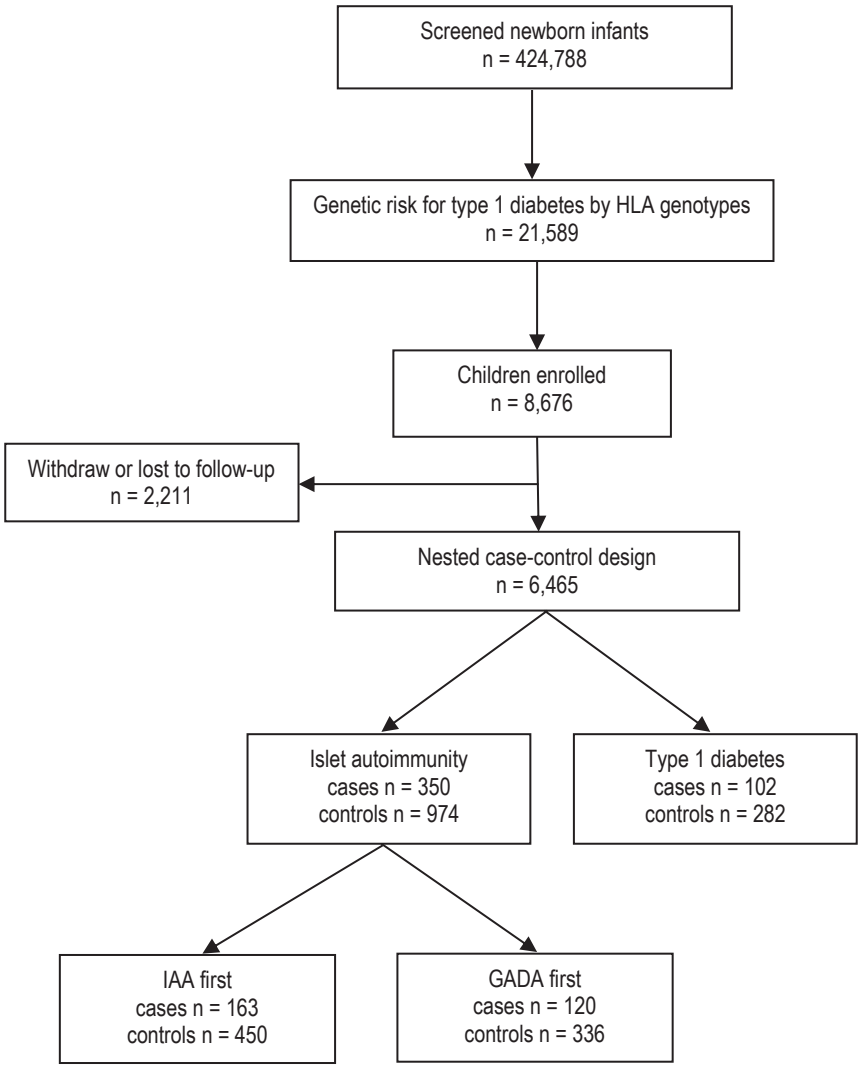

Figure 3. TEDDY Study participant flow chart

5.7.2 Maternal vitamin C, iron, nitrate, and nitrite intake and the risk of islet autoimmunity and type 1 diabetes in offspring (studies II & III)

The DIPP study flow chart of studies II and III is presented in Figure 4. The DIPP study sample included 4,943 children born in Tampere and Oulu University hospitals between October 1997 and September 2004. Maternal dietary data were available from 4,879 mothers because 64 mothers had twin pregnancies. For the analysis, separate outcome datasets were formed for islet autoimmunity (4,887 children) and type 1 diabetes (4,943 children). During the 15-year follow-up, 312 children developed islet autoimmunity at a median (interquartile range [IQR]) age of 3.5 (1.7–6.6) years, and 178 developed type 1 diabetes at the age of 7.1 (4.3–10.6) years. Among the 4,887 participants, the dropout rates in the autoantibody follow-up at the 1- and 5-year follow-ups were 10% and 34%, respectively.

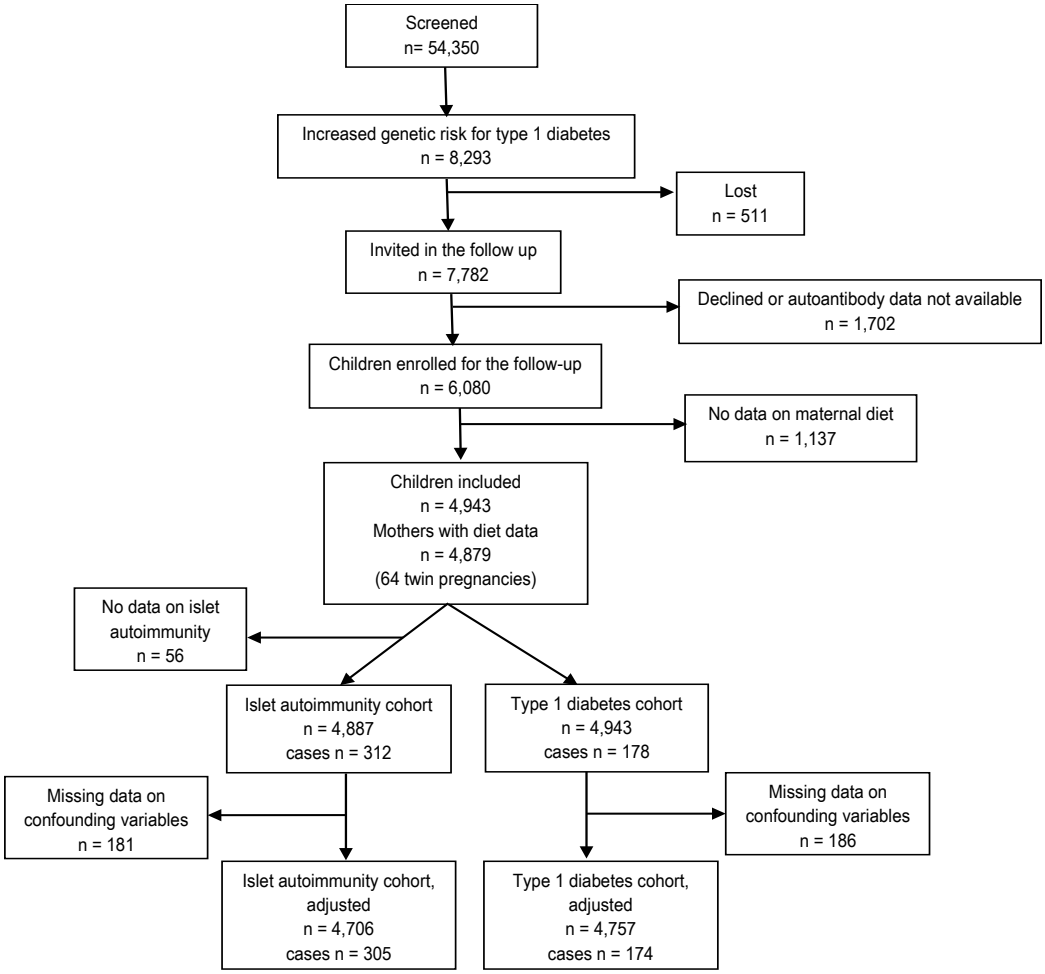

Figure 4. DIPP Study participant flow chart

5.8 Statistical analyses

In study I, a linear mixed-effects model adjusted for the case-control status was used to test whether the plasma ascorbic acid over time differed by background factors (country, age points, sex, breastfeeding at the age of 6 months vs. not, the study-specific SNPs, and the use of standard vs. long-distance protocol). The association between the plasma ascorbic acid status and the outcomes was analyzed using conditional logistic regression adjusted for HLA-DQ genotype (DR3/4 vs. other) and two principal components of ancestry to control for population stratification. The plasma ascorbic acid status variable includes plasma measures from all visits prior to and including the seroconversion visit, which is the first of two consecutive visits at which the child tested positive for an autoantibody, and for type 1 diabetes all visits prior to diagnosis. Growth variables—height, weight, and breastfeeding—have been associated with plasma ascorbic acid status and type 1 diabetes. Thus, mean height and weight z-scores prior to the outcome and breastfeeding status at 3 and 6 months were assessed in the study (Elding Larsson et al., 2016). To test the effect modification, an interaction term with the matching factors was included in the model. The log-linearity of the characteristics with each outcome was examined using the supremum test (Borgan & Zhang, 2015). All analyses were performed using SAS 9.4 (SAS Institute, Inc.). A two-sided p-value < 0.05 was considered statistically significant.

In studies II and III, a one-way analysis of variance (ANOVA) was used to test the differences in maternal vitamin C, iron, nitrate, and nitrite intake by confounding background factors. Differences in supplement use vs. nonuse by background variables were tested using the t-test and Pearson's chi-square test. The maternal intake of vitamin C, iron, nitrate, and nitrite was energy-adjusted using Willett's residual method for the analyses of outcomes (Willett et al., 1997). The dietary intake was analyzed as continuous variables and then categorized into quartiles. Two middle quartiles were combined, and the combination was used as the reference category. The use of supplements with vitamin C and iron at any time during pregnancy was categorized as yes/no. The Cox proportional hazards regression adjusted for sex (female vs. male), family history of type 1 diabetes (FDR vs. no), and HLA genotype (high vs. moderate risk) was used for the outcome analyses. Additional analysis was performed in study II, where the association between maternal nitrate and nitrite intake and the outcomes were further adjusted for the intake of dietary antioxidants: vitamin C, vitamin E, and selenium. Similarly, in study III, additional analyses, the association between maternal vitamin C and iron intake, were further adjusted for maternal education (none vs. vocational school/course,

secondary vocational education, or university studies/degree), pre-pregnancy BMI, and smoking during pregnancy (yes vs. no). To test whether protein intake modifies the association between nitrate and nitrite intake and the development of type 1 diabetes outcomes, an interaction term was used. The interaction term was also used to test whether total vitamin C intake modifies the association between total iron intake and outcomes.

Analyses in studies II and III were performed using SAS software version 9.3 and IBM SPSS Statistics version 25.0 (IBM Corporation, NY, USA). Statistical significance was set at 2-sided $P < 0.05$.

6 RESULTS

6.1 The TEDDY cohort

6.1.1 Characteristics of the TEDDY cohort

The background characteristics of participants by background variables in TEDDY study are presented in Table 14. In the nested case-control design within the TEDDY cohort, the mean plasma ascorbic acid status for islet autoimmunity cases and controls was 10.21 mg/l (SD 3.33) and 10.76 mg/l (SD 3.54), respectively. For the type 1 diabetes cases and controls, the mean plasma ascorbic acid status was 9.73 mg/l (SD 3.18) and 10.58 mg/l (SD 3.57), respectively. High weight was associated with islet autoimmunity (OR 1.23; 95% CI 1.07, 1.41), IAA first (OR 1.24; 95% CI 1.01, 1.51), and GADA first (OR 1.32; 95% CI 1.05, 1.65). Therefore, the association between plasma ascorbic acid status and outcomes was adjusted for the mean weight z-score. Breastfeeding was associated with lower plasma ascorbic acid status but not with the outcomes.

Table 14. Background characteristics of participants by background variables in TEDDY study, n (%)

Characteristic	Islet autoimmunity Cases, n = 350 (%)	Type 1 diabetes Cases, n = 102 (%)
Sex		
Female	157 (44.9)	47 (46.1)
Male	193 (55.1)	55 (53.9)
Country		
US	109 (31.2)	28 (27.5)
Finland	105 (30.0)	35 (34.3)
Germany	26 (7.4)	15 (14.7)
Sweden	110 (31.4)	24 (23.5)
Family history of type 1 diabetes		
FDR	76 (21.7)	36 (35.3)
GP	274 (78.3)	66 (64.7)

Abbreviations: FDR, first-degree relative of an individual with type 1 diabetes; GP, from the general population (no first-degree relative with type 1 diabetes)

6.1.2 Plasma ascorbic acid status, the risk of type 1 diabetes development and effect modification by vitamin C metabolism-related SNPs

Plasma ascorbic acid was associated with a decreased risk of islet autoimmunity and IAA first when adjusted for ancestry and HLA-DQ genotype (Table 15). When adjusted for mean weight z-score prior to the outcome, the association was significant only for IAA first (islet autoimmunity: OR 0.96; 95% CI 0.92, 1.00, IAA first: OR 0.93; 95% CI 0.88, 0.99).

None of the vitamin C metabolism-related SNPs modified the association between plasma ascorbic acid status and outcomes (Table 16).

Table 15. The association between plasma ascorbic acid status and vitamin C intake on the risk of islet autoimmunity and type 1 diabetes

	Islet autoimmunity N = 1,324, *n* = 350	IAA first N = 1,324, *n* = 163	GADA first N = 1,324, *n* = 120	Type 1 diabetes N = 384, *n* = 102
	OR (95% CI)[a]	OR (95% CI)[a]	OR (95% CI)[a]	OR (95% CI)[a]
Mean plasma ascorbic acid status (per 1 mg/L increase)	0.96 (0.92, 0.99)	0.94 (0.88, 0.99)	0.99 (0.93, 1.07)	0.93 (0.86, 1.02)

[a] Adjusted for two largest principal components for ethnicity and HLA genotype *DR3/4*.

Table 16. Effect modification between the vitamin C transport genes and plasma ascorbic acid status on the islet autoimmunity and type 1 diabetes risk

Gene SNP (minor allele)	Islet autoimmunity		Type 1 diabetes	
	% of minor allele, cases / controls	*p* Value[a]	% of minor allele, cases / controls	*p* Value[a]
SLC23A1 rs33972313 (A)	3.4 / 2.8	0.16	5.4 / 1.6	0.10
SLC2A1 rs1105297 (A)	33.7 / 32.8	0.09	33.8 / 32.6	0.17
SLC2A1 rs3754223 (A)	21.9 / 22.7	0.96	25.5 / 21.6	0.66
SLC2A2 rs5400 (A)	12.7 / 13.7	0.46	18.6 / 11.7	0.78

[b] Interaction between plasma ascorbic acid status with vitamin C transport gene SNP on the risk of islet autoimmunity and type 1 diabetes, adjusted for two largest principal components for ethnicity and HLA genotype *DR3/4*

6.1.3 Vitamin C metabolism-related SNPs and the risk of developing type 1 diabetes

In the TEDDY nested case-control study, none of the vitamin C metabolism-related SNPs were associated with islet autoimmunity, but *SLC2A2* rs5400 was associated with an increased risk of type 1 diabetes (Table 17). The association was significant even after adjusting for plasma ascorbic acid status, ethnicity, and HLA DR3/4 genotype (OR 1.77; 95% Cl 1.12, 2.80).

Table 17. Association between vitamin C metabolism-related SNPs and the risk of islet autoimmunity and type 1 diabetes

Gene SNP (minor allele)	Islet autoimmunity		Type 1 diabetes	
	% of minor allele, cases / controls	OR (95% CI)[a]	% of minor allele, cases / controls	OR (95% CI)[a]
SLC23A1 rs33972313 (A)	3.4 / 2.8	1.18 (0.70, 1.99)	5.4 / 1.6	2.52 (0.96, 6.59)
SLC2A1 rs1105297 (A)	33.7 / 32.8	1.04 (0.86, 1.26)	33.8 / 32.6	1.09 (0.75, 1.56)
SLC2A1 rs3754223 (A)	21.9 / 22.7	0.92 (0.74, 1.15)	25.5 / 21.6	1.40 (0.93, 2.11)
SLC2A2 rs5400 (A)	12.7 / 13.7	0.90 (0.69, 1.16)	18.6 / 11.7	1.66 (1.06, 2.60)

[a] Adjusted for two largest principal components for ethnicity and HLA genotype DR3/4

6.1.4 Vitamin C metabolism-related SNPs and plasma ascorbic acid status

The SLC23A1 rs33972313 minor allele carriers had lower mean plasma ascorbic acid status than non-carriers (mixed model regression parameter estimate (standard error): -2.22 (0.46), $p < 0.001$). None of the other studied SNPs was associated with plasma ascorbic acid status.

6.2 The DIPP cohort

6.2.1 Characteristics of the DIPP cohort

A total of 312 (6.4%) children developed islet autoimmunity at a median age of 3.5 (IQR 1.7–6.6) years, and 178 (3.6%) had type 1 diabetes at a median age of 7.1 (IQR 4.3–10.6) years during the 15-year follow-up. The dropout rates among the 4,887 children at 1- and 5-year autoantibody follow-up were 5.7% (279 children) and 30% (1,415 children), respectively.

6.2.2 Maternal nitrate, nitrite, vitamin C, and iron intakes and their food sources

Maternal vitamin C, nitrate, nitrite, and iron intake by background variables are presented in Table 18. The maternal primary dietary sources of nitrate, nitrite, vitamin C, and iron are presented in Figures 5-8. The mean (SD) maternal dietary intake of nitrate during pregnancy was 151 (97.4) mg/day, while intake of nitrite was 3.00 (1.06) mg/day, respectively. The mean (SD) intake of vitamin C from foods was 198 (116) mg/day, while vitamin C intake from supplements was 23 (82) mg/day, which comprised 11% of the total intake. A total of 1,555 mothers (32%) reported the use of dietary supplements containing vitamin C at any time during pregnancy. The mean (SD) intake of iron from foods during pregnancy was 17 (5) mg/day. The iron intake from supplements was 26 (33) mg/day, which was the main source of iron (62% of the total intake). Of the mothers, 3,375 (69%) reported the use of iron supplements at any time during pregnancy.

Table 18. Characteristics of mothers in relation to mean (standard deviation) total intake of vitamin C, iron, nitrate, and nitrite during 8th month of pregnancy.

