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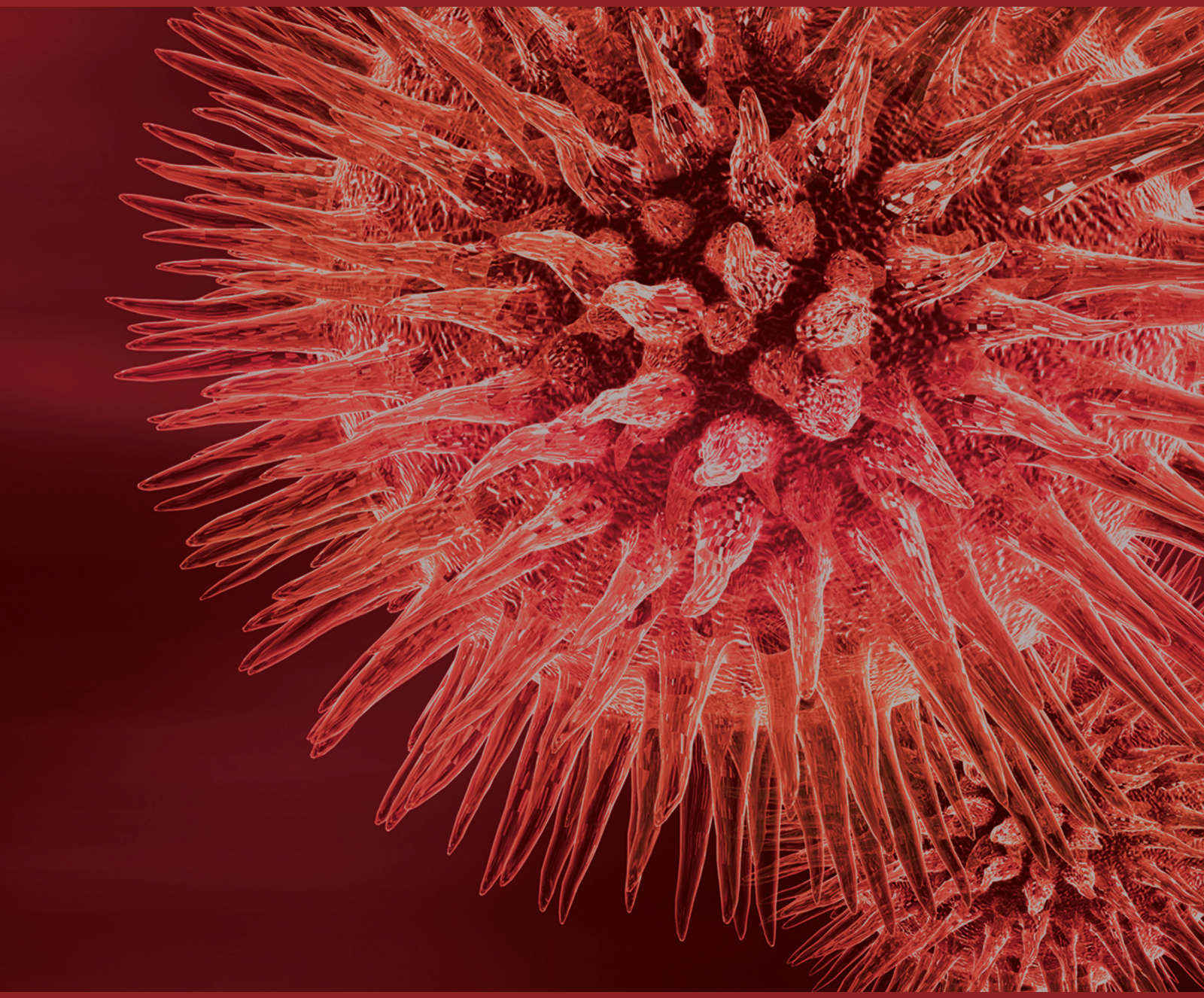
Environmental Trigger(s) of Type 1 Diabetes: Why Is It So Difficult to Identify?

Guest Editors: Kjersti S. Rønningen, Jill M. Norris, and Mikael Knip



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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 847906, 2 pages
http://dx.doi.org/10.1155/2015/847906

Editorial

Environmental Trigger(s) of Type 1 Diabetes: Why Is It So Difficult to Identify?

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Received 14 December 2014; Accepted 14 December 2014

Type 1 diabetes (T1D) is one of the most common chronic diseases with childhood onset, and the disease incidence has increased from two- to fivefold over the past half century from, as of yet, unknown reasons. T1D occurs when the body's immune system turns against itself, destroying in a very specific and targeted way the pancreatic beta cells. T1D results from poorly defined interactions between susceptibility genes and environmental determinants. In contrast to the rapid progress in finding T1D genes, the identification and confirmation of environmental trigger(s) remain a formidable challenge. The identification of environmental determinant(s) responsible for the development of or protection against T1D is crucial from that point of view that they may be modifiable and might ultimately be used in disease prevention. The high costs of the disease both to the society and to the affected individuals and their families imply that preventing a fraction of cases, or even delaying the onset, will be of high value.

This special issue contains original research articles as well as review articles that could stimulate the continuing efforts in the identification of exogenous triggers in the development of T1D. New data on environmental factors associated with progression to clinical T1D was of particular interest. Articles using bioinformatics to evaluate the number of cases (either autoantibody positivity or clinically recognized disease as the case definition) needed for the identification of a certain environmental trigger were as well very interesting. In addition, this special issue includes articles presenting autoantibody patterns in the prediction of T1D and cellular and molecular mechanisms in the disease pathogenesis. Here we give a short overview of the content of the current special issue.

In original research articles, the hygiene hypothesis and risk for T1D were studied prospectively in the Diabetes Autoimmunity Study in the Young (DAISY) by examining daycare attendance during the first 2 years of life. Altogether 1783 children were available with complete daycare and breastfeeding data. For the first time, the data obtained suggest that breastfeeding may modify the effect of daycare on the risk of T1D.