		Maternal intake from diet and dietary supplements							
		Nitrate, mg/day		Nitrite, mg/day		Vitamin C, mg/MJ/day		Iron, mg/MJ/day	
Characteristic	n	Mean (SD)	p-value[a]	Mean (SD)	p-value[a]	Mean (SD)	p-value[a]	Mean (SD)	p-value[a]
All mothers	4,879	151.0 (97.4)		3.0 (1.1)		18.9 (11.7)		3.8 (3.1)	
Maternal age, years			< 0.001		< 0.001		0.46		0.72
< 25	926	126.7 (90.0)		2.9 (1.1)		18.7 (11.1)		3.8 (3.4)	
25–29.9	1,700	148.0 (96.4)		2.9 (1.0)		18.8 (10.3)		3.7 (3.0)	
30–34.9	1,412	158.6 (94.6)		3.0 (1.0)		19.3 (13.4)		3.9 (3.2)	
≥ 35	841	171.9 (105.5)		3.1 (1.0)		19.1 (12.2)		3.8 (2.9)	
BMI in early pregnancy			0.56		< 0.001		0.01		< 0.001
< 25	3,024	150.4 (94.4)		2.9 (1.0)		19.3 (11.9)		4.0 (3.3)	
25–29.9	1,124	152.0 (90.2)		3.1 (1.1)		18.1 (10.5)		3.6 (2.9)	
≥ 30	434	154.0 (118.9)		3.1 (1.1)		18.7 (11.1)		3.4 (2.9)	
Missing	297								
Maternal vocational education[b]			< 0.001		0.03		0.001		0.40
None	294	115.1 (79.7)		3.1 (1.1)		18.2 (13.5)		3.6 (3.2)	
School or Course	1,291	139.2 (96.0)		3.1 (1.1)		18.4 (11.5)		3.9 (3.5)	
Secondary Education	2,067	149.9 (93.4)		3.0 (1.0)		18.9 (11.7)		3.8 (3.0)	
University Studies or Degree	1,098	170.0 (103.7)		2.9 (0.9)		20.1 (11.8)		3.8 (3.1)	
Missing	129								
Maternal smoking during pregnancy			< 0.001		0.37		0.10		0.04
Yes	467	120.2 (83.8)		3.0 (1.2)		18.1 (12.7)		3.5 (3.6)	
No	4,246	154.0 (98.1)		3.0 (1.0)		19.1 (11.7)		3.9 (3.1)	
Missing	166								
Maternal diabetes[c]			0.001		0.009		0.89		0.49
Yes	164	187.4 (134.2)		3.2 (1.2)		19.0 (11.9)		4.0 (3.1)	
No	4,612	149.7 (95.4)		2.9 (1.1)		19.0 (11.8)		3.8 (3.1)	
Missing	103								

[a] P values for difference between groups from one-way ANOVA.

[b] At the time of birth.

[c] Based on a questionnaire completed after birth. Type of diabetes not specified.

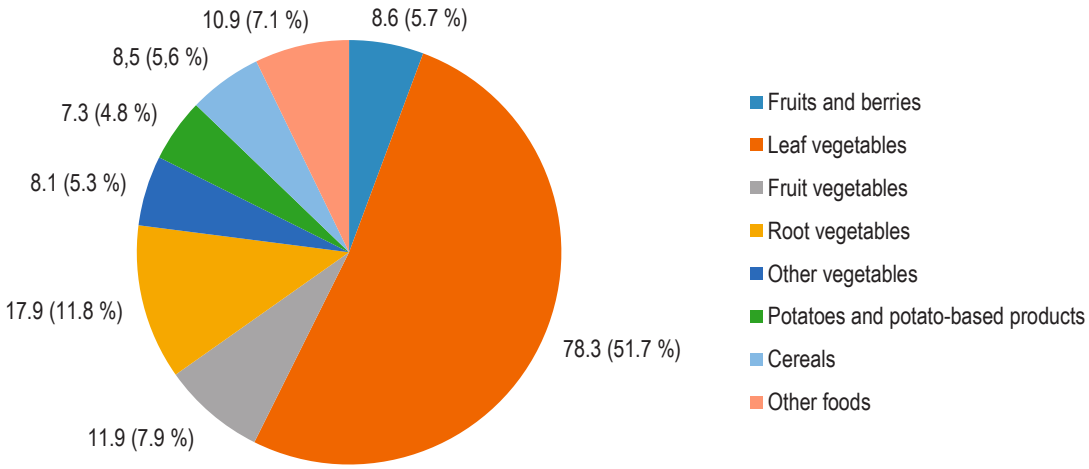

Figure 5. Maternal intake of nitrate during pregnancy from food groups, mg/day (% of total intake)

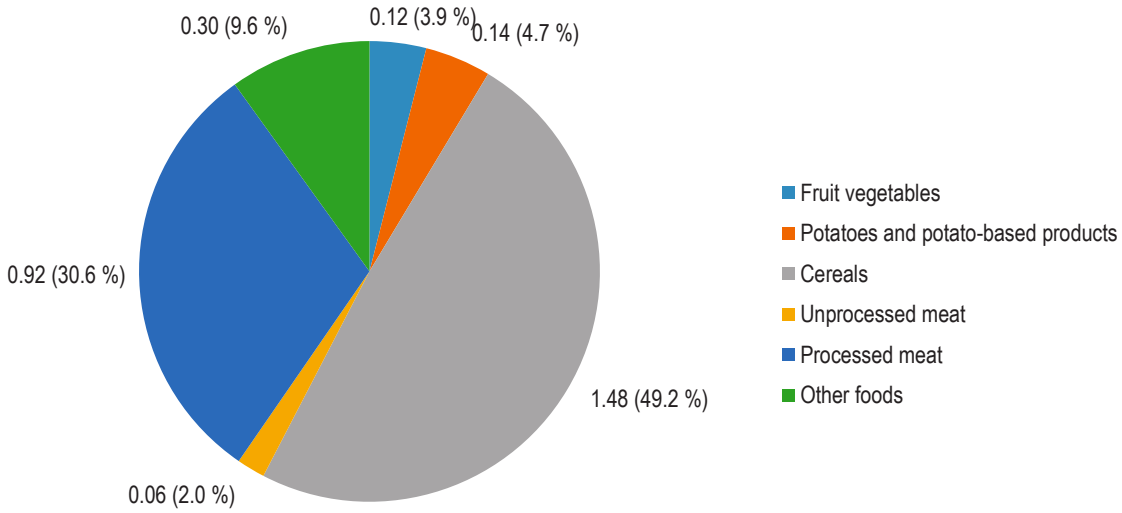

Figure 6. Maternal intake of nitrite during pregnancy from food groups, mg/day (% of total intake)

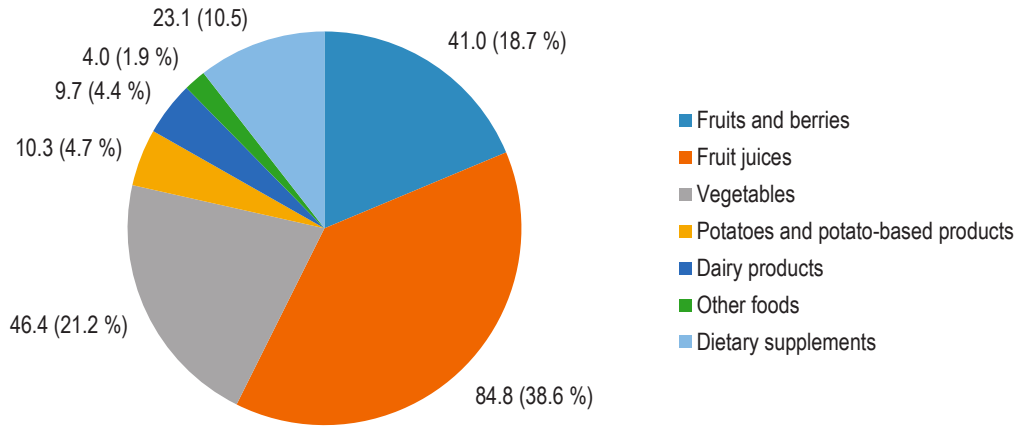

Figure 7. Maternal intake of vitamin C during pregnancy from food groups, mg/day (% of total intake)

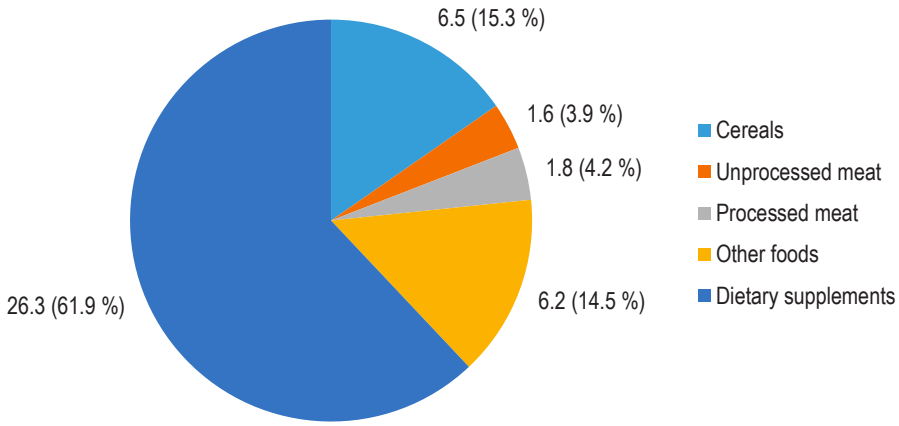

Figure 8. Maternal intake of iron during pregnancy from food groups, mg/day (% of total intake)

6.2.3 Maternal nitrate and nitrite intakes and the risk of developing type 1 diabetes in offspring

Maternal energy-adjusted intake of nitrate and nitrite during pregnancy was not associated with the risk of islet autoimmunity or type 1 diabetes (Figure 9). Results were similar when further adjusted for sex, family history of diabetes, and HLA genotype. Additional adjustments for the intake of dietary antioxidants (vitamin C, vitamin E, and selenium) did not change the results.

Maternal intake of protein during pregnancy did not modify the association between intake of nitrate or nitrite and the risk of islet autoimmunity (nitrate*protein interaction P = 0.23, nitrite*protein interaction: P = 0.99) or type 1 diabetes (nitrate*protein interaction P = 0.24, nitrite*protein interaction: P = 0.86).

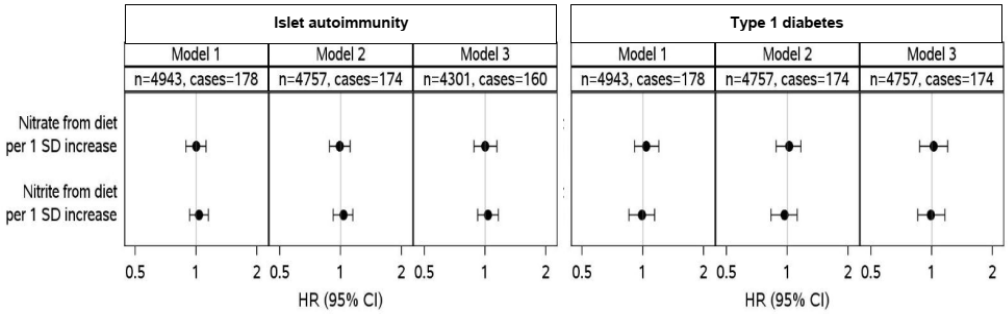

Figure 9. The association between maternal intake of nitrate and nitrite during pregnancy and the risk of islet autoimmunity and type 1 diabetes in offspring: Cox proportional hazard regression model.

Model 1: Energy adjusted with Willett's residual method

Model 2: Adjusted for energy residual method, sex, family history of diabetes, and HLA genotype

Model 3: Adjusted for energy residual method, sex, family history of diabetes, HLA genotype, and intake of vitamin C, vitamin E, and selenium

6.2.4 Maternal vitamin C and iron intake and the risk of development of type 1 diabetes in offspring

Maternal energy-adjusted vitamin C intake from food, dietary supplements, or combined during pregnancy was not associated with the development of islet autoimmunity or type 1 diabetes (Figure 10). Adjustments for sex, family history of diabetes, and HLA genotype did not change the results, nor did further adjustments for maternal education, pre-pregnancy BMI, and maternal smoking. Similarly, there was no association between energy-adjusted maternal intake of iron during pregnancy and the risk of islet autoimmunity or type 1 diabetes (Figure 10). The additional adjustment did not change the results. Energy-adjusted total vitamin C intake did not modify the association between total iron intake and the risk of islet autoimmunity (total iron intake*total vitamin C intake interaction P = 0.23) or type 1 diabetes (total iron intake*total vitamin C intake interaction P = 0.59).

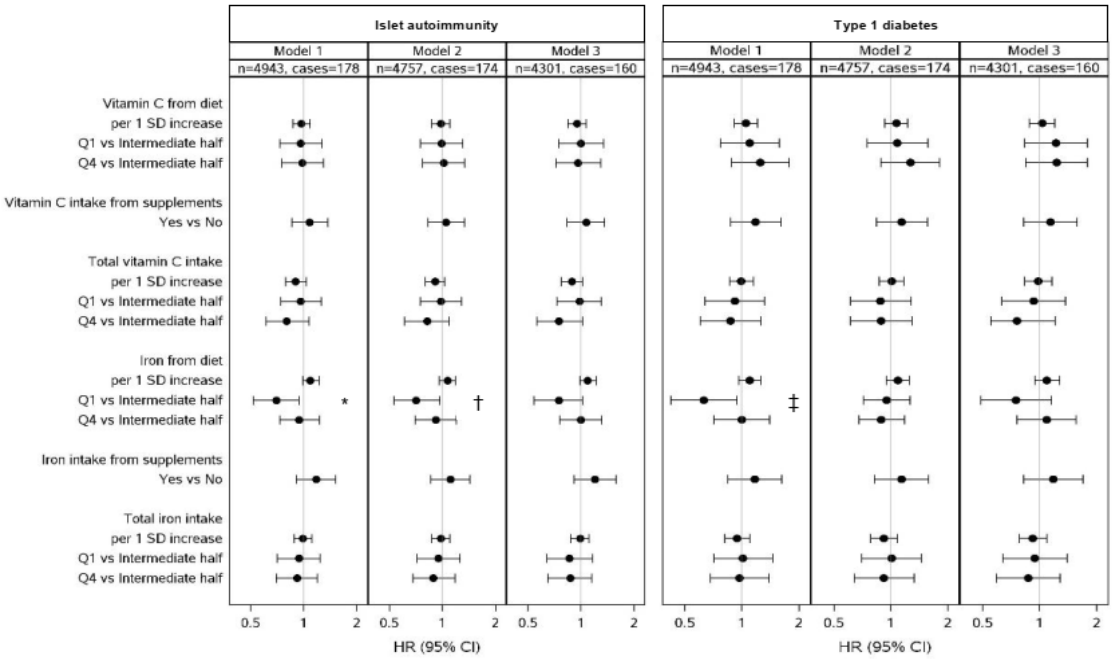

Figure 10. The association between maternal intake of vitamin C and iron during pregnancy and the risk of islet autoimmunity and type 1 diabetes in offspring: Cox proportional hazard regression model.

Model 1: Energy adjusted with Willett's residual method

Model 2: Adjusted for energy, sex, family history of diabetes, and HLA genotype

Model 3: Adjusted for energy residual method, sex, family history of diabetes, HLA genotype, maternal education, pre-pregnancy BMI, and smoking

* p = 0.06

† p = 0.09

‡ p = 0.06

7 DISCUSSION

7.1 Summary of results

In the present prospective multinational birth cohort study with children at risk for type 1 diabetes, a high plasma ascorbic acid status in childhood was associated with a decreased risk of islet autoimmunity and, specifically, the islet autoimmunity endotype, in which IAA is the first appearing autoantibody. In addition, a SNP in DHA transport gene *SLC2A2* rs5400 was associated with an increased risk of type 1 diabetes.

In the Finnish prospective birth cohort study, the maternal intake of nitrate, nitrite, iron, or vitamin C during pregnancy was not associated with the risk of islet autoimmunity or type 1 diabetes in children genetically at risk for type 1 diabetes.

7.2 Comparison of results with previous studies

7.2.1 Childhood plasma ascorbic acid status and the risk of islet autoimmunity and type 1 diabetes

In the present study, high plasma ascorbic acid was associated with a decreased risk of islet autoimmunity, particularly starting with IAA. Our novel finding suggests that high plasma ascorbic acid could protect against islet autoimmunity, particularly during infancy or in the early stages of autoimmunity development. The type of first-appearing autoantibody has been shown to vary depending on the age at seroconversion, and different autoantibodies may reflect different disease processes (Ilonen et al., 2013). IAA is usually the first autoantibody to appear during the first 2 years of age. In a previous DIPP study, certain dietary fatty acids were associated with the risk of islet autoimmunity, starting with IAA (Niinistö et al., 2017). Thus, there is evidence that dietary factors might play a role in autoantibody-specific islet autoimmunity.

7.2.2 Child's vitamin C metabolism-related SNPs and the risk of islet autoimmunity and type 1 diabetes

We assessed whether vitamin C metabolism-related SNPs modify the association between plasma ascorbic acid status and the risk of developing type 1 diabetes for the first time. Furthermore, our study was first to assessed whether these SNPs are associated with type 1 diabetes development.

The studied SNPs did not modify the association between plasma ascorbic acid and islet autoimmunity or type 1 diabetes. However, we observed that the minor allele for *SLC2A2/GLUT2* gene SNP rs5400 was associated with an increased risk of type 1 diabetes. This SNP has been associated with an increase in the risk of type 2 diabetes (Laukkanen et al., 2005; Willer et al., 2007), possibly via impaired glucose-stimulated insulin secretion. Interestingly, SNP rs5400 carriers have been suggested to have an increased preference for sugar intake, possibly due to altered glucose-sensing mechanisms and food intake regulation (Eny et al., 2008). A significant source of vitamin C during the early years of life might be fruit juices, which are also high in sugar, but we were unable to explore specific food groups at the time of this study. The association between SNP rs5400 and the risk of type 1 diabetes has not been previously studied; thus, further studies are needed to confirm whether our findings are due to chance.

Besides genotypes involved in vitamin C transport, genetic variations in proteins regulating oxidative stress and detoxification have been associated with vitamin C status such as glutathione S-transferase (GST), superoxide dismutase 2 (SOD2), and haptoglobin (HP) (Michels et al., 2013). However, these genes were not available in our current study.