The goal of the other DAISY study included was to demonstrate methods for identifying exposures that differentially influence the disease process at certain ages by testing for age-related heterogeneity. The DAISY study has followed 2547 children at increased risk for T1D from birth, and 188 have developed islet autoimmunity (IA). Ethnicity, maternal age, and erythrocyte membrane n-3 fatty acid levels demonstrated significant age-related associations with IA risk in children at increased risk of T1D. For example, increased n-3 fatty acid levels were associated with a significantly decreased risk of IA after the age of 4.25 years, but not before, which may have implications in the design of intervention studies.

In a study using carbohydrate counting in 22 cases compared to 15 controls on standard diabetic diet, it was shown that carbohydrate counting may be a functional method in patients affected by T1D.

In review articles, the role of maternal dietary essential fatty acids (EFA) was assessed in a review of studies on nonobese diabetic (NOD) mice. The paper concluded that,

among maternal factors, dietary n-6/n-3 EFA ratio during pregnancy and the lactation period may affect the severity of insulitis and the progression to autoimmune diabetes in the offspring.

In a review on Luminex and other multiplex high throughput technologies for the identification of environmental triggers of T1D, critical issues are discussed and potential solutions are offered for the development of optimal assays. Based on their own experiences, the authors conclude that multiplex technologies might be successfully used for the evaluation of different classes of environmental exposures and host responses in T1D pathogenesis.

Another review summarizes the association between environmental chemical exposure and T1D development. Chemicals may have direct toxic effects on insulin producing beta cells, they may have immunomodulatory effects, and/or they alter hormone levels affecting gut microbiota or intestinal permeability. Due to the lack of strong evidence for a single factor as the major trigger of T1D, it is tempting to propose that several factors have additive or synergistic effects, acting via several mechanisms and/or at different stages in the disease process.

One of the review articles focused on factors which have to be evaluated and decisions to be taken before starting a new prospective cohort study. Before the recruitment starts, it is essential to design the study in an optimal way to be able to identify important environmental factors. The paper titled "Environmental Trigger(s) of Type 1 Diabetes: Why So Difficult to Identify?" has been used for the discussion of Dr. Rønningen's own experiences. Most likely there are already sufficient prospective cohort data collected or under collection to identify the environmental trigger(s) of T1D. New valuable information on exogenous factors and their contribution associated with the development of beta cell autoimmunity and progression to T1D could be achieved by a huge international collaborative effort. Such an effort would make it possible to integrate demographic, genetic, autoimmune, and exposure data from the existing cohorts in Finland, Norway, Sweden, Germany, and Denver, Colorado, in addition to the TEDDY study generating comprehensive information on the role of environmental factors in the development of T1D both in a family cohort and in a population-based cohort.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 208947, 19 pages
http://dx.doi.org/10.1155/2015/208947

Review Article

Can Exposure to Environmental Chemicals Increase the Risk of Diabetes Type 1 Development?

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Received 28 June 2014; Accepted 14 September 2014

Academic Editor: Jill M. Norris

Type 1 diabetes mellitus (T1DM) is an autoimmune disease, where destruction of beta-cells causes insulin deficiency. The incidence of T1DM has increased in the last decades and cannot entirely be explained by genetic predisposition. Several environmental factors are suggested to promote T1DM, like early childhood enteroviral infections and nutritional factors, but the evidence is inconclusive. Prenatal and early life exposure to environmental pollutants like phthalates, bisphenol A, perfluorinated compounds, PCBs, dioxins, toxicants, and air pollutants can have negative effects on the developing immune system, resulting in asthma-like symptoms and increased susceptibility to childhood infections. In this review the associations between environmental chemical exposure and T1DM development is summarized. Although information on environmental chemicals as possible triggers for T1DM is sparse, we conclude that it is plausible that environmental chemicals can contribute to T1DM development via impaired pancreatic beta-cell and immune-cell functions and immunomodulation. Several environmental factors and chemicals could act together to trigger T1DM development in genetically susceptible individuals, possibly via hormonal or epigenetic alterations. Further observational T1DM cohort studies and animal exposure experiments are encouraged.

1. Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease with beta-cell destruction, resulting in insulin deficiency. A genetic predisposition seems to be necessary for developing the disease and is most often linked to genes in the HLA-complex [1]. About 90% of children with T1DM have the DR4-DQ8 haplotype and/or DR3-DQ2, and those who have both in combinations have the highest risk for T1DM. Islet autoantibodies are detected in ~90% of individuals at the time of diagnosis of T1DM, and these are directed against pancreatic proteins like insulin, glutamic acid decarboxylase (GAD), islet antigen 2 (IA-2), or zinc transporter 8 [2]. These autoantibodies generally appear in the circulation months to years before clinical onset.

It has become clear that environmental factors likely play a role in disease development, due to the facts that there has been an increasing incidence of type 1 diabetes in the last decades in many industrial countries and that there is less than 60% concordance of T1DM among monozygotic twins [3]. Factors like maternal age at delivery, infections in early life, deficiency of specific nutrients during pregnancy, and/or early childhood have been associated with risk of type 1 diabetes in observational studies [4, 5]. Other suggested environmental risk factors for T1DM are alterations in gut microbiota [6] and lack of general exposure to microbial factors (the "hygiene hypothesis") [7].

This review will focus on the possible impact of environmental chemicals on T1DM development. The observed associations are summarized in Table 1 and the suggested mechanisms are summarized in Figure 1. Where little or no data of direct relevance for T1DM are available, we briefly discuss relevant data on other immune mediated diseases or on T2DM.

We start with a brief overview of relevant study designs and models, before we discuss relevant studies with the

Table 1: Summary of studies reporting associations between exposure to environmental chemicals and endpoints relevant to T1DM development (T1DM, T2DM or beta-cell/immunomodulations). A "+" sign in front of the reference indicates positive association between chemical exposure and the respective endpoint (diabetes or beta-cell/immune modulations), while a "–" sign indicates no association between chemical exposure and T1DM or having inverse associations. "(+)" in front of the reference indicates that the exposure was measured in common drinking water, not for the individual, resulting in more uncertain conclusions.