7.2.3 Child's vitamin C metabolism-related SNPs and plasma ascorbic acid status

We observed that the minor allele for ascorbic acid transporter gene *SLC23A1* SNP rs33972313 was associated with lower plasma ascorbic acid status, which is in line with previous observation (Timpson et al., 2010). However, the SNP was not associated with islet autoimmunity or the development of type 1 diabetes, and the SNP did not modify the association between plasma ascorbic acid status or vitamin C intake and the risk of islet autoimmunity or type 1 diabetes. Our results suggests that high plasma ascorbic acid status might protect against islet autoimmunity even in minor allele carriers of *SLC23A1* SNP rs33972313.

7.2.4 Maternal intake of vitamin C during pregnancy and the risk of islet autoimmunity and type 1 diabetes

Maternal intake of vitamin C from diet and dietary supplements was not associated with the risk of islet autoimmunity or type 1 diabetes in our study. We assessed this association in a previous DIPP Nutrition Study, in which we also did not observe an association between maternal intake of vitamin C during pregnancy and the risk of islet autoimmunity (Uusitalo et al., 2008). However, in that study, type 1 diabetes was not explored as an outcome; the study included fewer mothers than in our current study, and the exclusive use of vitamin C supplements was not assessed.

Vitamin C absorption and excretion are efficiently regulated (Lykkesfeldt & Tveden-Nyborg, 2019). Intestinal absorption is enhanced and excretion in kidneys is suppressed during vitamin C deficiency and vice versa during sufficiency. The majority of the mothers in our study represent a well-nourished population, as only 5% of the mothers were vitamin C deficient at the time of dietary assessment. Thus, a higher intake of vitamin C might not provide extra benefits for type 1 diabetes prevention. However, we cannot conclude whether maternal intake of vitamin C would protect against islet autoimmunity or the development of type 1 diabetes in a vitamin C-deficient population. Fetus depends on maternal vitamin C intake, and it is required for normal child development (Lykkesfeldt & Tveden-Nyborg, 2019). Therefore, more studies are warranted, particularly in vitamin C-deficient populations.

Vitamin C has been suggested to function as a pro-oxidant in the presence of iron, although this association has been disputed based on *in vitro* studies on humans. (Valko et al., 2005). Vitamin C enhances the absorption of non-heme iron, which could contribute to increased iron status and further increased oxidative stress, but studies so far have shown that a high intake of vitamin C does not lead to iron overload (Gerster, 1999). This is in line with our current study, as we did not observe an interaction between maternal vitamin C and iron intake and the risk of islet autoimmunity or type 1 diabetes.

7.2.5 Maternal intake of nitrate and nitrite during pregnancy and the risk of islet autoimmunity and type 1 diabetes

Maternal intake of nitrate or nitrite during pregnancy was not associated with the risk of islet autoimmunity or type 1 diabetes in the offspring. Our current study provides new prospective evidence, as the only previous retrospective Childhood Diabetes in Finland study was conducted as far as 20 years ago (Virtanen et al., 1994). They observed

that the consumption of foods, which are a major source of nitrate and nitrite during pregnancy, was associated with an increased risk of type 1 diabetes in offspring. The high consumption of nitrate and nitrite is suggested to increase the endogenous formation of N-nitroso compounds in the gastrointestinal track (Kobayashi, 2018). Our current study included a more comprehensive dietary assessment, which could explain why we did not observe an association, as N-nitroso compound formation is influenced by other dietary components.

Vegetables and fruits naturally contains nitrate and nitrite but also other components such as vitamin C which is suggested to inhibit nitrosation (Bradbury et al., 2014; Wagner et al., 1985). Furthermore, N-nitroso compound formation in the gastrointestinal tract is influenced by gastric acidity and intestinal microbial flora (Kobayashi, 2018). Thus, the consumption of nitrate or nitrite does not exclusively lead to the formation of N-nitroso compounds.

Another potential mechanism for the endogenous formation of N-nitroso compounds is the use of nitrosatable drugs, which include common drugs, such as antibiotics and antihistamines. The ingestion of these drugs together with a nitrosating agent, such as nitrite, may enchase the formation of N-nitroso compounds. The use of nitrosatable drugs has been associated with an increased risk of preterm birth (Vuong et al., 2016, 2015), stillbirth (Thomsen et al., 2019), and neural tube defects (Brender et al., 2011). These results suggest that N-nitroso compound formation in the digestive tract of the mother during pregnancy might expose the unborn child to these compounds.

Nitrate and nitrite in the diet are sources of nitric oxide. Novel studies have also highlighted the potential dual role of nitric oxide. During islet inflammation, cytokine-induced nitric oxide production is suggested to induce DNA damage in beta cells and inhibit insulin secretion and oxidative metabolism (Oleson & Corbett, 2018). However, nitric oxide has also been suggested to protect beta cells from the aforementioned adverse outcomes. Whether sufficient vs. excess nitric oxide exposure results in different outcomes requires more studies.

Previous studies have suggested that childhood consumption of nitrate and nitrite might play a role in type 1 diabetes (Dahlquist et al., 1990; Virtanen et al., 1994). Furthermore, the Finnish Food Authority reported that some Finnish children exceeded the acceptable daily intake of nitrite due to the high consumption of processed meat products (Suomi et al., 2013, 2016). We could not assess childhood diet at the time of our current study, which is an important next step in the DIPP study. The EFSA reported that exposure to volatile nitrosamines in Finnish adults is equal to the European median, whereas in 3–9-year-old children, exposure is above the European

median (EFSA Panel on ANS et al., 2017). Thus, data on N-nitroso compounds intake during childhood could also be important in future studies.

7.2.6 Maternal intake of iron during pregnancy and the risk of islet autoimmunity and type 1 diabetes

We observed no association between maternal intake of iron from diet and dietary supplements and the risk of islet autoimmunity or type 1 diabetes in offspring. Increased iron status is suggested to result from excessive dietary iron since iron is required from diet (Sogaard et al., 2017). Excessive iron generates ROS accumulation and oxidative stress, to which beta cells are vulnerable. Since iron supplements are commonly used during pregnancy in our study, even though the majority of mothers were well-nourished, it could be possible that the majority of mothers already had sufficient iron status. However, we did not have data on maternal iron status. Similar to our study, the Danish National Birth Cohort Study found no association between the maternal use of pure iron supplements and the risk of type 1 diabetes in offspring, although dietary intake was not assessed (Thorsen et al., 2019). The researchers discussed that placental iron transport regulation or maternal iron absorption regulation via hepcidin might explain the findings. In contrast, the Norwegian Mother and Child Cohort Study observed that maternal use of iron supplements during pregnancy was associated with an increased risk of type 1 diabetes, but dietary intake was not (Stordal et al., 2018). Based on their secondary studies, the researchers suggested that the association might result from iron-induced maternal inflammation, as the maternal cytokines of the pro-inflammatory M1 macrophages increased in supplement users (Stordal et al., 2018). Excess iron due to supplement use has been observed to induce oxidative stress in placental tissue (Devrim et al., 2006). However, the implications for offspring have yet to be studied. A major limitation is that previous studies, including ours that assessed dietary iron intake and the risk of type 1 diabetes, did not include plasma iron status.

Another potential mechanism could be increased serum ferritin status (Milman et al., 1991; Preziosi et al., 1997). Cord serum ferritin status is suggested to be a strong predictor of iron status during the first 2 years of life (Hay et al., 2007), and a high cord blood iron concentration has been associated with an increased risk of type 1 diabetes in offspring (Kyvsgaard et al., 2017). The Norwegian study group observed a nonsignificant, although suggestive, association between increasing cord blood ferritin status and the risk of type 1 diabetes (Stordal et al., 2018). Our study did not include iron measurements from cord blood.

Genetics might also play a role in the association between iron and the development of type 1 diabetes. Hereditary (HFE-related) hemochromatosis is a disorder in which iron is absorbed in the intestine, resulting in an excessive pathological increase in iron stores. The disorder is caused by a mutation in the HFE gene. A Norwegian study suggested that the maternal HFE genotype might increase the risk of type 1 diabetes in offspring (Stordal et al., 2018). Increased iron stores due to HFE hemochromatosis could cause iron accumulation in the endocrine area of the pancreas and injury to beta cells (Cooksey et al., 2004; Huang et al., 2011).

Finally, excess iron has been suggested to alter the gut microbiota (Dostal et al., 2012) observed in patients with inflammatory bowel disease (Lee et al., 2017). However, the implications for healthy mothers and the development of type 1 diabetes in offspring require further study.

7.3 Strengths and limitations

The major strength of this study was the data from two large and well-defined birth cohorts containing children genetically at risk for type 1 diabetes. The prospective design ensures that the measurement of dietary exposure precedes type 1 diabetes outcomes. Furthermore, both cohorts assessed the development of islet autoimmunity following the emergence of specific type 1 diabetes-related autoantibodies. However, at the time of this study, specific autoantibody-initiated islet autoimmunity outcomes were available only in the TEDDY study.

Our study assessed plasma ascorbic acid status in childhood. Since plasma levels of DHA are very low and ascorbate radical is undetectable in healthy humans (Doseděl et al., 2021), ascorbic acid is an appropriate marker of plasma vitamin C status.

The FFQ used to assess maternal diet was validated (Erkkola et al., 2001). The validation study of the FFQ showed correlations of 0.65 for vitamin C, 0.60 for iron, 0.63 for nitrate, and 0.79 for nitrite in comparison to food records, indicating that the FFQ is appropriate for the assessment of these dietary factors in question. However, since FFQ tends to overestimate food, nutrient, and energy intake, we used energy-adjusted intake by the residual method in the outcome analyses (Willett et al., 1997).

Our food composition database considers the loss of vitamin C in vegetables and fruits during cooking, which can range from 20% to 90%, depending on vegetable or cooking time and method (Armstrong et al., 2019). Furthermore, the food composition database used in the study was updated recently by nitrate and nitrite contents in foods

based on recent assessments and literature (Foedevaredirektoratet., 1999; Laitinen et al., 1993; Suomi et al., 2013, 2016; Susin et al., 2006; Ysart et al., 1999).

We were able to include several potential confounding factors as adjustments in our analysis, such as the child's sex, HLA genotype, and family history of type 1 diabetes. In the TEDDY study analyses, we used principal component analysis for population stratification adjustment because SNPs are widely distributed in populations. In the DIPP study, we were also able to consider other dietary factors, such as antioxidants, and sociodemographic characteristics that could confound our results.

This study has some limitations. The array platform for measuring genetic information on SNPs might not accurately determine the target genes. The TEDDY study cohort included only children who developed type 1 diabetes at a very early age (mean age of diagnosis 29 months), and the follow-up time up to 10 years of age for type 1 diabetes was relatively short. Furthermore, plasma ascorbic acid status might be affected by other confounding factors not assessed in our study, such as endogenous stress and infection.

Besides cooking, we could not consider other processing of vegetables and fruits that could result in a loss of vitamin C, such as long storage (9–78% of loss), chilling (3–73%), and reheating (3–90%) (Armstrong et al., 2019). Nitrate content in vegetables can also decrease during washing (10–15%) and cooking (51%) (EFSA, 2008). Vitamin C and nitrate dissolve in the cooking liquid, and the consumption of the cooking liquid also influences intake. Furthermore, the nitrate content in vegetables varies depending on, for example, season, cultivation method (open field vs. greenhouse), use of fertilizers, and climate (Suomi et al., 2013).

The retrospective FFQ was used for the assessment of maternal diet during pregnancy since participating children were identified by cord blood genotype screening after birth. Since the maternal diet during pregnancy could not be recorded in real time, it could result in some recall bias. However, the validation study correlation coefficients were similar for the pregnancy FFQ, and a second FFQ was assessed 1 month after delivery (Erkkola et al., 2001). Furthermore, 8th month of pregnancy is a well identifiable time right before pregnancy leave, and it reflects the maternal diet throughout the pregnancy well. Although we did not have an accurate assessment of diet in early pregnancy, it is not known whether there is a critical period during pregnancy in relation to the development of autoimmune diseases.

Another limitation is that fruit and vegetable consumption may also be confounded by several socioeconomic and lifestyle factors not assessed in our study (Dehghan et al., 2007). Furthermore, our food composition database did not include nitrate content in drinking water, which could be high in some well waters in Finland (Ahonen et al.,

2008). However, nitrate content in Finnish drinking water in general is below the WHO standard (< 50 mg/l) (Ahonen et al., 2008; WHO, 2003). In addition, our food composition database did not include the N-nitroso compound contents in foods (Bahadoran et al., 2016).

Finally, observational studies explore associations, but they cannot confirm causality. Additionally, since the children included in the study have a genetic risk for type 1 diabetes, the generalizability of these results should be interpreted cautiously.

8 SUMMARY AND CONCLUSIONS

The current study provides novel evidence that a high plasma ascorbic acid status might protect against islet autoimmunity, starting with IAA, in children at risk for type 1 diabetes. Since there are no similar previous studies, these associations should be explored further.

Our study does not support the hypothesis that a high maternal intake of nitrate and nitrite during pregnancy increases the risk of islet autoimmunity and type 1 diabetes in offspring. However, the hypothesis is by no means conclusive. A more detailed assessment of exposure would require the assessment of maternal dietary intake of N-nitroso compounds, nitrate and nitrite intake from drinking water, and the use of nitrosatable drugs during pregnancy. As previous studies have suggested, the childhood consumption of nitrate and nitrite might play a role in type 1 diabetes, and the intake of nitrite and N-nitroso compounds is suggested to be higher than recommended in Finnish children. Therefore, the assessment of the association between nitrate, nitrite, and N-nitroso compound intake during childhood and the risk of islet autoimmunity and type 1 diabetes is an important next step.

Diet is the sole source of vitamin C. A more comprehensive assessment of vitamin C activity in the development of type 1 diabetes would require an assessment of both dietary intake and plasma status from both mother and child. Furthermore, the role of vitamin C metabolism-related genes on the risk of type 1 diabetes development requires further assessment.

It is apparent that evidence of an association between maternal iron intake and the development of type 1 diabetes is inconclusive. In our current study, we assessed dietary intake of iron during pregnancy, but it's implication on fetal iron status is more complex. The mothers in our study and previous studies are from well-nourished populations, and the use of iron supplements is common. Therefore, we cannot confirm our results for populations with iron deficiency. For a more comprehensive risk assessment of iron in the development of type 1 diabetes, iron biomarkers and possibly genetic data, in addition to dietary assessments, are needed. Our study was the first to explore the association between prenatal iron intake and the risk of islet autoimmunity and thus requires further study.

9 IMPLICATIONS FOR FUTURE DIRECTIONS

The current study has provided novel results and a foundation for further research. Here I present some of the planned next steps.

At the current TEDDY study, we were unable to assess vitamin C intake in childhood. We will be exploring the association between childhood dietary intake of vitamin C and the risk of islet autoimmunity and type 1 diabetes in the future TEDDY study. We will also assess whether the vitamin C metabolism-related genes modify the association between vitamin C intake and the risk of type 1 diabetes outcomes. In this study we have more comprehensive genetic data available which includes not only genotypes for vitamin C transport proteins but also proteins regulating oxidative stress and detoxification: glutathione S-transferase, superoxide dismutase 2, and haptoglobin.

During the current study, we updated our food composition database with nitrate and nitrite content of foods. Therefore, in the DIPP study, we will be exploring the association between nitrate and nitrite intake during childhood and the risk of islet autoimmunity and type 1 diabetes. In the statistical analyses we will be using joint model which provides more robust results in comparison to Cox regression. Joint model allows the inclusion of all food records available for each child which decreases potential bias caused by missing data.

In the DIPP Study, we will also explore the association between the consumption of vegetables, fruits, and berries in childhood and the risk of islet autoimmunity and type 1 diabetes. Since vegetables, fruits, and berries are major source of vitamin C and nitrate, this new study might provide interesting new findings that support the thesis and subsequent studies.

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PUBLICATIONS

PUBLICATION I

Plasma ascorbic acid (vitamin C) and the risk of islet autoimmunity and type 1 diabetes: The Environmental Determinants of Diabetes in the Young (TEDDY) Study

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ARTICLE

Plasma ascorbic acid and the risk of islet autoimmunity and type 1 diabetes: the TEDDY study

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Abstract

Aims/hypothesis We studied the association of plasma ascorbic acid with the risk of developing islet autoimmunity and type 1 diabetes and examined whether SNPs in vitamin C transport genes modify these associations. Furthermore, we aimed to determine whether the SNPs themselves are associated with the risk of islet autoimmunity or type 1 diabetes.

Methods We used a risk set sampled nested case–control design within an ongoing international multicentre observational study: The Environmental Determinants of Diabetes in the Young (TEDDY). The TEDDY study followed children with increased genetic risk from birth to endpoints of islet autoantibodies (350 cases, 974 controls) and type 1 diabetes (102 cases, 282 controls) in six clinical centres. Control participants were matched for family history of type 1 diabetes, clinical centre and sex. Plasma ascorbic acid concentration was measured at ages 6 and 12 months and then annually up to age 6 years. SNPs in vitamin C transport genes were genotyped using the ImmunoChip custom microarray. Comparisons were adjusted for HLA genotypes and for background population stratification.

Results Childhood plasma ascorbic acid (mean ± SD 10.76 ± 3.54 mg/l in controls) was inversely associated with islet autoimmunity risk (adjusted OR 0.96 [95% CI 0.92, 0.99] per +1 mg/l), particularly islet autoimmunity, starting with insulin autoantibodies (OR 0.94 [95% CI 0.88, 0.99]), but not with type 1 diabetes risk (OR 0.93 [95% Cl 0.86, 1.02]). The *SLC2A2* rs5400 SNP was associated with increased risk of type 1 diabetes (OR 1.77 [95% CI 1.12, 2.80]), independent of plasma ascorbic acid (OR 0.92 [95% CI 0.84, 1.00]).

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Research in context

What is already known about this subject?

- Vitamin C may prevent oxidative damage in beta cells, protecting against islet autoimmunity and type 1 diabetes; however, longitudinal evidence is lacking

What is the key question?

- Is plasma ascorbic acid (vitamin C) associated with the risk of islet autoimmunity and type 1 diabetes in children who are at increased genetic risk of type 1 diabetes?

What are the new findings?

- In this nested case–control study, childhood plasma ascorbic acid status was inversely associated with islet autoimmunity risk

How might this impact on clinical practice in the foreseeable future?

- Increased plasma ascorbic acid may protect against islet autoimmunity in children at genetic risk for type 1 diabetes

Conclusions/interpretation Higher plasma ascorbic acid levels may protect against islet autoimmunity in children genetically at risk for type 1 diabetes. Further studies are warranted to confirm these findings.
Data availability The datasets generated and analysed during the current study will be made available in the NIDDK Central Repository at https://www.niddkrepository.org/studies/teddy.

Keywords Autoimmunity · Plasma ascorbic acid · Single nucleotide polymorphism · SNP · Transporter genes · Type 1 diabetes · Vitamin C

Abbreviations

DHA	Dehydroascorbic acid
GADA	GAD autoantibody
IAA	Insulin autoantibody
IA-2A	Autoantibody to tyrosine phosphatase-related islet antigen 2
NIST	National Institute of Standards and Technology
SLC2A	Solute carrier family 2
SVCT	Sodium L-ascorbic acid transporter
TEDDY	The Environmental Determinants of Diabetes in the Young

Introduction

Oxidative stress may play a role in the pathogenesis of type 1 diabetes for several reasons. The cells in pancreatic islets are more vulnerable to oxidative damage than many other cells due to the low activity of free-radical detoxifying and redox-regulating enzymes such as catalase, superoxide dismutase and glutathione peroxidase [1]. It has been hypothesised that dietary antioxidants might improve the islets' capacity to cope with oxidative stress (e.g. induced by hyperglycaemia) [1–4].

Vitamin C (ascorbic acid) is a water-soluble vitamin obtained from vegetables, fruits and berries [5]. As a dietary antioxidant vitamin C might protect against the development of type 1 diabetes [6]. However, only two case–control studies have investigated the issue. In an Australian study, use of vitamin C supplements was less frequent in children with type 1 diabetes before onset [7]. On the other hand, a Swedish study found no differences in dietary vitamin C intake before onset between type 1 diabetes cases and controls [8]. The association between plasma ascorbic acid concentration and islet autoimmunity or the subsequent development of type 1 diabetes has not been investigated. Plasma ascorbic acid represents the most accurate measure of available vitamin C in the body [9, 10].

Genetic variation in vitamin C metabolic pathways causes inter-individual differences in plasma and tissue ascorbic acid availability [9], which might similarly contribute to type 1 diabetes risk. The metabolism of ascorbic acid is regulated by key proteins called sodium L-ascorbic acid transporters (SVCTs). Two isoforms, hSVCT1 and hSVCT2, encoded by the genes *SLC23A1* and *SLC23A2*, respectively, control the active transport of ascorbic acid across cell membranes and uptake to tissues [11]. hSVCT1 expression is confined to epithelia in renal, intestinal and hepatic tissues, while hSVCT2 is responsible for tissue-specific uptake.

The SNP rs33972313, a low-frequency missense variant in *SLC23A1*, has been consistently associated with lower circulating ascorbic acid status [11]. Other SNPs in *SLC23A1*

(intronic SNPs rs6596473 and rs4257763, and promoter SNP rs10063949) have also been associated with ascorbic acid concentration, but less consistently. Furthermore, intronic SNPs (rs6053005 and rs1279683) in *SLC23A2* have been associated with ascorbic acid concentration [11–14]. Less is known about the importance of the second pathway, the solute carrier family 2 (SLC2A; also called GLUT) family proteins that transport dehydroascorbic acid (DHA) into cells where it is converted into ascorbic acid [15].

The aim of this study was to examine the association of plasma ascorbic acid concentration with the risk of islet autoimmunity and type 1 diabetes in children with a high genetic risk of type 1 diabetes. Furthermore, we studied the association of plasma ascorbic acid with the risk of islet autoimmunity relative to the first autoantibody to be observed (either insulin autoantibody [IAA] or GAD autoantibody [GADA], referred to hereafter as IAA first and GADA first, respectively), because the type of autoantibody appearing first may reflect different disease processes [16–18]. In addition, we examined whether vitamin C metabolism genes available on the ImmunoChip are associated with, or modify, the association between plasma ascorbic acid and the development of islet autoimmunity and type 1 diabetes. This included a protein-coding missense SNP in *SLC23A1* (rs33972313), two intronic *SLC2A1* (also known as *GLUT1*, 1p34.2) SNPs (rs1105297 and rs3754223), and an *SLC2A2* (also known as *GLUT2*, 3q26.2) SNP (rs5400) in the dehydro-L-ascorbic acid transport genes.

Methods

Study design The study was performed as a nested case–control study within The Environmental Determinants of the Diabetes in the Young (TEDDY) study. The TEDDY study is an international multicentre observational cohort study that prospectively follows children from birth in the search for environmental factors involved in the development of islet autoimmunity and subsequent type 1 diabetes in genetically susceptible children (based upon HLA genotype), as described in detail previously [19, 20]. Written informed consents have been obtained for all study participants, from a parent or primary caretaker, separately, for genetic screening and participation in prospective follow-up visits. The study is funded by the National Institutes of Health (NIH) and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and approved by local Institutional Review Boards and monitored by an External Advisory Board. The TEDDY study is conducted in clinical research centres in the USA (Colorado, Georgia/Florida, Washington State), Finland, Sweden and Germany.

Study population Between 1 September 2004 and 28 February 2010, a total of 424,788 new-born infants were screened for type 1 diabetes-associated HLA genotypes. Eligibility criteria for initial contact was one of the following HLA class II genotypes: *HLA-DR3/4*; *HLA-DR4/4*; *HLA-DR4/8*; *HLA-DR3/3* and *HLA-DR4/4*. Infants with HLA-DR genotypes *HLA-DR4/1*, *HLA-DR4/13*, *HLA-DR4/9* and *HLA-DR3/9* were included only if they had a first-degree relative (i.e. mother, father or sibling) with type 1 diabetes [21]. Of the eligible infants, 21,589 had type 1 diabetes genetic risk based on HLA genotyping and 8676 children were enrolled. Children were followed every 3 months with a scheduled clinic visit until the age of 4 years, and every 6 months thereafter until being diagnosed with type 1 diabetes. Children with islet autoimmunity were followed every 3 months throughout the study. Of the enrolled children, 2211 (25.5% of the 8676) were withdrawn or lost to follow-up by 6 years of age at the time of the design.

Outcomes The primary outcomes in this study were persistent confirmed islet autoimmunity and the diagnosis of type 1 diabetes. Persistent confirmed islet autoimmunity was defined by appearance of one or more islet cell autoantibodies (IAA, GAD, or autoantibody to tyrosine phosphatase-related islet antigen 2 [IA-2A], also known as insulinoma antigen-2 antibody) confirmed at two consecutive visits. Type 1 diabetes diagnosis was based on American Diabetes Association criteria [22]. Different autoantibodies may be associated with different disease processes [16–18] and therefore secondary analyses were conducted regarding timing of autoantibodies: IAA first and GADA first.

Nested case–control design

The current study was performed with risk set sampling described previously [23]. The study was conducted in two nested case–control designs within the TEDDY study: (1) for islet autoimmunity outcome; and (2) for type 1 diabetes outcome. Three matched controls were selected per islet autoimmunity and type 1 diabetes case. Children were matched for family history of type 1 diabetes, clinical centre and sex. The nested case–control study sets were based on the data collected as of 31 May 2012 [23].

The islet autoimmunity outcome analysis included 350 cases with median seroconversion age of 23 months (range 6–72 months) and 974 matched controls. Islet autoimmunity cases were defined as a participant with persistent islet autoimmunity. A control was defined as a participant who had not developed persistent islet autoimmunity by the time that the corresponding matched case had done, plus 45 days. The islet autoimmunity case–control set used for the statistical analyses consisted of 3371 plasma samples taken for ascorbic acid

measurement. Three hundred and sixty-five samples failed the laboratory's internal quality control. Samples from controls were processed only when the matched case had an available sample at a corresponding visit. The mean ascorbic acid level for each child was calculated from all available measurements.

Type 1 diabetes outcome analysis consisted of 102 cases with median age of 31 months at diagnosis (range 8–75 months) and 282 matched controls. A control for a type 1 diabetes case was defined as a participant who had not developed type 1 diabetes by the time the corresponding matched case had done so, plus 45 days. Of the 350 islet autoimmunity cases, 74 were also analysed as type 1 diabetes cases. Those islet autoimmunity cases who developed type 1 diabetes but had seroconverted already before the first plasma sample was collected for ascorbic acid measurement (collection started at 6 months of age) were included only in the type 1 diabetes analysis. Thus, 28 type 1 diabetes cases were included only for type 1 diabetes analysis.

Secondary outcome analyses were performed within the islet autoimmunity nested case–control design. The islet autoimmunity dataset included 163 IAA first cases with median seroconversion age of 18 months (range 6–72 months) and 450 controls. Within the islet autoimmunity dataset there were 120 GADA first cases with median seroconversion age of 28 months (range 6–68 months) with 336 controls.

Plasma ascorbic acid measurements Plasma samples for ascorbic acid measurement were collected at the age of 6 months and 12 months, and then annually until 6 years of age or until and including the time of seroconversion of the islet autoimmunity cases, and for type 1 diabetes cases the visit just preceding the type 1 diabetes diagnosis (with corresponding time for matched controls). Stabilisers were added to plasma samples intended for ascorbic acid analysis before freezing to minimise degradation of ascorbic acid [24]. After sample collection at the clinical centres, 50 μl of sodium citrate plasma (in BD Vacutainer CPT Cell Preparation Tubes [Becton Dickinson, Franklin Lakes, NJ, USA]) was transferred into cryovials and 0.2 ml of 5% (wt/vol.) trichloroacetic acid and 200 mg disodium EDTA was added, with subsequent freezing at −70°C. A long-distance protocol was developed for the collection of blood samples from families living away from their nearest TEDDY study clinic (most frequently being the case in Germany compared with the other countries). In the long-distance collection protocol, blood samples were obtained by a family paediatrician and transported within 24 h to the TEDDY study clinic site. Case samples were paired with matched control samples and randomly placed within a batch before samples were transported to the laboratory.

Ascorbic acid measurements were performed at the Biochemistry Laboratory, Genomics and Biomarkers Unit, National Institute for Health and Welfare (THL), Helsinki, Finland. Ascorbic acid was determined by an ion-paired, reversed-phase, high-performance liquid chromatographic method using electrochemical detection, as described [24]. Isoascorbic acid was used as internal standard for the quantification of ascorbic acid. The laboratory staff were blinded to the case–control status of samples. The laboratory included three internal quality control samples of three ascorbic acid levels in each run (altogether nine samples). Precision within the project, expressed as the CV of the quality control samples, was 9.2–12.6% at a concentration range of 4.6–11.2 mg/l. During the project, the laboratory participated three times in an external quality assessment scheme (National Institute of Standards and Technology (NIST) Micronutrients Measurement Quality Assurance Program for Total Ascorbic Acid). The results were in excellent concordance with NIST values.

Genotyping Illumina Infinium ImmunoChip custom microarray was used for SNP genotyping, based upon robust genome-wide association analyses (GWAS) in 12 autoimmune diseases (including type 1 diabetes). The ImmunoChip array contained 195,806 SNPs that were genotyped on TEDDY study DNA samples. Principal components analysis (PCA) using EIGENSTRAT software (Department of Genetics, Harvard Medical School, Boston, MA, USA) was performed using each unrelated TEDDY study participant to estimate ancestry, with the two most significant principal components used as covariates in analytical models. For our primary hypothesis on SNPs in ascorbic acid pathways, *SLC23A1* (rs33972313), *SLC2A1* (rs1105297 and rs3754223) and *SLC2A2* (rs5400) were on the ImmunoChip, passed quality control metrics and were selected for analysis.

Statistical analyses A linear mixed effects model adjusted for the case–control status was used to examine whether plasma ascorbic acid over time was different by the use of standard vs long-distance protocol, country, SNPs and other risk factors for type 1 diabetes. The random effects for participant were nested within the random effects for case–control set in the model. Weight z score and height z score were derived from Centers for Disease Control and Prevention standardised growth charts.

Plasma ascorbic acid was analysed up to the case's event age specific to each nested case–control set. The measures were from all visits prior to and including the first of two consecutive visits at which the child tested positive for an autoantibody for islet autoimmunity analysis and prior to the diagnosis for type 1 diabetes analysis. The mean of the corresponding measures for each participant (childhood ascorbic acid) was compared between matched case and control children. Conditional logistic regression was used to assess the association between the characteristics of interest and the case–control status. Interaction term with the matching factors was tested for the effect modification. All analyses were adjusted for HLA genotype (*HLA-DR3/4* vs other) and two principal components of

ancestry to control for population stratification. The log-linearity of the characteristic with each outcome was examined using the supremum test [25]. All analyses were performed using SAS 9.4 (SAS Institute, Cary, NC, USA). A two-sided p value <0.05 was considered statistically significant.

Results

Background characteristics The mean and SD of childhood plasma ascorbic acid concentrations are shown according to matching variables in islet autoimmunity and type 1 diabetes case–control children in Table 1. The mean (± SD) plasma ascorbic acid concentration for islet autoimmunity cases and controls was 10.21 ± 3.33 mg/l and 10.76 ± 3.54 mg/l, respectively. For the type 1 diabetes cases and controls, the mean (± SD) plasma ascorbic acid concentration was 9.73 ± 3.18 mg/l and 10.58 ± 3.57 mg/l, respectively.

Plasma ascorbic acid concentrations did not differ by the use of standard protocol vs long-distance protocol. As potential risk factors for type 1 diabetes, we examined growth variables (mean height and weight z score prior to the outcome), as well as breastfeeding status at 3 and 6 months. Breastfeeding was associated with lower plasma ascorbic acid status (mixed model regression variables estimate [SE]: −0.96 [0.26], $p < 0.001$ for breastfeeding vs not breastfeeding at 3 months and −0.77 [0.23], $p < 0.001$ for breastfeeding vs not breastfeeding at 6 months) but no significant association was found with the outcomes. Higher weight or height z scores were associated with lower plasma ascorbic acid concentration (−0.28 [0.10], $p = 0.006$ and −0.12 [0.04], $p = 0.001$, respectively). Because weight was also associated with islet autoimmunity (OR [95% CI]: 1.23 [1.07, 1.41] for any islet autoimmunity; 1.24 [1.01, 1.51] for IAA first; 1.32 [1.05, 1.65] for GADA first), we adjusted for mean weight z score in the models examining ascorbic acid concentration vs outcomes.

Primary outcomes: islet autoimmunity and type 1 diabetes Childhood plasma ascorbic acid status was inversely associated with islet autoimmunity (OR 0.96 [95% CI 0.92, 0.99], $p = 0.04$) but the association with type 1 diabetes risk was not significant (OR 0.93 [95% CI 0.86 1.02], $p = 0.11$) (Table 2). Adjustment for mean weight z score prior to the outcome showed a similar pattern: islet autoimmunity OR 0.96 (95% CI 0.92, 1.00), $p = 0.06$; type 1 diabetes OR 0.93 (95% CI 0.86, 1.02), $p = 0.11$. All the outcome analyses were adjusted for ethnicity and *HLA-DR3/4* genotype. The association between plasma ascorbic acid concentration and risk of islet autoimmunity and type 1 diabetes was not modified by the participant *HLA-DR3/4* genotype, clinical centre, sex or family history of type 1 diabetes.

Table 1 Mean childhood plasma ascorbic acid in islet autoimmunity and type 1 diabetes cases and controls

Matching variable	Islet autoimmunity			Type 1 diabetes		
	No. (%) of cases	Plasma ascorbic acid concentration (mg/l)[a]		No. (%) of cases	Plasma ascorbic acid concentration (mg/l)[a]	
		Cases	Controls		Cases	Controls
Clinical centre						
Colorado	51 (14.6)	11.7 ± 3.0	12.2 ± 3.3	15 (14.7)	12.0 ± 2.5	12.2 ± 3.1
Georgia	24 (6.9)	12.5 ± 3.5	12.3 ± 3.4	6 (5.9)	13.1 ± 4.2	13.6 ± 4.1
Washington State	34 (9.7)	11.4 ± 4.3	11.8 ± 4.4	7 (6.9)	9.8 ± 3.5	11.6 ± 3.2
Finland	105 (30.0)	10.5 ± 2.8	10.7 ± 3.0	35 (34.3)	9.8 ± 2.7	10.7 ± 3.6
Germany	26 (7.4)	9.2 ± 2.5	9.7 ± 3.4	15 (14.7)	10.2 ± 3.2	10.3 ± 3.8
Sweden	110 (31.4)	8.7 ± 3.0	9.5 ± 3.6	24 (23.5)	7.9 ± .9	8.8 ± 3.0
Sex						
Female	157 (44.9)	10.0 ± 3.4	10.7 ± 3.5	47 (46.1)	10.2 ± 3.1	10.2 ± 3.1
Male	193 (55.1)	10.4 ± 3.2	10.8 ± 3.7	55 (53.9)	9.7 ± 3.3	11.2 ± 4.0
FDR/GP status						
FDR	76 (21.7)	10.8 ± 3.1	11.1 ± 3.7	36 (35.3)	10.8 ± 2.8	11.0 ± 3.8
GP	274 (78.3)	10.0 ± 3.4	10.7 ± 3.5	66 (64.7)	9.5 ± 3.4	10.6 ± 3.6

Plasma ascorbic acid concentrations are presented as mean ± SD

[a] Mean childhood plasma ascorbic acid; includes measures from all visits prior to and including the seroconversion visit, which is the first of two consecutive visits at which the child tested positive for an autoantibody. To convert ascorbic acid concentration to μmol/l, multiply values in mg/l by 5.678

FDR, first-degree relative of an individual with type 1 diabetes; GP, from the general population (no first-degree relative with type 1 diabetes)

Table 2 Risk of type 1 diabetes-related outcomes associated with childhood plasma ascorbic acid

Outcome	OR (95% CI)[a]	*p* value
Islet autoimmunity (cases, *n* = 350)	0.96 (0.92, 0.99)	0.041
Type 1 diabetes (cases, *n* = 102)	0.93 (0.86, 1.02)	0.109
IAA first (cases, *n* = 163)	0.94 (0.88, 0.99)	0.028
GADA first (cases, *n* = 120)	0.99 (0.93, 1.07)	0.988

Data are presented as OR (95% CI) per 1 mg/l increase in childhood ascorbic acid concentration

Mean childhood plasma ascorbic acid includes measures from all visits prior to and including the seroconversion visit, which is the first of two consecutive visits at which the child tested positive for an autoantibody, and for type 1 diabetes all visits prior to diagnosis. To convert ascorbic acid concentration to μmol/l, multiply values in mg/l by 5.678

[a] Adjusted for two largest principal components for ethnicity and *HLA-DR3/4* genotype

Secondary outcomes: IAA first and GADA first Childhood plasma ascorbic acid status was inversely associated with the risk of IAA first (OR 0.94 [95% CI 0.88, 0.99], $p = 0.03$). Adjustment for mean weight *z* score prior to the outcome produced similar results (OR 0.93 [95% CI 0.88, 0.99], $p = 0.03$). Plasma ascorbic acid concentration was not associated with GADA first (OR 0.99 [95% CI 0.93, 1.07], $p = 0.99$). All the outcome analyses were adjusted for ethnicity and *HLA-DR3/4* genotype. The *HLA-DR3/4* genotype did not modify the association between plasma ascorbic acid concentration and the risk of IAA first or risk of GADA first.