Subgroup of chemicals	Environmental chemical	T1DM epidemiology	T1DM animal studies	T2DM epidemiology	Beta-cell modulations	Immuno modulations	Likelihood for T1DM influence
Polychlorinated biphenyls	PCB	+ Longnecker et al. 2001 [16] + Langer et al. 2002 [18] – Rignell-Hydbom et al. 2010 [17]		+ Everett et al. 2011 [22] + Carpenter 2006 [21]		+ Schmidt and Bradfield 1996 [23]	+++ (–)
Dioxins	TCDD/dioxin		– Shinomiya et al. 2000 [28]	+ Cranmer et al. 2000 [39]	+ Martino et al. 2013 [25]	– Rohlman et al. 2012 [30]	++ (–)
			– Kerkvliet et al. 2009 [29]	+ Pelclová et al. 2006 [40]	+ Kurita et al. 2009 [26]	– Li and McMurray 2009 [33] – Schulz et al. 2012a, b [34, 35]	
				– Warner et al. 2013 [41]	+ Kim et al. 2009 [27]	– Hanieh 2014 [32] + Ishimaru et al. 2009 [36] + Mustafa et al. 2011a, b [37, 38]	
Organochlorides (pesticides)	DDT/DDE	– Rignell-Hydbom et al. 2010 [17]		+ Codru et al. 2007 [43]		+ Yang et al. 2012 [44]	++ (–)
				+ Philibert et al. 2009 [45]		– Li and McMurray 2009 [33]	
Polybrominated biphenyls (Flame retardants)	PDBE			+ Taylor et al. 2013 [46] + Turyk et al. 2009 [47] + Lee et al. 2011 [48] – Turyk et al. 2009 [47] + Llm et al. 2008 [52]	+ Zhang et al. 2013 [49]	+ Hennigar et al. 2012 [50] + Turyk et al. 2008 [51]	++
Perfluorinated alkyl substances	PFAS			+ Lind et al. 2014 [53]	+ Lin et al. 2009 [54] + Lv et al. 2013 [56]	+ Grandjean et al. 2012 [55] + Granum et al. 2013 [57] + Borg et al. 2013 [58]	++
Endocrine disruptors	BPA		+ Bodin et al. 2013 [59]; Bodin et al. 2014 [60]	+ Aekplakorn et al. 2014 [61] + Ahmadkhaniha et al. 2014 [63] – Kim and Park 2013 [65] + Sabanayagam et al. 2013 [67] + Shankar and Teppala 2011 [68] + Silver et al. 2011 [69] + Sun et al. 2014 [70]	+ Song et al. 2012 [62] + Soriano et al. 2012 [64] + Nadal et al. 2009 [66]	+ Bodin et al. 2014 [60]	++++
	Triclosan					+ Paul et al. 2009 [71] + Zorrilla et al. 2009 [72] + Koeppe et al. 2013 [73]	+

Table 1: Continued.

Subgroup of chemicals	Environmental chemical	TIDM epidemiology	TIDM animal studies	T2DM epidemiology	Beta-cell modulations	Immuno modulations	Likelihood for T1DM influence
	Phthalates			+ Huang et al. 2014 [74] + James-Todd et al. 2012 [76] + Kim et al. 2013 [78] + Lind et al. 2012 [80] + Stahlhut et al. 2007 [81] + Svensson et al. 2011 [82] + Trasande et al. 2013 [83]		+ Mankidy et al. 2013 [75] + Vetrano et al. 2010 [77] + Sarath Josh et al. 2014 [79]	+
Metals	Arsenic			+ Rager et al. 2014 [84] + Tsai et al. 1999 [87] + Bräuner et al. 2014 [90] + Lee and Kim 2013 [92] + Mahram et al. 2013 [94]	+ Douillet et al. 2013 [85] + Lu et al. 2011 [88] + Yang et al. 2012 [44]	+ Dangleben et al. 2013 [86] + Ahmed et al. 2012 [89] + Banerjee et al. 2009 [91] + Lu et al. 2014 [93]	++
	Organotins				+ Miura et al. 1997 [95]; Miura et al. 2012 [96] + Zuo et al. 2014 [97] − Matsui et al. 1984 [98]		+
Nitroso compounds	Nitrates and nitroso amines	+ Dahlquist et al. 1990 [99] + Benson et al. 2010 [101] − Samuelsson et al. 2011 [103] − Cherian et al. 2010 [104] (+) Kostraba et al. 1992 [105] (+) Parslow et al. 1997 [106] (+) van Maanen et al. 1999 [107] (+) Helgason 1991			+ Helgason and Jonasson 1981 [100] + Wilson et al. 1983 [102]		++ (+)
	Streptozotocin		+ Szkudelski 2001 [108] + Leiter 1982 [110] + Rossini et al. 1977 [112] + Rerup 1970 [113]		+ Schnedl et al. 1994 [109] + Wang and Gleichmann 1998 [111] + Lenzen 2008 [11]		++
	Alloxan				+ Lenzen 2008 [11] + Szkudelski 2001 [108] + Eizirik et al. 1994 [114]		++
	Bafilomycin		+ Hettiarachchi et al. 2004 [115]		+ Myers et al. 2003 [116] + Hettiarachchi et al. 2006 [117]		++
	Vacor				+ Myers et al. 2003 [116] + Esposti et al. 1996 [118] + Taniguchi et al. 1989 [119] + Wilson and Gaines 1983 [120]		+
	Cereulides				+ Virtanen et al. 2008 [121] + Vangoitsenhoven et al. 2014 [122]		+

Table 1: Continued.