SNPs and outcomes and effect modification by SNPs The *SLC23A1* rs33972313 minor allele carriers had lower mean plasma ascorbic acid concentration than non-carriers (mixed model regression variable estimate [SE]: −2.22 [0.46], $p < 0.001$). However, the SNP was not associated with the risk of islet autoimmunity or type 1 diabetes (Table 3), nor with IAA first or GADA first (data not shown). Furthermore, *SLC23A1* rs33972313 did not modify the association between plasma ascorbic acid and the risk of islet autoimmunity or type 1 diabetes (Table 3), IAA first (interaction $p = 0.43$) or GADA first (interaction $p = 0.10$). DHA transport gene SNPs in *SLC2A1* (rs1105297 and rs3754223) were not associated with plasma ascorbic acid concentration ($p = 0.32$ and $p = 0.76$) or the risk of islet autoimmunity, type 1 diabetes, IAA first or GADA first, either alone or in conjunction with the plasma ascorbate status. They did not modify the association between plasma ascorbic acid and the risk of islet autoimmunity or type 1 diabetes (Table 3), IAA first or GADA first (data not shown).

SLC2A2 rs5400 was not associated with plasma ascorbic acid concentration ($p = 0.54$) or the risk of islet autoimmunity, IAA first or GADA first; however, *SLC2A2* rs5400 was associated with increased risk of type 1 diabetes (OR 1.66 [95% CI 1.06, 2.60], $p = 0.028$) (Table 3). The association remained even after adjusting for plasma ascorbic acid in addition to the two largest principal components for ethnicity and *HLA-DR3/4* genotype. In this model, rs5400 was associated with increased risk of type 1 diabetes (OR 1.77 [95% CI 1.12, 2.80], $p = 0.015$), while plasma ascorbic acid concentration showed no association (OR 0.92 [95% CI 0.84, 1.00], $p = 0.058$). The *SLC2A2* rs5400 SNP did not modify the association of plasma ascorbic acid status with the risk of islet autoimmunity or type 1 diabetes, IAA first or GADA first.

Discussion

In this relatively large prospective study, mean ascorbic acid concentration in plasma was inversely associated with the risk of islet autoimmunity, but not type 1 diabetes, in children with increased genetic risk of type 1 diabetes. The association between ascorbic acid and type 1 diabetes was, however, only marginally different and showed a stronger point estimate compared with islet autoimmunity. The SNPs investigated in our study (i.e. SNPs in ascorbic acid or dehydroascorbic acid transport genes) did not modify the observed associations with islet autoimmunity and the SNPs themselves were not associated with islet autoimmunity. However, the presence of the minor alleles in *SLC2A2* rs5400 appeared to increase the risk of type 1 diabetes. Furthermore, SNP rs33972313 in *SLC23A1* was associated with lower plasma ascorbic acid concentrations, in line with previous studies, and appeared to increase type 1 diabetes risk, although the finding was of borderline significance ($p = 0.06$).

One of the strengths of our study is the large multinational study sample with multiple measurements of plasma ascorbic acid as well as genetic information. To our best knowledge, our study is the first one to assess prospectively whether plasma ascorbic acid concentration (and genetic variation related to ascorbic acid) is associated with development of islet autoimmunity and type 1 diabetes. Measurement of ascorbic acid concentration in the plasma may more accurately reflect availability to tissues, as compared with dietary intake measurements. Previous case–control studies have assessed ascorbic acid intake from diet and supplements using questionnaire [7, 8]. Another strength is that we were able to investigate the endpoints of IAA first and GADA first separately; this is important because they may reflect different disease processes. IAA usually appears during the first to second year of life, whereas GADA usually appears at 3–5 years of age or even later [26, 27]. In other words, we were able to study associations of plasma ascorbic acid at very early stages of autoimmunity development. A limitation of our study is the use of array platform for measuring genetic information on SNPs, as this might not determine the target genes accurately.

Table 3 Risk of islet autoimmunity and type 1 diabetes associated with ascorbic acid transport gene polymorphisms and effect modification between the genes and childhood plasma ascorbic acid on the islet autoimmunity and type 1 diabetes risk

Gene	SNP (minor allele)	Islet autoimmunity				Type 1 diabetes			
		% of minor allele, cases/controls	OR (95% CI)[a]	*p* value[a]	*p* value[b]	% of minor allele, cases /controls	OR (95% CI)[a]	*p* value[a]	*p* value[b]
SLC23A1[c]	rs33972313 (A)	3.4/2.8	1.18 (0.70, 1.99)	0.533	0.158	5.4/1.6	2.52 (0.96, 6.59)	0.060	0.101
SLC2A1[c]	rs1105297 (A)	33.7/32.8	1.04 (0.86, 1.26)	0.690	0.094	33.8/32.6	1.09 (0.75, 1.56)	0.661	0.175
SLC2A1[c]	rs3754223 (A)	21.9/22.7	0.92 (0.74, 1.15)	0.473	0.959	25.5/21.6	1.40 (0.93, 2.11)	0.107	0.665
SLC2A2[c]	rs5400 (A)	12.7/13.7	0.90 (0.69, 1.16)	0.408	0.456	18.6/11.7	1.66 (1.06, 2.60)	0.028	0.785

[a] Adjusted for two largest principal components for ethnicity and *HLA-DR3/4* genotype

[b] Indication of interaction of childhood plasma ascorbic acid with the number of transport gene SNP alleles on the risk of islet autoimmunity and type 1 diabetes, adjusted for two largest principal components for ethnicity and *HLA-DR3/4* genotype

[c] Genetic data were missing from two islet autoimmunity cases, one type 1 diabetes case and five controls

Furthermore, besides HLA type and ethnicity, we could not take into account other potential confounding factors that might affect ascorbic acid status (e.g. endogenous stress such as infection or genetic variation of proteins regulating oxidative stress such as glutathione *S*-transferases and haptoglobin [9, 10]). Other limitations of our study are that it included only children who developed type 1 diabetes at a very early age (mean age of diagnosis 29 months) and that the follow-up time (up to 6 years of age) for type 1 diabetes was relatively short.

Our prospective study shows that higher plasma ascorbic acid concentration was associated with lower risk of islet autoimmunity, in particular with lower risk of IAA first. The results indicate that vitamin C might be protective, particularly during the early stages of autoimmunity development or early in life. This is in line with previous findings linking other early dietary exposures to IAA [16, 28].

Our study confirms previous findings that carrying the *SLC23A1* gene SNP rs33972313 minor alleles results in reduced plasma ascorbic acid concentration [11]. The presence of the minor 'A' allele, results in a change from the valine (Val/GTG) form to the methionine (Meth/ATG) form [11]. This alters the function the vitamin C transport proteins, impairing the active transport of ascorbic acid via decreased renal reabsorption, increased excretion and altered dose–response of plasma ascorbic acid [29].

Our study included SNPs in genes involved in the transport of DHA as well as ascorbic acid. Genetic variation in ascorbic acid transport would appear more important, because vitamin C mainly enters cells as L-ascorbic acid. However, vitamin C is also taken up as DHA, which is the oxidised form of ascorbic acid and is reduced back to ascorbic acid intracellularly. DHA transport occurs through five glucose transporters encoded by the *SLC2A* solute carrier gene family [30–33]. SNPs in any of these transport-protein-encoding genes could affect cellular ascorbate status and they are therefore all of interest in our study.

None of the SNPs investigated modified the associations between plasma ascorbic acid and the outcomes. In addition, our results do not imply any association between the SNPs and islet autoimmunity. However, a common variant in the *SLC2A2* gene (rs5400) was associated with an increased risk of type 1 diabetes, while a less frequent variant in *SLC23A1* (rs33972313) showed an association of borderline statistical significance. More studies are needed to corroborate the findings and to investigate the underlying mechanisms, because they may be related to other functions of these proteins.

Of the SNPs investigated, only rs33972313 in the *SLC23A1* gene has been shown to reduce ascorbic acid status [11]. SLC2A proteins are well-known glucose transporters, with the proteins encoded by *SLC2A1* and *SLC2A3* being more essential for pancreatic glucose transport in humans, compared with proteins encoded by *SLC2A2*, because they are expressed at higher levels. A previous study found an association between *SLC2A2* SNP rs5400 and an increased risk of type 2 diabetes [34] but we are not aware of studies linking the SNP to type 1 diabetes.

In this study, children who were not breastfed at 3 or 6 months of age had higher plasma ascorbic acid concentration compared with children who were breastfed for longer. This may result from early introduction of complementary foods containing higher amounts of vitamin C. Although a child's plasma ascorbic acid status might be affected by maternal intake of vitamin C, there are no studies assessing the association between maternal intake of vitamin C during lactation and a child's risk of islet autoimmunity and type 1 diabetes. The mother's adequate vitamin C status during pregnancy is important to the development of the fetus [35] and may affect the child's risk of developing type 1 diabetes. However, in a previous study, maternal vitamin C intake during pregnancy was found not to be associated with the child's risk of islet autoimmunity or type 1 diabetes in a population with adequate vitamin C intake [36]. It should be noted that the associations

between ascorbic acid and type 1 diabetes outcomes may be different at different intake levels or plasma concentrations and inter-individual differences could also play a role. Different results may be observed in studies performed in vitamin C-deficient populations or in supplementation trials.

It has been suggested that higher weight gain during infancy and/or childhood is related to increased risk of islet autoimmunity and type 1 diabetes [37, 38]. In the current study, mean weight prior to islet autoimmunity was associated with islet autoimmunity outcomes but adjustment for weight did not change the association between plasma ascorbic acid concentration and the outcomes.

The associations observed in this study are novel and relatively weak. Further studies are therefore needed to corroborate the findings.

Conclusions Higher plasma ascorbic acid may reduce the risk of islet autoimmunity in children with increased genetic risk of type 1 diabetes. Furthermore, genetic variation in vitamin C and glucose transporters might play a role in the development of type 1 diabetes. Further studies are warranted to elucidate the role of vitamin C in type 1 diabetes development.

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Data availability The datasets generated and analysed during the current study will be made available in the NIDDK Central Repository at https://www.niddkrepository.org/studies/teddy.

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Contribution statement MM contributed to the study design, analysis, interpretation of data, critical revision of the manuscript and the drafting of the manuscript. IE contributed to the study design, analysis, interpretation of data and critical revision of the manuscript, and supervised ascorbic acid laboratory analyses. H-SL performed statistical analysis and contributed to the interpretation of data and revision of the manuscript. SN contributed to the study design, analysis, interpretation of data and critical revision of the manuscript. UU, CAA, SH, HP, SSR, WH, JT, ÅL, AGZ, MR, JPK and JMN contributed to the acquisition and interpretation of the data and critically reviewed the manuscript. SMV contributed to the study concept and design, analysis, acquisition and interpretation of data, drafting of the manuscript and critical revision of the manuscript. All authors approved the final version of the article. MM, H-SL and SMV are the guarantors of this work, had full access to all the data in the study, and take responsibility for the integrity of the data and the accuracy of the data analysis.

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PUBLICATION II

Maternal Nitrate and Nitrite Intakes during Pregnancy and Risk of Islet Autoimmunity and Type 1 Diabetes: The DIPP Cohort Study.

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Maternal Nitrate and Nitrite Intakes during Pregnancy and Risk of Islet Autoimmunity and Type 1 Diabetes: The DIPP Cohort Study

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ABSTRACT

Background: High dietary intake of nitrate and nitrite might increase the risk of type 1 diabetes. To our knowledge, no earlier prospective study has explored whether maternal dietary intake of nitrate and nitrite during pregnancy is associated with the risk of type 1 diabetes in the offspring.

Objective: Our aim was to study association between maternal intake of nitrate and nitrite during pregnancy and the risk of islet autoimmunity and type 1 diabetes in the offspring.

Design: Children born between 1997 and 2004 at Oulu and Tampere University Hospitals in Finland and carrying increased human leukocyte antigen (HLA)–conferred risk for type 1 diabetes were followed in the Type 1 Diabetes Prediction and Prevention (DIPP) study from 3 mo of age. Islet autoantibodies were screened at 3- to 12-mo intervals from serum samples. Of 4879 children, 312 developed islet autoimmunity and 178 developed type 1 diabetes during a 15-y follow-up. Maternal intake of nitrate and nitrite during the eighth month of pregnancy was assessed after birth using a validated self-administered FFQ. Cox proportional hazards regression was used for the statistical analyses.

Results: Maternal intake of nitrate and nitrite during pregnancy was not associated with the child's risk of islet autoimmunity [nitrate: HR 0.99 (95% CI: 0.88, 1.11); nitrite: HR 1.03 (95% CI: 0.92, 1.15)] or type 1 diabetes [nitrate: HR 1.02 (95% CI: 0.88, 1.17); nitrite: HR 0.97 (95% CI: 0.83, 1.12)] when adjusted for energy (residual method), sex, HLA risk group, and family history of diabetes. Further adjustment for dietary antioxidants (vitamin C, vitamin E, and selenium) did not change the results.

Conclusion: Maternal dietary intake of nitrate or nitrite during pregnancy is not associated with the risk of islet autoimmunity or type 1 diabetes in the offspring genetically at risk for type 1 diabetes. *J Nutr* 2020;150:2969–2976.

Keywords: pregnancy, islet autoimmunity, type 1 diabetes mellitus, cohort, child, diet, nitrate, nitrite, N-nitroso compounds

Introduction

Type 1 diabetes results from the destruction or malfunction of pancreatic β cells mediated by autoimmune mechanisms (1, 2). Environmental triggers such as diet during early childhood (3, 4) and pregnancy (5, 6) might influence the disease development in childhood. High intake of nitrate and nitrite from the diet could increase their endogenous conversion to N-nitroso compounds such as nitrosamine and nitrosamide (7), which could potentially be toxic to β cells (8). Endogenously, 5–7% of total ingested nitrate is reduced to nitrite by salivary bacteria (9). In the stomach, nitrite is further reduced to nitric oxide, which has several biological functions such as smooth muscle dilation. However, excess nitrite can also increase the formation of N-nitroso compounds (7).

Nitrate and nitrite are naturally found inorganic compounds. Main dietary sources of nitrate are vegetables such as leafy greens, root vegetables, tubers, and drinking water. Nitrate's reduced form, nitrite, is used as a preservative food additive in

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various processed meats such as ham, sausages, and bacon (10). Besides endogenous formation, N-nitroso compounds also occur in foods such as processed meats and beer (11). In vitro, animal, and observational studies suggest that high intake of nitrate, nitrite, and N-nitroso compounds from the diet could increase the risk of type 1 diabetes (8, 12, 13). Ecological surveys and case-control studies in humans assessing intake of nitrate and nitrite from drinking water have given inconsistent results (14). Case-control studies have suggested that high dietary intake of nitrites (15, 16) and N-nitroso compounds (15) in childhood could increase the risk of type 1 diabetes, but results are inconsistent (17).

High consumption of red meat and processed meat during childhood could increase the risk of type 1 diabetes due to high intake of nitrite and N-nitroso compounds (18–20). In a case-control study, the risk of type 1 diabetes was higher in children whose intake of both N-nitroso compounds and protein from meat was high in comparison to only high intake of N-nitroso compounds (15). Undigested protein residues in the gut are converted to nitrosatable compounds, such as phenols, indoles, ammonia, amines, and amides, via microbial fermentation. When these compounds react with a nitrosating compound such as nitrite, it could enhance formation of N-nitroso compounds. (21).

High maternal intake of nitrite during pregnancy might also increase the risk of type 1 diabetes in the offspring (16), but this has not been assessed in prospective studies. The plasma nitrite concentration of a newborn infant is lower than that of an adult, but before birth, the maternal and fetal blood nitrite concentration is similar due to passive exchange of anions across the placenta (22). Thus, high maternal nitrate and nitrite intakes could increase maternal plasma nitrite concentration and expose the fetus to high nitrite concentrations. However, little is still known about the regulation of nitrite exchange via the placenta. In a prospective cohort study, maternal intake of red meat and processed meat products during pregnancy was not associated with islet autoimmunity or type 1 diabetes (23), but whether nitrate and nitrite per se increase the risk has not been explored in prospective studies. Furthermore, whether maternal intake protein modifies the association between maternal nitrate or nitrite intakes and the risk of type 1 diabetes development in offspring has not been studied. As dietary antioxidants such as vitamin C and vitamin E inhibit the formation of N-nitroso compounds from nitrate and nitrite, they were used as putative confounders in the analysis (24, 25).