Subgroup of chemicals	Environmental chemical	T1DM epidemiology	T1DM animal studies	T2DM epidemiology	Beta-cell modulations	Immuno modulations	Likelihood for T1DM influence
Air pollution	Particulate matter			+ Eze et al. 2014 [123] + Hathout et al. 2001 [125] + Brook et al. 2013 [127]		+ Danielsen et al. 2011 [124] + den Hartigh et al. 2010 [126] + Yan et al. 2011 [128]	++
	Ozone	+ Hathout et al. 2006 [129]				+ Bass et al. 2013 [130]	+++
	Carbon monoxide			+ Janghorbani et al. 2014 [131] + Dales et al. 2012 [132] − Nikolic et al. 2014 [133]			+
	Maternal/smoking	+ Hathout et al. 2006 [129] − Dahlquist and Kallen 1992 [136] − Hjern and Söderström 2008 [138] − Ievins et al. 2007 [139] − Johansson et al. 2008 [140] − Marshall et al. 2004 [141] − Rasouli et al. 2013 [135] − Robertson and Harrild 2010 [142]		+ Thiering et al. 2011 [134] + Persson et al. 2000 [137]	+ Rasouli et al. 2013 [135]		+++ (−)
	PAH polycyclic aromatic hydrocarbon			+ Zhao et al. 2014 [183]		+ Nadeau et al. 2010 [144] + den Hartigh et al. 2010 [126] + Danielsen et al. 2011 [124] + Perreault et al. 2013 [145]	++

[+]Beta-cell toxicity or immunomodulation.
[++]Beta-cell toxicity and immunomodulation.
[+++]Beta-cell toxicity or immunomodulation and T1DM human or animal study.
[++++]Beta-cell toxicity and immunomodulation and T1DM human or animal study.

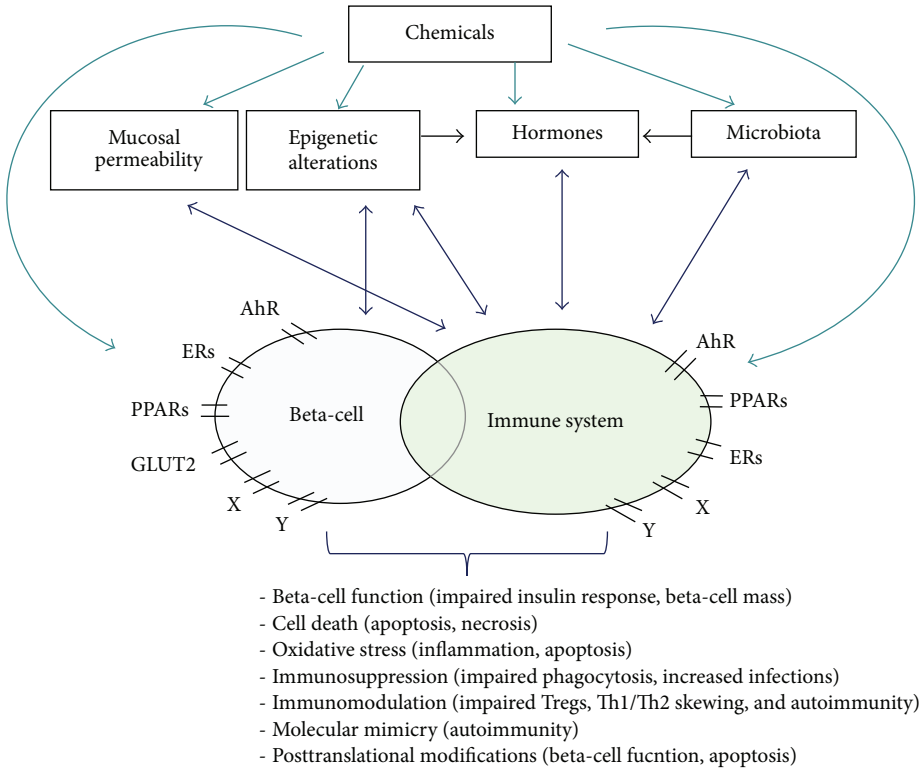

Figure 1: Mechanisms suggested to be involved in pathways of T1DM development after exposure to environmental chemicals via food/gut, air/lungs, and skin. Chemicals can act directly on beta or immune cells, by binding to receptors (X and Y-receptors could, for instance, be adrenergic-, purinergic-, or scavenger receptors) or after uptake in the cells by pinocytosis, endocytosis, or diffusion. Chemicals can also affect factors like mucosal permeability, the microbiome, or the hormone balance, all shown to interact with the immune system. Several chemicals have been shown to induce epigenetic changes. Chemical exposures can further lead to apoptosis or cell death, increased oxidative stress, impaired insulin response, altered immune function or immunosuppression, molecular mimicry, and posttranslational modifications.

specific environmental chemicals, followed by a review of some potential mechanisms involved.

2. Study Designs Used in T1DM Research

The possible impact of environmental risk factors on T1DM development has been analysed in epidemiological studies by comparing the serum or urine levels of the chemical or their metabolites/biomarkers in T1DM patients and healthy controls. Interpretation of such studies must take into account the possibility that exposure may have occurred at or after diagnosis, by considering kinetics of the biomarkers and the ability of biomarkers to reflect actual exposure. Potential risk factors that could induce islet autoimmunity (presence of islet autoantibodies) or the progression from autoimmunity to development of disease can be investigated in longitudinal epidemiological studies with serial serum samples available from early childhood (from before the presence of autoantibodies and after seroconversion) up to diagnosis of T1DM. To our knowledge, chemicals have been determined in few prospective studies of type 1 diabetes in humans. Epidemiological studies have been limited by the low incidence of T1DM and the difficulty in accurately assessing exposures in epidemiological studies of large size.

Some epidemiological studies use proxies for environmental chemical exposures (for instance by self-report in questionnaires) or use ecological study designs where exposure is not determined at the individual level but only by region or country, and these must be interpreted with caution.

Animal models allow for controlled exposures of the chemical in question and are important for establishing causal relationships and the mechanistic mode of action. The most commonly used models for T1DM development are the nonobese diabetic (NOD) mouse and the Bio Breeding (BB) rat demonstrating spontaneous insulitis, influx of autoimmune cells into pancreatic islets attacking insulin producing beta-cells, and T1DM development [8–10]. Another frequently used model to induce diabetes in mice is by multiple low dose administrations of the beta-cell specific toxin streptozotocin, where the beta-cells are destroyed and the animals rapidly develop diabetes [11]. Increased levels of serum glucose and insulitis in pancreatic sections are examples of T1DM features in both animal models, whereas hyperinsulinemia and insulin resistance in other animal strains generally are signs of type 2 diabetes (T2DM) development. Other models of T1DM, including knock-out variants of the ones mentioned above, are reviewed elsewhere [12].

In vitro models are suitable to investigate direct effects on specific cell types, including receptor interactions. The

most commonly used *in vitro* beta-cell systems for diabetes research are the rat beta-cell lines (INS-1E, RIN-m5F) [13, 14], the mouse beta-cell line (MIN6) [15] and primary islets and single beta-cells isolated from human, mouse, and rat pancreas. Decreased glucose secretion and increased apoptotic signaling are examples of T1DM-related mechanisms in beta-cells.

3. Environmental Chemicals

This review focuses on environmental chemicals that (i) have been found to contaminate food, water, and air and (ii) have been reported to influence the function of beta-cells or the immune system. These components include persistent organic pollutants (POPs like PCBs, dioxins, pesticides, and flame retardants), endocrine disruptors (bisphenol A, phthalates, and triclosan), certain metals (arsenic, organic derivatives of tin), N-nitroso compounds, bacterial toxins, ambient air pollution (such as ozone, particulate matter, and polycyclic aromatic hydrocarbons), and tobacco smoke. Some of these are persistent organic pollutants, which are resistant to environmental degradation and therefore accumulate in nature and the food chain. Other chemicals, including many of the endocrine disruptors (such as bisphenol A and phthalates) have a short half-life in the environment and have low bioaccumulation in humans.

3.1. Polychlorinated Biphenyls (PCBs). There are 209 configurations of organochlorides with 1 to 10 chlorine atoms, classified as persistent organic pollutants. PCBs have been used as dielectric and coolant fluids in electrical equipment and can be found in marine food and wild animals due to accumulation in fatty tissue in the food chain.

In a prospective study on pregnant women with diabetes (primarily type 1), PCB serum levels were associated with the disease [16]. Another epidemiological study, however, showed tendency of an inverse association between maternal serum levels of PCB-135 or p,p′-DDE during pregnancy and T1DM development in the child, but this was not statistically significant [17]. In support of PCB effects on autoimmunity, employees working at a PCB production factory had higher prevalence of antiglutamic acid decarboxylase (anti-GAD) autoantibodies in their serum compared to controls [18].

Animal studies reveal induced insulin resistance, indicating T2DM development, after exposure to a mixture of persistent organic pollutants that mimics the relative abundance of organic pollutants present in crude salmon oil [19, 20], and there are several studies indicating associations between serum PCB levels and T2DM in humans [21, 22]. Dioxins and dioxin like PCBs act via the aryl hydrocarbon receptor (AhR) and can cause oxidative stress, apoptosis, and increased inflammation during metabolization/detoxification of the chemical [23].

3.2. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). TCDD is primarily formed as a byproduct in the manufacturing of materials requiring the use of chlorinated phenols and during the combustion of chlorinated chemical products. TCDD is a persistent organic pollutant that has been used in herbicides like Agent Orange [24]. Humans are mostly exposed through intake of marine food and game due to accumulation of the chemical in fatty tissue in the food chain.

There are no epidemiological studies investigating associations between TCDD exposure and T1DM. However, TCDD has been shown to be highly toxic for INS-1E rat pancreatic beta-cells regarding survival and ultrastructure via activation of the aryl hydrocarbon receptor (AhR) [25]. Further, experimental studies have shown that TCDD exposure in C57BL/6J mice impaired glucose-stimulated secretion of insulin from the islets via the AhR signaling pathway [26]. TCDD has also been shown to induce calcium influx via T-type channels, regulating vesicular trafficking, such as lysosomal and secretory granule exocytosis, indicating that TCDD might exert adverse effects on beta-cells by stimulating continuous insulin release resulting in beta-cell exhaustion in an INS-1 rat beta-cell line [27].

On the other hand, in the NOD mouse model, TCDD has been shown to prevent T1DM development when administered from 8 weeks of age, a time point after the spontaneous insulitis development is normally initiated (starting from 4 weeks of age in the NOD mice [28]), due to increased number of regulatory T-cells in the pancreas and reduced insulitis [29]. The immunosuppressive effect of TCDD has been shown to be due to activation of the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor in CD4+ Th17 T-cells, and upregulation of IL-22 expression [30]. IL-22 is secreted by Th17 cells and is highly present in various autoimmune diseases, but whether IL-22 is mediating the inflammation itself, or is a byproduct of the inflammation is depending on the tissue and overall cytokine setting [31]. In agreement with this, in another murine model for autoimmunity, systemic lupus erythematosus (SLE), TCDD appears to promote differentiation of regulatory T-cells via AhR and inhibiting Th17 cells and cause immunosuppressive effects [32, 33]. Both models indicate a therapeutic effect of AhR activation in autoimmunity development in adult animals. In line with the immunosuppressive effects of TCDD via Tregs stimulation following AhR ligation/activation, TCDD has also demonstrated suppressive (preventive) effects in rodent allergy models [34, 35]. Interestingly, other AhR ligands did not have this suppressive effect on the allergy development, suggesting that the effect via AhR is ligand specific [34]. On the other hand, TCDD administered during gestation induced adult autoimmunity in different mice strains [36–38], suggesting that exposure to chemicals during critical developmental stages *in utero* may possibly promote the development of autoimmune diseases, including T1DM, later in life.

Human serum levels of TCDD have been associated with increased insulin plasma levels and T2DM, although there are some conflicting results from epidemiological studies [39–42].

3.3. Dichlorodiphenyltrichloroethane (DDT) and Dichlorodiphenyldichloroethylene (DDE). The organochloride DDT and its metabolite DDE have been used as insecticides triggering spasms via the opening of neuronal ion channels

and are persistent chemicals that accumulate in fatty tissue in the food chain.