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The data in the current study are not publicly available due to the protection of the identity of the study participants and their clinical data, but data are available from the corresponding author on reasonable request.

Abbreviations used: BW, body weight; DiMe, Childhood Diabetes in Finland; DIPP, Type 1 Diabetes Prediction and Prevention; HLA, human leukocyte antigen; ICA, islet cell antibody.

The aim of this prospective birth cohort study was to investigate associations between the maternal intake of dietary nitrate and nitrite during pregnancy and the risk of islet autoimmunity and type 1 diabetes in children genetically susceptible to type 1 diabetes. Furthermore, we explored whether maternal protein intake modifies the association between nitrate and nitrite intakes and type 1 diabetes outcomes.

Subjects and Methods

Subjects

The present study was a part of the Type 1 Diabetes Prediction and Prevention (DIPP) nutrition study. The DIPP nutrition study is a part of the larger DIPP study, a multidisciplinary prospective population-based cohort study, in which all newborn infants at Oulu, Tampere, and Turku University Hospitals in Finland are screened for human leukocyte antigen class II histocompatibility antigen, DQ beta 1 (*HLA-DQB1*)-conferred susceptibility to type 1 diabetes using cord blood samples provided that the parents give their informed consent (26). All infants carrying a high or moderate genetic risk are invited to a follow-up study. Follow-up visits were scheduled for 3, 6, 12, 18, and 24 mo and thereafter annually up to 15 y of age or at the onset of type 1 diabetes. The present study comprises mothers of children born between October 1997 and September 2004 in the Oulu and Tampere University Hospitals. The present report includes 4887 children with data on islet autoimmunity and 4943 children with data on type 1 diabetes. Maternal diet during pregnancy was assessed by a FFQ, and data were available for 4879 pregnancies due to twin pregnancies. The flowchart of participation is presented in **Figure 1**. The study adheres to the Declaration of Helsinki, and the local ethics committees approved the study protocol. Families gave their written informed consent for the genetic testing of the newborn infant and for their participation in the follow-up study.

Islet cell antibodies (ICAs) were screened at 3- to 12-mo intervals up to 15 y of age (27). If a participant was observed to test positive for ICAs, all available samples from such a subject were analyzed for insulin autoantibodies, glutamic acid decarboxylase, and islet antigen 2 autoantibodies. Islet autoimmunity was defined by repeated positivity for ICAs and at least one other autoantibody. Type 1 diabetes was defined according to World Health Organization criteria (28).

Genetic methods

Human leukocyte antigen DQ genotyping using panels of sequence-specific oligonucleotide probes has been described previously (26). The *HLA-DQB1 (*02/*03:02)* genotype represents "high" and *HLA-DQB1*03:02/x* (x $\neq$**02, *03:01, *06:02*) indicates "moderate" risk for type 1 diabetes.

Dietary methods

The mothers completed a validated 181-item semiquantitative FFQ concerning their diet during pregnancy (29). The FFQs were mailed to the mothers after delivery and checked at the child's 3-mo follow-up visit. Mothers were asked retrospectively after delivery to describe their diet during the eighth month of pregnancy, which is the last month preceding maternity leave in Finland (29). The FFQ comprised a list of 181 food items and mixed dishes. Open-frequency categories were used in increasing order: not at all and number of times per month, week, or day. The serving sizes chosen for each item were based on commonly used portions identified during earlier Finnish dietary studies, and for some foods (e.g., eggs and beverages), natural units were used. Information about supplement use during the whole pregnancy was collected. Mothers were instructed to record the dietary supplements with brand names; manufacturers of the supplements; amounts of supplements per day, week, or month; and the month of pregnancy when the supplements were used.

Maternal individual nutrient intakes were calculated using the data gathered by FFQs. The calculation was made with the in-house software

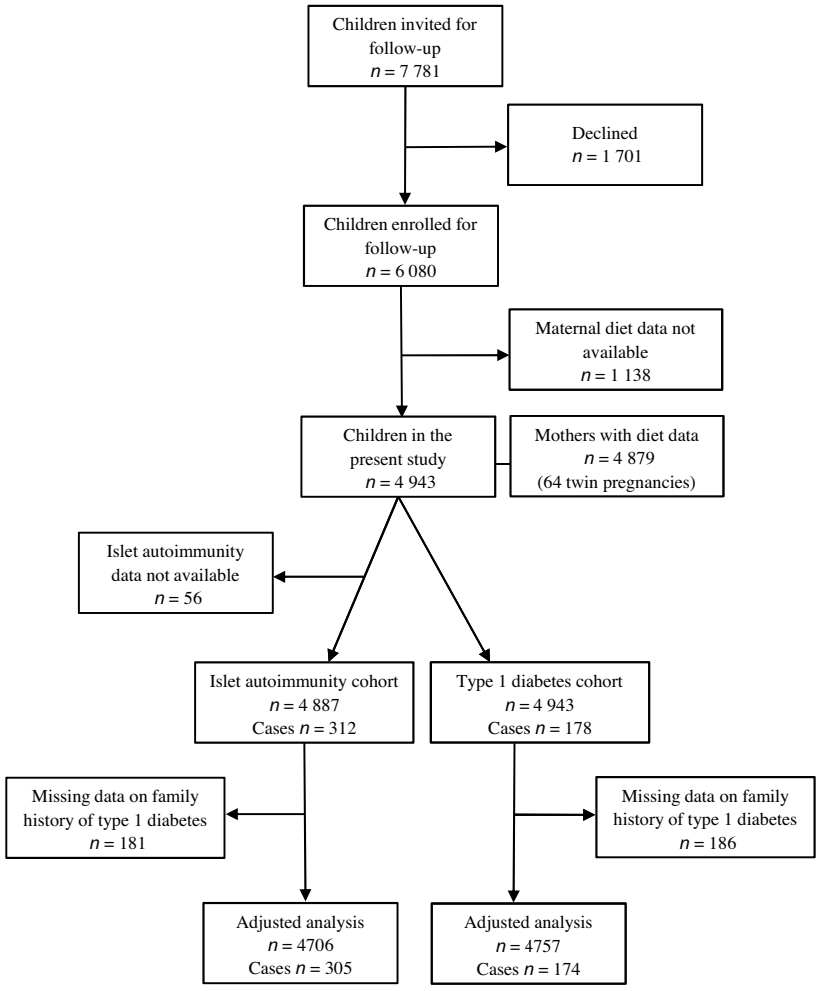

FIGURE 1 Participant flowchart.

(Finessi) of the Finnish Institute for Health and Welfare using the Finnish national food composition database (Fineli) as the source of food composition data (30). Energy from dietary fiber was included in the energy from carbohydrates and in the total energy. FFQs with >10 missing items were excluded. Questionable values were double-checked on the original FFQ and the database.

Finnish Customs Laboratory and Finnish Food Authority analyzed nitrate and nitrite values of various vegetables and meat products during 2008–2012 (9, 31). The data of these analyses were used to add nitrate and nitrite values to the respective food items in the Fineli database. The nitrate or nitrite values of foods, which were not analyzed in the abovementioned analyses, were determined from scientific literature (32–35). The latest scientific literature from 2000 onward had the highest priority followed by literature from 1980–2000. Analytical values representing predominantly European food items were preferred. Values not found from literature were derived from aggregation, recipe calculation, or imputation from similar foods.

Sociodemographic characteristics

Information on type 1 diabetes status of the first-degree relatives, child sex, and maternal education was collected from parents after delivery using a structured questionnaire. Information on the gestational age, birth weight and length, and maternal smoking during pregnancy was acquired from the medical birth registers of the university hospitals.

Statistical methods

One-factor ANOVA or *t* test was used to study the differences in maternal nitrate/nitrite intake and background variables. Maternal age, BMI (in kg/m^2) in early pregnancy, and weight gain rate during pregnancy were categorized into quartiles or tertiles (BMI) for the analysis. Maternal intake of nitrate and nitrite was analyzed as a continuous variable. Cox proportional hazards regression was applied to estimate associations between maternal intake of nitrates and nitrites and the risk of islet autoimmunity and type 1 diabetes in the offspring. Analyses were adjusted for energy using Willett's residual method (36), maternal education, child's genetic susceptibility to type 1 diabetes, and family history of type 1 diabetes. In a second model, maternal intake of dietary antioxidants (vitamin C, vitamin E, and selenium) was used as additional adjustments. Furthermore, we tested whether protein intake modifies the association between both nitrate and nitrite intakes and the development of type 1 diabetes outcomes, including the interaction term protein × nitrate/nitrite in the Cox proportional hazards regression models. SAS software version 9.3 (SAS Institute) was used in the outcome analyses. Analyses concerning

TABLE 1 Nitrate and nitrite intakes of mothers during month 8 of pregnancy, stratified by various characteristics, Type 1 Diabetes Prediction and Prevention nutrition study, Finland[1]

Variable	*n*	Nitrate, mg/d	*P* value[2]	Nitrite, mg/d	*P* value[2]
All mothers	4879	151 ± 97.4		3.00 ± 1.06	
Age, y			<0.001		<0.001
<24	926	127 ± 90.0		2.93 ± 1.12	
25–29	1700	148 ± 96.4		2.94 ± 1.05	
30–34	1412	159 ± 94.6		3.04 ± 1.04	
≥35	841	172 ± 105		3.12 ± 1.04	
Missing	0				
BMI in early pregnancy, kg/m^2			0.74		<0.001
<25	3025	150 ± 94.4		2.95 ± 1.02	
25–29.9	1123	152 ± 90.2		3.07 ± 1.10	
≥30	434	154 ± 119		3.13 ± 1.13	
Missing	297				
Weight gain rate, g/wk			0.48		0.78
First quarter <0.33	1136	153 ± 104		3.01 ± 1.11	
Second quarter 0.33–0.41	1137	154 ± 94.5		2.98 ± 1.06	
Third quarter 0.42–0.51	1137	149 ± 92.0		2.98 ± 1.01	
Fourth quarter ≥0.52	1136	148 ± 92.0		3.02 ± 1.04	
Missing	333				
Vocational education[3]			<0.001		0.001
None	294	115 ± 79.7		3.06 ± 1.14	
Vocational school or course	1291	139 ± 96.0		3.05 ± 1.18	
Secondary vocational education	2067	150 ± 93.4		3.02 ± 1.05	
University studies or degree	1097	170 ± 104		2.89 ± 0.89	
Missing	130				
Smoking during pregnancy			<0.001		0.91
Yes	467	120 ± 83.8		3.00 ± 1.20	
No	4246	154 ± 98.1		3.00 ± 1.05	
Missing	166				
Diabetes[4]			<0.001		0.003
Yes	164	187 ± 134		3.24 ± 1.18	
No	4611	150 ± 95.4		2.99 ± 1.06	
Missing	103				

[1] Values are means ± SDs.
[2] *P* values for difference between groups from one-factor ANOVA or *t* test.
[3] At the time of birth.
[4] Data on maternal diabetes from questionnaire completed after birth. Type of diabetes not specified.

background characteristics were done using IBM SPSS Statistics version 25.0 (IBM Corporation). Statistical significance was set at 2-sided $P < 0.05$.

Results

Overall, 312 children (6.8%) developed islet autoimmunity at a median (IQR) age of 3.5 (1.7–6.6) y, and 178 (3.6%) developed type 1 diabetes at a median (IQR) age of 7.1 (4.3–10.6) y during the 15-y follow-up. During the autoantibody follow-up of 4887 participants, the dropout rates were 279 children (5.7%) at 1 y and 1415 children (30%) at 5-y follow-up. Mean (SD) maternal intake of nitrate was 151 (97.4) mg/d and nitrite 3.00 (1.06) mg/d (**Table 1**). Intake of nitrate was higher in older mothers, nonsmokers, and well-educated mothers than younger mothers, smokers, and mothers with poor education (Table 1). Intake of nitrite was higher in older mothers, well-educated mothers, and mothers with high BMI.

The main sources of nitrate were leaf vegetables (78.3 mg/d; 51.7% of total intake), root vegetables (17.9 mg/d; 11.8%), and fruit vegetables (11.9 mg/d; 7.9%). The main sources of nitrite were cereals 1.48 mg/d (49.2%) followed by processed meat products (0.92 mg/d; 30.6%) (**Table 2**).

Maternal intake of nitrate and nitrite during pregnancy was not associated with child's risk of islet autoimmunity or type 1 diabetes in an unadjusted model (not shown) or in a model adjusted for energy by Willett's residual method, sex, family history of diabetes, and HLA genotype (**Table 3**). The results remained similar in a model that was further adjusted for the intake of dietary antioxidants (vitamin C, vitamin E, and selenium) (Table 3). Maternal intake of protein during pregnancy did not modify the association between intake of nitrate or nitrite and the risk of islet autoimmunity (nitrate × protein interaction, $P = 0.23$; nitrite × protein interaction, $P = 0.99$) or type 1 diabetes (nitrate × protein interaction, $P = 0.24$; nitrite × protein interaction, $P = 0.86$).

Discussion

In our prospective cohort, the maternal dietary intake of nitrate and nitrite during pregnancy was not associated with the risk of islet autoimmunity or type 1 diabetes in the offspring. Maternal

TABLE 2 Maternal intake of nitrate and nitrite during pregnancy from food groups, Type 1 Diabetes Prediction and Prevention nutrition study, Finland[1]

Food groups	Nitrate mg/d	% of total	Nitrite mg/d	% of total
Fruit and berries[2]	8.63 ± 6.69	5.70	0.05 ± 0.07	1.59
Fruit juices	5.46 ± 6.82	3.61	0 ± 0	0
Other sweetened fruit drinks	0.76 ± 1.39	0.50	0.03 ± 0.06	1.05
Vegetables				
Leaf vegetables	78.3 ± 79.5	51.7	0.05 ± 0.05	1.59
Fruit vegetables	11.9 ± 9.93	7.88	0.12 ± 0.11	3.86
Root vegetables	17.9 ± 17.7	11.8	0.04 ± 0.04	1.23
Other vegetables[3]	8.07 ± 10.1	5.33	0.03 ± 0.04	1.11
Legumes, nuts, seeds, and soy products	0.31 ± 1.44	0.21	<0.01 ± 0.01	0.08
Potatoes and potato-based products	7.28 ± 3.45	4.81	0.14 ± 0.07	4.70
Dairy products	0.89 ± 0.65	0.59	0.05 ± 0.04	1.65
Cereals	8.46 ± 3.15	5.59	1.48 ± 0.55	49.2
Egg and egg dishes	0 ± 0	0	0 ± 0	0
Fish and fish dishes	0.16 ± 0.27	0.10	0.02 ± 0.03	0.68
Meat and meat dishes (beef, pork, lamb, poultry, game)				
Unprocessed meat	0.45 ± 0.23	0.30	0.06 ± 0.03	2.05
Processed meat[4]	2.19 ± 1.58	1.44	0.92 ± 0.66	30.6
Other foods[5]	0.67 ± 0.55	0.44	0.02 ± 0.01	0.64
All foods	151 ± 97.4	100	3.00 ± 1.06	100

[1] Mean ± SD intake of 4879 mothers.
[2] Including canned and dried fruit and berries.
[3] Cabbages, onions, mushrooms, and canned vegetables.
[4] Sausages and cured meat products.
[5] Fats, oils, beverages, sugars, sweets, condiments, and dietary supplements.

intake of protein did not modify the association between intake of nitrate or nitrite and the risk of islet autoimmunity or type 1 diabetes.

A strength of our study is that it was conducted in a well-defined birth cohort of individuals with increased genetic risk of type 1 diabetes. Our study is, as far as we are aware, the first study to explore prospectively whether maternal intake of nitrate and nitrite during pregnancy is associated with type 1 diabetes development as previous studies have been ecological surveys or case-control studies (14). Furthermore, our study explored the association between maternal nitrate and nitrite intakes and the risk of islet autoimmunity, which has not been studied previously, to our knowledge. We used a regularly updated national food composition database in which nitrate and nitrite contents were updated specifically for the current study. The validation study of the FFQ used in our survey suggested that the FFQ is appropriate for estimation of nitrate and nitrite intakes from food. Intake calculated from FFQ compared with food records showed a correlation of 0.63 for nitrate and 0.79 for nitrite (29). In addition, our study took into account the intake of dietary antioxidants, which could confound association between nitrate and risk of type 1 diabetes-related outcomes (15, 37–39).

A major limitation in our study was the imprecision in the calculation of nitrate content from vegetables. The food composition database used in the current study does not take into account the cooking or food preparation losses for nitrate or nitrite (9). Washing leaf vegetables decreases the nitrate content ~10–15% while cooking decreases the nitrate content in vegetables and potatoes ~51% depending on cooking method (40). Since nitrate is water soluble, consuming or discarding the cooking liquid also affects the exposure. Our food composition database also did not include nitrate or nitrite content of drinking water. Another limitation in our study was that the children's diet during infancy was not available for the current study.