In a nested case-control study maternal serum levels of p,p′-DDE during pregnancy and T1DM development in the child, there was no significant association with T1DM; however the T1DM cases had a tendency of lower p,p′-DDE levels than control subjects (as mentioned above for PCB) [17].

Serum levels of DDT and DDE are both associated with the development of T2DM [33, 43, 45–47]. It has been shown that DTT activates AhR-signaling and can induce apoptosis in murine embryonic neuronal cells, but there are no reports available about beta-cell toxicity [146].

3.4. Polybrominated Diphenyl Ethers (PDBE). PDBEs are bioaccumulating persistent chemicals used as flame retardants in building materials, textiles, furnishings, and electronics. Exposure to humans is mainly via ingestion of food and by inhalation of indoor air and they can act as an endocrine disruptors.

There are no epidemiological studies investigating associations between PDBE exposure and T1DM in humans. In a rat exposure study 2,2′,3,3′,4,4′,5,5′,6,6′-decabromodiphenyl ether (BDE209) exposure was shown to induce hyperglycemia, decrease insulin, glutathione, and superoxide dismutase serum levels and increase TNFα serum levels, probably via induction of oxidative damage and was further correlated to changes in rat liver cell MHC and TNFα transcripts that possibly could be involved in T1DM development [49]. In a study on porcine alveolar cells, a mix of PDBE, DE-71 was shown to induce lower levels of proinflammatory cytokine release compared to control, indicating that PDBEs may suppress innate immunity [50].

PDBEs have been suggested to be associated with altered thyroxin hormone levels, and there are conflicting reports on association with T2DM in humans [47, 48, 51, 52].

3.5. Perfluorinated Alkyl Substances (PFAS). PFAS have attractive lipid and water repelling properties and are therefore used in fire-fighting foam, textiles, kitchen ware, and food packaging materials. Human exposure to PFAS is mainly through diet via marine food and game.

An epidemiological study has reported that increased serum level of the perfluorononanoic acid (PFNA) in human adolescents is associated with decreased blood insulin and beta-cell function [54].

PFAS exposure *in utero* appears to modulate the immune response in children, resulting in reduced immune responses to vaccines and increased infections in early childhood [55, 57]. In a human cumulative health risk assessment report PFAS are suggested to be immunotoxic, although the mechanisms are unknown and possible multiple [58]. Further, elevated PFNA serum level was also associated with diabetes in an elderly population supporting the view that PFAS can alter glucose metabolism in humans and induce T2DM [53]. Lv et al. [56] reported that PFAS exposure in rats during gestation and lactation altered glucose tolerance in adult offspring.

3.6. Bisphenol A (BPA). BPA is used in the production of polycarbonate plastic and epoxy resins coating the inside of metal cans and can leak from the plastic into food. Human exposure is ubiquitous, as BPA metabolites are measured in more than 90% of children and adults in westernized countries [147]. BPA is rapidly metabolized and more than 99% is secreted in the urine within 4 hours [148], making detection in human blood samples variable and inconsistent at the limit of detection.

No human study of BPA exposure and T1DM development has been performed. Using the NOD mouse model, BPA was found to increase the spontaneous T1DM development after both long term postnatal exposure and short term prenatal and early life exposure [59, 60]. A very high BPA exposure (resembling 15 mg/kg/day) showed tendency to a preventive effect, which possibly could be explained by different mechanisms dominating at higher BPA exposure, such as an increased insulin secretion or estrogenic compensation mechanisms. These studies suggest that BPA acts by impairing macrophage function, resulting in impaired clearance of apoptotic cells, a feature common for several autoimmune diseases. BPA was also seen to modulate immune responses in lymphoid tissue in the mice and to impair islet morphology and beta-cell function in isolated rat pancreatic islets [60, 62].

Epidemiological studies have shown both positive and no associations to T2DM [61, 63, 65, 67–69]. In addition, BPA exposure has also been associated with asthma development in both human epidemiological studies and animal experimental studies [149–152]. It has been shown that BPA induces insulin secretion, in both human and mouse beta-cells via ERβ activation, possibly contributing to T2DM development [64, 66]. Further animal studies have shown induced insulin resistance and T2DM in mice [66, 145, 153–157].

3.7. Triclosan. Triclosan is a chlorinated aromatic compound that has anti-inflammatory effects, suppressing microbial-pathogen recognition pathway molecules and chronic mediators of inflammation and is used as antimicrobial agent in soap, toothpaste, clothes, and suture material for medical surgery [158].

There are no epidemiological studies investigating associations between triclosan exposure and T1DM or T2DM; however, triclosan exposure has been associated with increased risk of sensitization, rhinitis, and food allergy [159–161]. As an endocrine disruptor, triclosan has been shown to decrease thyroid hormone levels in humans and in rats [71–73]. Treatment with tri-iodothyronine (T3) in the BB rat reduced T1DM incidence and increased beta-cell mass in diabetes free Wistar rats [162], indicating that modulation of thyroid hormone levels may affect T1DM development in genetically susceptible animals.

3.8. Phthalates. Phthalates are commonly used as plasticizers and in a variety of consumer products, like paint and cosmetics. Phthalates are rapidly biodegradable endocrine disruptors and human exposure is mainly through diet via contamination from plastic into food and via inhalation of

phthalates in dust in indoor air. Uptake via the skin from cosmetic products is also contributing to systemic exposure.

No epidemiological studies have so far investigated associations between phthalate exposure and T1DM. There are, however, epidemiological studies showing associations with phthalate exposure and insulin resistance and T2DM [62, 74, 76, 78, 80–83]. Phthalates can induce sustained oxidative stress and inflammation via activation of AhR, ER, and/or binding to peroxisome proliferator activated receptor PPARs [75, 77, 79, 163, 164]. Phthalate exposure is also associated with asthma development and Th2 deviation, possibly via epigenetic modulations [164–167].

3.9. Arsenic. Arsenic is often contaminating drinking water from private wells, in high levels especially throughout South East-Asia and Latin-America but also in lower levels in parts of the United States, Australia, and Europe [86, 168].

There are no epidemiological studies investigating associations between arsenic exposure and T1DM development. Arsenic exposure has been shown to impair the immune system in humans and animal models [86, 89, 91] and to alter the gut microbiome diversity, microbiome metabolic profiles as well as inhibiting the glucose stimulated insulin release in mice [85, 93]. Further, prenatal arsenic exposure has been associated with increased miRNAs (miR-107 and miR-126) involved in signaling pathways related to diabetes [84, 169] and another possible mechanism for diabetes development can be a direct negative effect on beta-cell functions and apoptosis due to arsenic exposure seen in MIN6 pancreatic murine and RIN-m5F rat beta-cell lines [44, 88].

Epidemiological studies have reported an association between arsenic exposure and T2DM [87, 90, 92, 94, 170].

3.10. Organotin Compounds. Organotin compounds are used as stabilizers in the production of polyvinyl chloride, and triphenyltin compounds are used as antifungal agents [171].

There are no present epidemiological study investigating associations between organotin compounds and diabetes in humans. However, triphenyltin exposure has been shown to cause hyperglycemia in rabbits and hamsters, possibly due to inhibitory effects on insulin secretion by decreasing the glucose-induced rise in intracellular Ca^{2+} in pancreatic beta-cells, as shown in triphenyltin exposed hamsters [95, 96]. Triphenyltin exposure did not affect diabetes development in rats and mice [98], although it has recently been shown that tributyltin chloride induces pancreatic islet cell apoptosis in male KM mice [97].

3.11. N-Nitroso Compounds. N-nitroso compounds are present in processed food [172] but can also be formed in the gastrointestinal tract when nitrates from food or water are converted to nitrites and reacts with amines. These compounds are shown to be toxic to pancreatic beta-cells [102].

Higher levels of nitrates in drinking water have been associated with increased incidence of T1DM [105–107], although case-control studies on children's dietary intake of nitrates show conflicting results [99–101, 103, 104, 173–178]. Nitrosamines in food additives have been associated with a higher risk for T1DM development [99–101].

An exposure study with smoked/cured mutton containing N-nitroso compounds, fed at the time of mating, during gestation and in early life in the normal nondiabetic mouse strain CD1 showed development of diabetes in the offspring, more pronounced in male offspring compared to females (16% compared to 4%) [100].

3.12. Vacor. N-nitroso-compounds have previously been used as pest control chemicals and the rodenticide Vacor is shown to specifically decrease beta-cell functions by inhibiting mitochondrial ATP production and suppressed glucose-induced insulin secretion in isolated rat pancreatic islets and beta-cells [116, 118–120].

3.13. Streptozotocin. Streptozotocin is a naturally occurring glucosamine-nitrosourea compound produced by the soil microbe *Streptomyces achromogenes* causing destruction of beta-cells via DNA fragmentation, activating poly ADP-ribosylation, formation of superoxide radicals, hydrogen peroxide, and liberation of nitric oxide [11, 108, 113]. It is exclusively taken up by beta-cells via the glucose transport protein GLUT2 due to its similarity to glucose and the toxicity is therefore specific to beta-cells [109, 111]. Multiple low dose exposure of streptozotocin is used in animal studies to induce beta-cell destruction associated with pancreatic insulitis and subsequent T1DM-like symptoms [108, 110, 112]. Another toxic glucose analogue used in rodents to induce diabetes is alloxan, an oxygenated pyrimidine derivative. Alloxan generates reactive oxygen species (ROS), superoxide radicals, hydrogen peroxide, and, in a final iron-catalysed reaction step, hydroxyl radicals that together with increased cytosolic calcium concentration induce beta-cell death [11, 108, 114].

3.14. Bafilomycin. Bafilomycin from *Streptomyces*-infected vegetables has been shown to specifically decrease beta-cell function, seen as reduction of islet size and beta-cell mass after injection in mice [116, 179]. Bafilomycin exposure *in utero*, but not after birth, significantly accelerated the onset and incidence of diabetes in NOD mice [115], indicating that naturally occurring environmental toxicants possibly could influence T1DM risk. However, such association has not been investigated in epidemiological T1DM studies. Furthermore, high dose bafilomycin exposure was shown to promote cell death whereas low dose induced insulin secretion in the MIN6 mouse pancreatic cell line [117].

3.15. Cereulide. Cereulide is a toxin produced by certain strains of *Bacillus cereus*, a bacterium connected to emetic food poisonings from raw milk and industrially produced baby food [180].

There are no epidemiological studies investigating associations between cereulide exposure in human and diabetes development.

Cereulide has, however, been shown to cause necrotic cell death in porcine pancreatic Langerhans islets in cell culture [121] and to induce mitochondrial stress markers (p53

upregulated modulator of apoptosis, Puma, and CCAAT/-enhancer-binding protein homologous protein, CHOP) and apoptosis in mouse (MIN6) and rat (INS-1E) beta-cell lines, as well as in mouse islets [122].

3.16. Air Pollution. Cumulative exposure to ozone and sulphate in ambient air in Southern California has been associated with T1DM development [129]. Animal ozone exposure experiments, however, revealed induced glucose intolerance in rats [130]. Further, carbon monoxide (CO) has been associated with T2DM [131, 132]. Interestingly, carbon monoxide has been used as treatment of T1DM in the NOD mouse model due to its anti-inflammatory and antiapoptotic properties [133].

Exposure to particulate matter (PM) induces formation of reactive oxygen species in human lung endothelial cells and circulating monocytes, leading to DNA damage and inflammation [124, 126].

Fine particulate matter (PM2.5) has been associated with diabetes in rats, by intratracheal instillation, enhanced insulin resistance, and visceral inflammation in rats fed a high fat diet but not a normal chow [70, 128]. In humans, air pollution measured as outdoor PM < 10 μm in aerodynamic diameter (PM10) and nitrogen dioxide (NO2) has been shown to be associated with T2DM [123, 125] and decreased insulin sensitivity [127].