In our study, maternal intake of nitrate and nitrite during pregnancy was not associated with the risk of type 1 diabetes development, which is not in line with the previous Childhood Diabetes in Finland (DiMe) case-control study (16). In addition to the different study design, the maternal intake of nitrate and nitrite focused on a different time period and was asked

TABLE 3 HRs (95% CIs) for the association between maternal intake of nitrate and nitrite during pregnancy and the risk of islet autoimmunity and type 1 diabetes in the offspring[1]

	Islet autoimmunity		Type 1 diabetes	
Characteristic	Model 1 $n = 4706$ (305)[2]	Model 2 $n = 4706$ (305)[2]	Model 1 $n = 4757$ (174)[2]	Model 2 $n = 4757$ (174)[2]
Nitrate from diet				
per 1 SD	0.99 (0.88, 1.11)	1.00 (0.88, 1.14)	1.02 (0.88, 1.17)	1.02 (0.87, 1.20)
Nitrite from diet				
per 1 SD	1.03 (0.92, 1.15)	1.03 (0.92, 1.16)	0.97 (0.83, 1.12)	0.99 (0.85, 1.16)

[1] Values are HRs (95% CIs) analyzed using Cox proportional hazard regression model. Model 1: adjusted for energy with residual method, sex, family history of diabetes, and human leukocyte antigen genotype. Model 2: like model 1 and further adjusted for the intakes of vitamin C, vitamin E, and selenium.
[2] n represents total number of children, and number in parentheses represents numbers of children with the outcome.

later in life when the offspring had developed type 1 diabetes. In the DiMe study, the consumption frequency of the most important dietary sources of nitrate and nitrite was inquired, whereas the current study included detailed calculation of total nitrate and nitrite intakes using a recently updated food composition database. In the DiMe study, maternal intake of nitrate and nitrite from diet focused on the time of conception while the FFQ in the current study represented the dietary intake during the eighth month of pregnancy. Our validation study showed that mothers were able to report their food consumption reliably for the eighth month of pregnancy even if the assessment was done after delivery (29). The eighth month of pregnancy is a very well-identifiable time point for Finnish pregnant women as most of the women work and are requested to start the pregnancy leave 1 mo before estimated delivery. For a person, it is difficult to estimate food consumption during periods of changing diet like during the whole pregnancy as nausea, for example, in the beginning of pregnancy is common. We think that this eighth month of pregnancy reflects also earlier pregnancy. Furthermore, it is not known whether there are critical time points during pregnancy that are related to the development of autoimmune diseases in offspring.

The maternal dietary protein intake did not modify the risk between nitrate or nitrite intakes and type 1 diabetes outcomes in our study. A previous Swedish case-control study suggested that the combination of high intake of N-nitroso compounds and protein from meat in childhood could further increase the risk of type 1 diabetes in comparison to high intake of N-nitroso compounds alone (15). Our study assessed the intake of nitrate and nitrite, not N-nitroso compounds, and thus the exposure was different, which might contribute to different outcomes. Furthermore, we explored total protein intake instead of protein from specific dietary sources. In the European Food Safety Authority report, the median total exposure to volatile nitrosamines from meat products was 2.5 ng/kg of body weight (BW) per day in Finnish children aged 3–9 y, which was above the European median, 2.0 ng/kg (41). In the Finnish adult population, the intake was 0.9 ng/kg, which is same as the European median (41). Furthermore, we found no association between maternal consumption of processed meat during pregnancy and the risk of type 1 diabetes outcomes in our previous study (23). Thus, the childhood consumption of processed meat products may play a bigger role than maternal consumption.

In our study, the mean daily nitrite intake was 3 mg, and the last measured mean maternal weight during pregnancy was 79 kg, which corresponds to 0.04 mg/kg BW/d. This is similar to a recent European Food Safety Authority report of 0.04 mg/kg BW/d in a Finnish adult population (41). In our study, the mean nitrate intake was rather high (151 mg/d) compared with previous studies in adults. An earlier Finnish study reported a nitrate intake of 77 mg/d based on dietary history interview method (42). In a Danish survey, mean nitrate intake was 61 mg/d, and in a Dutch study, 80 mg/d was measured from duplicate 24-h diet samples (43, 44). Different nitrate intake estimates between studies may be explained by methodologic differences.

In our study, the main sources of nitrate were leaf and root vegetables, which contributed to more than three-fourths of the daily intake. In our previous study, the maternal consumption of vegetables during pregnancy was not associated with the risk of islet autoimmunity (5), which is in line with our current study. Surprisingly, the main source of nitrite was cereals, comprising almost half of the daily intake, followed by processed meat, with 31% of the daily intake. Maternal consumption of cereals during pregnancy was not associated with the risk of islet autoimmunity in our previous study (5). However, 2 recent studies have observed that maternal (45) and childhood (46) intake of gluten-containing cereals might increase the risk of type 1 diabetes development. Although the nitrite content in cereals is lower than in processed meats, the proportion of cereals in the diet is higher than of that of meat in DIPP study mothers (47). Although processed meats are generally suggested as the main source of nitrite (41, 48), cereals also have been observed to be the main source of nitrite in the adult population (49). Studies analyzing nitrate and nitrite content in cereals in Europe are rather limited, and nitrite content in cereals in Finland has not been analyzed, to our knowledge. Therefore, we cannot rule out the possibility of overestimation.

Our study did not include the contents of N-nitroso compounds in foods as the Fineli food composition database used in our study does not contain these values. In addition to endogenous formation, the direct exposure to N-nitroso compounds from diet and drinking water could also influence the risk of type 1 diabetes (15, 19). Food preparation can induce N-nitroso compound formation in the food before consumption (e.g., cooking nitrite-containing bacon at high temperature) (50). Thus, future studies should consider both endogenous and exogenous exposure to these compounds. Furthermore, the maternal intake of N-nitroso compounds in association with type 1 diabetes in offspring has not been previously studied, to our knowledge.

In our study, we could not consider the maternal use of nitrosatable drugs. These drugs, along with nitrosating compounds such as nitrite from the diet, may enhance the endogenous formation of N-nitroso compounds. Prenatal use of these drugs has been associated with several unfavorable pregnancy outcomes (51–53), but their association with type 1 diabetes development in offspring has not been studied, to our knowledge. Nitrosatable drugs include antibiotics and antihistamines, for example. Since these drugs are rather commonly used, their use during pregnancy might be important to consider in future studies.

In conclusion, our prospective study suggests that maternal intake of nitrate and nitrite is not associated with the risk of islet autoimmunity or type 1 diabetes in the offspring.

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PUBLICATION III

Maternal Vitamin C and Iron Intake during Pregnancy and the Risk of Islet Autoimmunity and Type 1 Diabetes in Children: A Birth Cohort Study

Mattila M, Hakola L, Niinistö S, Tapanainen H, Takkinen HM, Ahonen S, Ilonen J, Toppari J, Veijola R, Knip M, Virtanen SM.

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Maternal Vitamin C and Iron Intake during Pregnancy and the Risk of Islet Autoimmunity and Type 1 Diabetes in Children: A Birth Cohort Study

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Abstract: Our aim was to study the associations between maternal vitamin C and iron intake during pregnancy and the offspring's risk of developing islet autoimmunity and type 1 diabetes. The study was a part of the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) prospective birth cohort including children genetically at risk of type 1 diabetes born between 1997–2004. The diets of 4879 mothers in late pregnancy were assessed with a validated food frequency questionnaire. The outcomes were islet autoimmunity and type 1 diabetes. Cox proportional hazards regression analysis adjusted for energy, family history of diabetes, human leukocyte antigen (HLA) genotype and sex was used for statistical analyses. Total intake of vitamin C or iron from food and supplements was not associated with the risk of islet autoimmunity (vitamin C: HR 0.91: 95% CI (0.80, 1.03), iron: 0.98 (0.87, 1.10)) or type 1 diabetes (vitamin C: 1.01 (0.87, 1.17), iron: 0.92 (0.78, 1.08)), neither was the use of vitamin C or iron supplements associated with the outcomes. In conclusion, no association was found between maternal vitamin C or iron intake during pregnancy and the risk of islet autoimmunity or type 1 diabetes in the offspring.

Keywords: pregnancy; nutrition; vitamin C; ascorbic acid; iron; islet autoimmunity; type 1 diabetes; birth cohort

1. Introduction

Dietary factors during the fetal period, infancy and childhood are implicated to trigger, inhibit or modify the autoimmune processes leading to type 1 diabetes [1]. Vitamin C (ascorbic acid) and iron are essential micronutrients that human body cannot produce, and therefore, they are needed from the diet [2,3]. Vitamin C might have a protective role against type 1 diabetes due to its antioxidant properties [4,5]. In contrast, an excessive amount of iron might lead to the generation of oxygen radicals and increased inflammation and, thus, increase the risk of type 1 diabetes [3,6]. However, prospective studies assessing these nutrients in the disease process of type 1 diabetes are scarce.

In two retrospective case-control studies, the child's intake of dietary vitamin C was not associated with type 1 diabetes [7,8], whereas in one study, dietary vitamin C supplementation was associated with decreased risk of type 1 diabetes [9]. A recent study conducted by The Environmental Determinants of Diabetes in the Young (TEDDY) Study group suggested that high plasma ascorbic acid status during childhood could decrease the risk of islet autoimmunity [10]. The maternal intake of vitamin C during pregnancy and the risk of islet autoimmunity have so far been analyzed only in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) Nutrition Study by us [11]. The intake of vitamin C from diet only or from diet and vitamin C supplementation combined was not associated with the risk of developing islet autoimmunity. We or, to our knowledge, others have not reported the association of maternal vitamin C intake with clinical type 1 diabetes before.

High cord blood iron concentration [12] and maternal use of iron supplementation during pregnancy [13] have been linked to increased risk of type 1 diabetes. Mechanisms are not yet known, but iron overload was suggested to generate reactive oxygen species that might lead to beta-cell apoptosis or ferroptosis, i.e., cell death depended on iron [3]. Beta cells are lacking sufficient antioxidant enzymes that are available in other tissues [14,15]. Vitamin C enhances the absorption of non-heme iron from diet [16]. Furthermore, in pregnant women, the use of vitamin C supplements in addition to iron supplements has increased maternal plasma iron status [17]. However, the interaction between vitamin C and iron intake on the risk of type 1 diabetes development has not been studied previously.

Our aim was to study the associations between maternal intake of vitamin C and iron during pregnancy as well as use of dietary supplements with vitamin C or iron on the risk of islet autoimmunity and type 1 diabetes in a prospective birth cohort. We hypothesized that high maternal vitamin C intake is associated with decreased risk, while high iron intake is associated with increased risk of islet autoimmunity and type 1 diabetes in the offspring. Since vitamin C enhances the absorption of iron, we also studied interaction between maternal iron and vitamin C intake on the risk of outcomes.

2. Materials and Methods

The DIPP Study is a large population-based birth cohort study of children with human leukocyte antigen (HLA)-conferred genetic risk of type 1 diabetes [18]. In the DIPP Nutrition Study within the DIPP cohort, 7782 children born in the Tampere and Oulu University Hospitals between October 1997 and September 2004 were invited for follow-up. Children with the genotypes HLA-DQB1*02/*03:02 and DQB1*03:02/x (x stands for alleles other than DQB1*02 or DQB1*06:02) were eligible for the follow-up. At the time of screening, 99% of the Finnish population was of ethnic Finnish origin. Migrant children with parents that could not speak either Finnish or Swedish and those with severe diseases or anomalies were excluded. The children were invited to study visits at the age of 3, 6, 12, 18 and 24 months and then annually up to the age of 15 years or until type 1 diabetes diagnosis. The visit interval of autoantibody positive children was 3 months. In the current report, the inclusion criteria for the analyses was the available maternal dietary assessment. Parents gave written informed consent for both genetic testing of their newborn infant from cord blood sample and for participation in the follow-up. The study adheres to the Declaration of Helsinki, and the ethics committees of Oulu and Tampere University Hospitals approved the study protocol (ETL 97193M).

2.1. Maternal Vitamin C and Iron Intake

The mothers were asked to report their diet during pregnancy with a validated 181-item semi-quantitative food frequency questionnaire (FFQ) [19]. These pregnancy FFQs were sent to the mothers after delivery and checked at the 3-month follow-up visit. Mothers were asked to answer retrospectively about their diet during the eighth month of pregnancy (the last month preceding maternity leave in Finland) [19]. The frequency (not at all, number of times per month, week or day) and the amounts (units of common serving sizes) of consumed foods were inquired. General units were used for some foods such as eggs and beverages. Mothers were also asked about the use of nutritional supplements over the whole time of pregnancy. The name of supplement, the manufacturer and the dosage per day, week or month were asked. The vitamin C and iron intake from vitamin C and iron only supplements and multivitamin supplements were combined in the calculation. Individual vitamin C and iron intakes were calculated based on the Finnish Food Composition Database (Fineli) using the in-house software (Finessi) of the Finnish Institute for Health and Welfare, Finland [20]. The nutrient intakes were calculated from unprocessed vegetables and fruits, which does not consider the loss of vitamin C due to processing and cooking. Energy from dietary fiber was included in the total energy. FFQs with >10 missing items were excluded. Any implausible values were double checked on the original FFQ and from the database.

2.2. Definiton of Type 1 Diabetes-Related Outcomes

Children were screened for islet cell autoantibodies (ICA) at intervals of 3–12 months as described before [18]. When participant had seroconversion for ICA for the first time, all of the previous and subsequent samples were analyzed for insulin autoantibodies (IAA), glutamic acid decarboxylase antibodies (GADA) and islet antigen-2 antibodies (IA-2A). ICA was quantified by a standard indirect immunofluorescence method, IAA, GADA and IA-2A with specific radiobinding assays. Transplacentally transferred autoantibodies were not considered as the child's endogenous autoantibodies. The definition for islet autoimmunity was repeated positively for ICA and at least one biochemical autoantibody (IAA, GADA, IA-2A) or having type 1 diabetes (one child was diagnosed with type 1 diabetes without information on autoantibody positivity). Data on the diagnosis of type 1 diabetes were obtained in May 2015 from Finnish Pediatric Diabetes Register and University Hospitals. The diagnosis of type 1 diabetes was defined according to World Health Organization criteria [21]. In the present study, children were considered free from type 1 diabetes if they were not found in the register. Of the children invited, two datasets were formed. The islet autoimmunity cohort included 4887 children, and the type 1 diabetes cohort included 4943 children. Data on maternal diet during pregnancy were available for 4879 pregnancies, as 64 mothers had twin pregnancies. The mothers who were invited to the study but had insufficient data on maternal diet were more likely to be in the lowest or highest age category, more likely to be smokers and had more previous deliveries and a less education than those mothers with dietary data [22].

2.3. Genetic Methods

HLA-DQ was genotyped using panels of sequence-specific oligonucleotide probes, as described before [18]. Genotypes HLA-DQB1 (*02/*03:02) represent "high" and HLA-DQB1*03:02/x (x $\neq$ *02, *03:01, *06:02) "moderate" risk for type 1 diabetes.

2.4. Background Characteristics

Information on maternal education, diabetes (unspecified type) and family history of diabetes among the first-degree relatives was collected with a questionnaire after the delivery. Information on the offspring's sex, maternal age and maternal smoking during pregnancy was obtained from the birth registers of the hospitals. Maternal BMI was determined from the mother's weight at the first prenatal visit as described previously [23].

2.5. Statistical Methods

Differences in maternal vitamin C and iron intake (in mg/MJ) between groups of potentially confounding background characteristics were tested using one-way ANOVA. Differences in characteristics between supplement users and non-users were tested using t-test and Pearson Chi-square test. In the analyses exploring the risk of islet autoimmunity and type 1 diabetes, maternal vitamin C and iron intakes from diet and total intake (including the intake from supplements) were energy-adjusted using Willett's residual method [24]. Maternal vitamin C and iron intake were analyzed both as continuous and categorized variables. The intakes were categorized into quartiles, from which the combined two middle quartiles were used as the reference category. The use of supplements with vitamin C and iron at any time during pregnancy was categorized as yes/no. The risk of islet autoimmunity and type 1 diabetes was assessed with Cox proportional hazards regression analysis.

The main analyses were energy-adjusted with the Willett's residual method and further adjusted for sex (boy or girl), family history of diabetes (yes or no) and HLA genotype (high or moderate risk). Another model was also adjusted for maternal education, pre-pregnancy BMI and smoking.

We tested whether child's sex modified the association between the vitamin C and iron intake and the outcomes. The results indicated no interaction, and therefore, main analyses included girls and boys together. To test whether total vitamin C intake modified the association between total iron intake and the outcomes, an interaction term was added into the model.

SAS software version 9.3 (SAS Institute, Cary, NC, USA) and IBM SPSS Statistics version 25.0 (IBM Corporation, Armonk, NY, USA) were used in the analyses. Statistical significance was set at 2-sided $p < 0.05$.

3. Results

3.1. Background Characteristics

Altogether 312 (6.4%) children developed a positive islet autoimmunity at a median age of 3.5 (IQR 1.7–6.6) years, and 178 (3.6%) had type 1 diabetes at the median age of 7.1 (IQR 4.3–10.6) years during the 15-year follow-up. The dropout rates among the 4887 children at 1- and 5-year autoantibody follow-up were 5.7% (279 children) and 30% (1415 children), respectively.

Maternal total vitamin C and iron intake by background variables are presented in Table 1. Mothers with lower education had lower vitamin C intake during pregnancy than those with higher education (Table 1). Mothers who smoked had lower iron intake than non-smokers. Lower maternal BMI in early pregnancy was associated with higher iron intake and non-linearly with vitamin C and iron intake (Table 1).

Mothers who used supplementation with vitamin C (1555 mothers, 32%) had lower BMI (23.8 vs. 24.5 kg/m^2, $p < 0.001$), higher education (overall $p < 0.001$), were more often non-smokers (92 vs. 89%, $p = 0.001$) and had a higher intake of dietary iron (16.9 vs. 16.5 mg/day, $p < 0.001$) compared to those who did not use supplementation with vitamin C.

Mothers who used iron supplementation during pregnancy (3375 mothers, 69%) had lower BMI (23.9 vs. 25.2 kg/m^2, $p < 0.001$), were more often highly educated (overall $p < 0.001$) and non-smokers (92% vs. 86%, $p < 0.001$) and had a higher total energy (11.8 vs. 11.5 MJ/day, $p = 0.01$) and higher iron intake from food (16.2 vs. 15.8 mg/day, $p < 0.001$), in comparison to those who did not use iron supplementation.

Table 1. Characteristics of 4879 mothers in relation to mean (standard deviation) total intake of vitamin C during and iron 8th month of pregnancy.

	Maternal Intake during Pregnancy		
		Vitamin C, mg/MJ	Iron, mg/MJ
Characteristic	***n***	**Mean (SD)**	**Mean (SD)**
Maternal age, years			
≤24	926	18.7 (11.1)	3.8 (3.4)
25–29.9	1700	18.8 (10.3)	3.7 (3.0)
30–34.9	1412	19.3 (13.4)	3.9 (3.2)
≥35	841	19.1 (12.2)	3.8 (2.9)
p-value [b]		0.46	0.72
Maternal BMI in early pregnancy, kg/m^2			
<25	3025	19.3 (11.9)	4.0 (3.3)
25–29.9	1123	18.1 (10.5)	3.6 (2.9)
≥30	434	18.7 (11.1)	3.4 (2.9)
Missing	297		
p-value [a]		0.01	<0.001
Maternal weight gain rate, kg/week [c]			
1st quarter < 0.33	1136	19.4 (13.6)	3.9 (3.2)
2nd quarter 0.33–0.41	1137	18.4 (9.8)	3.9 (3.3)
3rd quarter 0.42–0.52	1137	18.8 (10.9)	3.8 (3.0)
4th quarter > 0.52	1136	19.1 (11.3)	3.8 (3.1)
Missing	333		
p-value [a]		0.14	0.48
Maternal vocational education [d]			
None	294	18.2 (13.5)	3.6 (3.2)
Vocational School or Course	1291	18.4 (11.5)	3.9 (3.5)
Secondary Vocational Education	2067	18.9 (11.7)	3.8 (3.0)
University Studies or Degree	1097	20.1 (11.8)	3.8 (3.1)
Missing	130		
p value [a]		0.001	0.40
Maternal smoking during pregnancy			
Yes	467	18.1 (12.7)	3.5 (3.6)
No	4246	19.1 (11.7)	3.9 (3.1)
Missing	166		
p value [a]		0.10	0.04
Maternal diabetes [d]			
Yes	164	19.0 (11.9)	4.0 (3.1)
No	4611	19.0 (11.8)	3.8 (3.1)
Missing	104		
p value [a]		0.89	0.49

[a] total intake based on diet and dietary supplements during 8th month of pregnancy. [b] *p* values for difference between groups from one-factor ANOVA. [c] At the time of birth. [d] Based on a questionnaire completed after birth. Type of diabetes not specified.

3.2. Intake and Dietary Sources of Vitamin C and Iron

Mean (SD) maternal intake of vitamin C from foods during pregnancy was 198 (116) mg/day and from supplements 23 (82) mg/day. The most important sources of vitamin C were juices, vegetables, fruits, and dietary supplements (Table 2). A total of 240 (5%) of the mothers consumed less than 70 mg/day of vitamin C, which was the recommended intake of pregnant mothers in Finland during the time of dietary assessment. Only five (0.1%) mothers had insufficient vitamin C intake < 20 mg/day.

Mean (SD) maternal intake of iron from foods during pregnancy was 17 (5) mg/day and from supplements 26 (33) mg/day. Dietary supplements, cereals and meats were the main source of iron (Table 2).

Table 2. Maternal intake of vitamin C and iron during pregnancy from food groups, DIPP Nutrition Study, Finland.

Vitamin C and Iron Intake from Food Groups	Mean Vitamin C mg/day	% of Intake	Mean Iron mg/day	% Of Intake
Fruits and berries [1]	41.0	18.7	0.69	1.6
Fruit juices	84.8	38.6	0.28	0.6
Other sweetened fruit drinks	2.1	1.0	0.32	0.7
Vegetables	46.5	21.2	1.14	2.7
Leaf vegetables	3.4	1.6	0.19	0.5
Fruit vegetables	20.9	9.5	0.31	0.7
Root vegetables	8.7	4.0	0.29	0.7
Other vegetables [2]	13.4	6.1	0.35	0.8
Legumes, nuts, seeds and soy products	0.8	0.4	0.32	0.8
Potatoes and potato-based products	10.3	4.7	0.83	1.9
Dairy products	9.7	4.4	0.54	1.3
Cereals	0.01	0	6.52	15.3
Egg and egg dishes	0	0	0.70	1.6
Fish and fish dishes	0	0	0.23	0.5
Meat and meat dishes (beef, pork, lamb, poultry, game)	0.5	0.2	3.42	8.1
Unprocessed meat	0	0	1.64	3.9
Processed meat [3]	0.5	0.2	1.78	4.2
Other foods [4]	0.6	0.3	1.21	2.8
Dietary supplements [5]	23.1	10.5	26.31	61.9
Total	219.4	100	42.50	100

[1] Including canned and dried fruits and berries. [2] Cabbages, onions, mushrooms and canned vegetables. [3] Sausages and cured meat products. [4] Fats, oils, beverages, sugars, sweets and condiments. [5] Contains vitamin C or iron exclusively supplements and multivitamins.

3.3. Maternal Vitamin C and Iron Intake and Risk of Type 1 Diabetes-Realted Outcomes

Energy-adjusted maternal intake of vitamin C or iron from food and total intake (from food and supplements together) during pregnancy were not associated with islet autoimmunity or type 1 diabetes (Table 3). Results were similar for both continuous and categorized intake. Further adjustment for sex, family history of diabetes and HLA genotype did not change the risk significantly (Table 3) nor did the adjustment for maternal education, pre-pregnancy BMI and smoking (Supplementary Table S1). The maternal use vs. non-use of dietary supplements with vitamin C during pregnancy was not associated with the risk of islet autoimmunity (HR 1.08 (95% CI 0.86, 1.37), $p = 0.51$) or type 1 diabetes (1.18 (0.87, 1.61), $p = 0.28$). Adjustment for sex, family history of diabetes and HLA genotype did not change the results for islet autoimmunity (1.05 (0.83, 1.33), $p = 0.67$) or type 1 diabetes (1.14 (0.84, 1.56), $p = 0.39$). Further adjustment for maternal education, pre-pregnancy BMI and smoking did not change the results for islet autoimmunity (1.07 (0.83, 1.36), $p = 0.61$) or type 1 diabetes (1.14 (0.82, 1.58), $p = 0.43$). Maternal use of supplements with iron during pregnancy was not associated with the risk of islet autoimmunity (1.18 (0.91, 1.51), $p = 0.21$) or type 1 diabetes (1.17 (0.84, 1.62), $p = 0.36$). Adjustment for sex, family history of diabetes and HLA genotype did not change the results for islet autoimmunity (1.11 (0.86, 1.43), $p = 0.43$) or type 1 diabetes (1.14 (0.82, 1.58), $p = 0.45$). Further adjustment for maternal education, pre-pregnancy BMI and smoking did not change the results for islet autoimmunity (1.20 (0.91, 1.58), $p = 0.20$) or type 1 diabetes (1.18 (0.82, 1.69), $p = 0.37$).

3.4. Interaction Analyses

We observed no interaction between energy-adjusted total vitamin C intake and total iron intake on the risk of islet autoimmunity or type 1 diabetes.

Table 3. Hazard ratios (HR) and 95% confidence intervals (95% CI) for the association between maternal intake of vitamin C and iron during pregnancy and the risk of islet autoimmunity and type 1 diabetes in the offspring.

	Islet Autoimmunity				Type 1 Diabetes			
	Model 1 *n* = 4887 (312) [a]		Model 2 *n* = 4706 (305) [a]		Model 1 *n* = 4943 (178) [a]		Model 2 *n* = 4757 (174) [a]	
	HR [b] (95% CI)	*p*-Value	HR [b] (95% CI)	*p*-Value	HR [b] (95% CI)	*p*-Value	HR [b] (95% CI)	*p*-Value
Vitamin C from diet								
per 1 SD increase	0.97 (0.87, 1.09)	0.62	0.98 (0.87, 1.10)	0.73	1.05 (0.91, 1.21)	0.51	1.07 (0.93, 1.23)	0.37
Q_1	0.96 (0.73, 1.27)	0.96	0.99 (0.75, 1.30)	0.98	1.10 (0.77, 1.58)	0.47	1.08 (0.75, 1.57)	0.41
Q_2 and Q_3	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q_4	0.98 (0.75, 1.29)		1.02 (0.77, 1.34)		1.25 (0.88, 1.77)		1.27 (0.89, 1.81)	
Total vitamin C intake [c]								
per 1 SD increase	0.90 (0.79, 1.03)	0.12	0.91 (0.80, 1.03)	0.14	0.99 (0.86, 1.15)	0.94	1.01 (0.87, 1.17)	0.92
Q_1	0.96 (0.74, 1.26)	0.32	0.98 (0.75, 1.28)	0.36	0.92 (0.64, 1.32)	0.74	0.88 (0.61, 1.28)	0.73
Q_2 and Q_3	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q_4	0.80 (0.61, 1.07)		0.82 (0.61, 1.09)		0.87 (0.60, 1.26)		0.89 (0.61, 1.29)	
Iron from diet								
per 1 SD increase	1.09 (0.99, 1.22)	0.09	1.07 (0.96, 1.19)	0.22	1.10 (0.96, 1.26)	0.17	1.09 (0.95, 1.25)	0.23
Q_1	0.70 (0.52, 0.94)	0.06	0.71 (0.53, 0.97)	0.09	0.63 (0.42, 0.94)	0.06	0.95 (0.72, 1.26)	0.71
Q_2 and Q_3	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q_4	0.94 (0.73, 1.23)		0.92 (0.70, 1.20)		1.00 (0.71, 1.40)		0.89 (0.68, 1.18)	
Total iron intake [c]								
per 1 SD increase	0.99 (0.88, 1.11)	0.82	0.98 (0.87, 1.10)	0.71	0.94 (0.81, 1.10)	0.46	0.92 (0.78, 1.08)	0.30
Q_1	0.94 (0.71, 1.24)	0.79	0.95 (0.72, 1.26)	0.71	1.01 (0.71, 1.45)	0.98	1.01 (0.70, 1.45)	0.89
Q_2 and Q_3	1 (ref)		1 (ref)		1 (ref)		1 (ref)	
Q_4	0.92 (0.70, 1.20)		0.89 (0.68, 1.18)		0.97 (0.68, 1.39)		0.92 (0.64, 1.33)	

Abbreviations: Q1–Q4, quarter. [a] n represents total number in the analyses and number in parenthesis represents numbers of children with the outcome. [b] HRs (95% CI) are from Cox regression analysis (*p* values from Wald test). [c] Total vitamin C and total iron intake from diet and dietary supplements. Model 1: Energy adjusted with the Willett's residual method. Model 2: As model 1 and further adjusted for sex, family history of diabetes HLA genotype.

4. Discussion

In this large prospective birth cohort of children with increased genetic risk for type 1 diabetes, the maternal intake of vitamin C or iron during pregnancy and use of supplementation with vitamin C or iron were not associated with the risk of islet autoimmunity or type 1 diabetes.

The strengths of this study include a large study population, carefully collected data on maternal dietary intake and use of supplementation, use of well-maintained food composition database, as well as regular assessment of autoantibodies and type 1 diabetes. In the validation study of the FFQ used in our study, the FFQ in comparison to food records (10 days) showed a correlation of 0.65 for vitamin C and 0.60 for iron [19], suggesting that the FFQ is appropriate for the estimation of vitamin C and iron intake. Furthermore, no previous studies have explored the association between both dietary and supplementary intake of iron and the risk of islet autoimmunity.

Our study also had some limitations. The validation study of the FFQ did not include the use of dietary supplements [19]. Furthermore, food storage and processing can affect the vitamin C content in the foods, which cannot be totally taken into account in the food composition databases. Another limitation is that the vitamin C intake is linked to fruit and vegetable consumption, which may be confounded by several socioeconomic and lifestyle factors [25]. Additionally, we did not have plasma vitamin C and iron status available in our study, which could have provided us with an additional and, likely, a more accurate biomarker of these nutrients in the body [25,26].

Our results do not support the hypothesis that higher intake of vitamin C during pregnancy would protect from islet autoimmunity or type 1 diabetes. As far as we know, this is the only prospective study so far to explore the maternal intake of vitamin C and the risk of clinical type 1 diabetes outcome. In an earlier study with fewer mothers and children, we explored the association between maternal vitamin C intake and islet autoimmunity outcome [11]. Results were similar than in the present study but the use of vitamin C supplements per se were not studied in the previous study. The current study was carried out in a well-nourished population, and the mean vitamin C intake in mothers was more than triple the amount of the recommended 70 mg/day during pregnancy [27]. Only 240 (5%) mothers had an intake below the recommended intake, and only five (0.1%) mothers had a vitamin C intake under 20 mg/day in which plasma ascorbic acid status begins to deteriorate [28]. However, the FFQ in our study is likely to overestimate the intake of foods, which are considerable sources of vitamin C such as vegetables and fruits [19]. In addition, the same maternal characteristics were associated with non-participation and lower intakes of vitamin C, suggesting the final study population may have a higher intake of vitamin C compared to those who did not participate in the follow-up. Therefore, we cannot conclude whether vitamin C would be associated with risk of islet autoimmunity or type 1 diabetes in populations with lower vitamin C intakes. Our study did not include plasma ascorbic acid status, which is suggested to represent vitamin C function more accurately than dietary intake since absorption, transport and physiological requirements of vitamin C vary between individuals [2,25]. Plasma ascorbic acid status should be considered in future studies as high plasma ascorbic acid status in childhood has been associated with reduced risk of islet autoimmunity in a previous study [10].

Our results do not support the hypothesis that high maternal intake of iron from the diet or supplements would increase the risk of islet autoimmunity or type 1 diabetes in the offspring. Two previous prospective studies exploring maternal iron supplement use and the risk of clinical type 1 diabetes gave inconsistent results. While the Danish National Birth Cohort Study found no association between maternal iron supplement use during pregnancy and type 1 diabetes in the offspring [29], the Norwegian Mother and Child Cohort Study observed that maternal use of any iron-containing supplements was associated with increased risk of type 1 diabetes [13]. Mothers in our study and previous studies were from well-nourished populations and the use of iron supplements was common. The Finnish dietary recommendation states that iron supplementation may

be needed after the first trimester to maintain an iron balance of 500 mg iron storage [27,30]. Hence, some of the mothers in our study may have used supplements without any actual need. Similarly to the Norwegian cohort study, iron intake from food was not associated with type 1 diabetes risk in the current report [13].

Although we did not find association between maternal iron intake type 1 diabetes development, iron is an important nutrient for further studies. Mechanisms between maternal iron exposure and fetal outcomes might be complex. Maternal use of iron supplement during pregnancy may induce oxidative stress in the placenta, but the implication for the offspring has yet to be explored [13,31]. Maternal iron bioavailability is regulated by hepcidin, which suppresses the excess maternal iron flow in the circulation when iron supplements are taken regularly [32,33]. Fetal hepcidin and iron transport proteins in the placenta might also protect fetuses from iron overload [33]. In addition, epigenetic mechanisms may play an important role since, e.g., maternal hemochromatosis gene (HFE) genotypes have been associated with increased risk of type 1 diabetes [13,34]. Polymorphisms in the HFE gene induces hereditary hemochromatosis and increased iron stores, which could cause iron accumulation in the endocrine pancreas and beta-cell injury [35,36].

5. Conclusions

In conclusion, maternal vitamin C or iron intake during pregnancy were not associated with the risk of developing islet autoimmunity or type 1 diabetes in a prospective cohort of children carrying increased genetic susceptibility to type 1 diabetes.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6643/13/3/928/s1, Table S1: Maternal intake of vitamin C and iron during pregnancy and hazard ratios (HR) and 95% confidence intervals (95% CI) for islet autoimmunity and type 1 diabetes in the offspring, additional adjustments.

Author Contributions: M.M., L.H., S.N. and S.M.V. conceptualized and designed the study and interpreted the data. M.M. and L.H. drafted the initial manuscript. M.M., H.-M.T. and H.T. performed statistical analysis. S.A. coordinated and supervised the data collection and processing. J.I., J.T., R.V. and M.K. designed and supervised the DIPP study. S.N. and S.M.V. reviewed the manuscript. M.M. is the guarantor of this work and had full access to the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data is available from the corresponding author on reasonable request. The data are not publicly available due to the protection of the identity of the study participants and their clinical data.

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Supplementary Table S1. Maternal intake of vitamin C and iron during pregnancy and hazard ratios (HR) and 95% confidence intervals (95% CI) for islet autoimmunity and type 1 diabetes in the offspring, additional adjustments.

	Islet Autoimmunity		**Type 1 Diabetes**	
	Model 3 *n* = 4253 (284)[a]		**Model 3** *n* = 4301 (160)[a]	
	HR[b] (95% CI)	***P*-value**	**HR[b] (95% CI)**	***P*-value**
Vitamin C from diet				
per 1 SD increase	0.95 (0.84, 1.07)	0.42	1.03 (0.88, 1.20)	0.73
Q_1	1.00 (0.75, 1.34)	0.96	1.22 (0.83, 1.79)	0.44
Q_2 and Q_3	1 (ref)		1 (ref)	
Q_4	0.96 (0.72, 1.29)		1.23 (0.84, 1.79)	
Total vitamin C intake[c]				
per 1 SD increase	0.89 (0.77, 1.02)	0.10	0.81 (0.83, 1.03)	0.15
Q_1	0.98 (0.73, 1.30)	0.17	0.93 (0.63, 1.37)	0.73
Q_2 and Q_3	1 (ref)		1 (ref)	
Q_4	0.75 (0.56, 1.02)		0.76 (0.55, 1.21)	
Iron from diet				
per 1 SD increase	1.09 (0.98, 1.22)	0.11	1.09 (0.94, 1.27)	0.23
Q_1	0.75 (0.54, 1.02)	0.17	0.75 (0.49, 1.15)	0.30
Q_2 and Q_3	1 (ref)		1 (ref)	
Q_4	1.00 (0.76, 1.31)		1.09 (0.76, 1.56)	
Total iron intake[c]				
per 1 SD increase	0.99 (0.87, 1.11)	0.82	0.92 (0.78, 1.09)	0.34
Q_1	0.86 (0.64, 1.16)	0.47	0.94 (0.64, 1.40)	0.78
Q_2 and Q_3	1 (ref)		1 (ref)	
Q_4	0.87 (0.65, 1.15)		0.87 (0.59, 1.28)	

Abbreviations: Q1-4, quarter

[a] *n* represents total number in the analyses and number in parenthesis represents numbers of children with the outcome

[b] HRs (95% CI) are from Cox regression analysis (*P* values from Wald test)

[c] Total vitamin C and total iron intake from diet and dietary supplements

Model 3: Adjusted for energy residual method, sex, familial diabetes, HLA genotype, maternal education, pre-pregnancy BMI, and smoking