3.17. Tobacco Smoke. Maternal smoking during pregnancy has been associated with decreased T1DM development [135, 136, 138–142], although passive smoking was more frequent in children with T1DM in one study [129].

An association between prenatal and postnatal tobacco smoke and increased insulin resistance has been shown in 10 year old children [134]. Further studies have shown increased risk of T2DM due to maternal smoking, as well as increased insulin resistance and increased risk of T2DM development due to direct smoking in adults [137].

3.18. Polycyclic Aromatic Hydrocarbons (PAHs). PAHs are found in fossil fuels and tar deposits and are produced during incomplete combustion of organic matter and thus are abundant in air pollution. In addition, considerable PAH exposure is experienced from dietary sources [181].

We are not aware of any studies on PAH and risk of T1DM in humans. Animal and human *in vitro* cell studies link PAH exposure to the generation of oxidative stress, DNA damage and inflammation via activation of the aryl hydrocarbon receptor (AhR) in the metabolism and secretion of the PAHs by CYP enzymes [23, 124, 126, 182].

The impaired regulatory T cell (Treg) function associated with human PAH and ambient air pollution exposure, explained by increased methylation of the transcription factor Foxp3 in Tregs [144], may be a plausible mechanism for promoting T1DM development, although this has not yet been investigated. Epidemiological studies have shown association between urinary PAH levels and T2DM development [143].

4. Mechanisms for Chemical-Induced Triggering of T1DM

4.1. Toxic Effects on Beta-Cells. Direct effects on beta-cell function or viability could be a mechanism of environmental chemical for contributing to autoimmunity. Suggested mechanisms leading to beta-cell apoptosis are related to altered mitochondrial functions and induction of oxidative stress. Other mechanisms than apoptosis leading to reduced beta cell mass include impairment of beta-cell replication, by cAMP suppression via α_2-adrenergic receptors and thereby reducing total beta-cell mass [183]. It has also been shown that adenosine receptor agonists acting through the adenosine receptor A2aa, increased beta-cell proliferation and accelerated restoration of normoglycemia in zebrafish [184]. Regarding ATP purinoceptors, increased beta-cell apoptosis has been reported in P2X(7) knock-out mice [185]. Glucose is shown to induce ATP release in a mouse beta-cell line and ADP activation of P2Y(13) receptors to inhibit insulin release [186]. In rodent as well as human pancreatic beta-cells, extracellular ATP has been proposed as a paracrine signal amplifying glucose-induced insulin secretion via P2X(3) receptor activation [187]. Further, it has been reported that ATP activation of P2X(7) receptors in peritoneal mouse macrophages mediated free fatty acid release, substrate for many enzymes including cyclooxygenases that promote inflammation [188]. Environmental chemicals could possibly induce extracellular accumulation of ATP following Th2-type inflammatory responses, similar to what has been shown for airborne fungal allergens in naïve mice [189]. Activation of estrogen receptors ERα can cause enhancement of glucose-induced insulin biosynthesis, reduction in islet toxic lipid accumulation and promote beta-cell survival from pro-apoptotic stimuli, and activation of ERβ can increase glucose induced insulin secretion in both rodent and human beta-cells [190]. Activation of AhR can induce oxidative stress, DNA damage and inflammation [23]. Chemicals influencing the gap junctions between beta-cells could increase toxicity and susceptibility to cytokine induced apoptosis, as shown when downregulating connexin36 in INS1E-cells, suggested to be involved in Ca^{2+} homeostasis within the endoplasmatic reticulum ER [191].

BPA, PFAS, TCDD, streptomycin, alloxan, N-nitro-so compounds, streptozotocin, zinc, organotins, and bafilomycin are all shown to cause alterations in beta-cell function and structure and/or apoptosis in animal studies [44, 54, 59, 60, 62, 88, 102].

4.2. Immunomodulation. In addition to direct effects on beta-cell numbers and function and glucose-insulin balance, environmental chemicals may affect T1DM development by modulating the function of innate and adaptive immune cells. Recurring infections in early childhood could trigger the immune system and possibly boost autoimmunity, and enteroviral infections in early life are associated with T1DM development [192, 193]. As an example PFAS exposure *in utero* appears to modulate the immune response in children, resulting in reduced vaccine responses and increased infections in early childhood [55, 57]. The increased risk of

infections may indirectly give increased risk of enteroviral infections triggering T1DM development in children with auto antibody positivity. Other chemicals, such as for instance PAHs, are reported to reduce the numbers and function of regulatory T-cells [144, 194], cells that are important in the suppression of the autoreactive T-cells that are key players in the induction of autoimmunity. Corsini et al. [195] showed that several PFAS decrease LPS-induced cytokine secretion in human peripheral blood leucocytes and Brieger et al. [196] showed a direct increased cytotoxicity to human NK cells by PFAS (PFOA) exposure. Altered cytokine secretion, reduced regulatory T-cells or Th17 cells by environmental chemicals could be plausible explanations for a modified immune response and the development of autoimmunity. It has been shown that exposure not only to PFAS, but also to BPA, phthalates, arsenic, PCB, and air pollution can alter the cytokine balance in human and mice cells *in vitro* and a shift in cytokine balance is further associated with development of autoimmunity [59, 60, 195, 197–202].

Another suggested immunological process linking viral infections to T1DM onset is molecular mimicry [203]. There is a high degree of homology between human Glutamic Acid Decarboxylase GAD65, a pancreatic enzyme considered to be an important autoantigen involved in T1DM development, and a heat shock protein from the *Mycobacterium avium subspecies paratuberculosis*, MAP Hsp65, and it has been shown that T1DM patients can have antibodies against MAP Hsp65 [204]. It has been suggested that there is a cross-reactivity between MAP Hsp65 and GAD65, implying that biological mimicry potentially could be a mechanism of triggering TIDM. It has also been reported that antibodies from T1DM patients recognizing MAP3865c epitopes from the *Mycobacterium avium* could cross-react with ZnT8, another autoantigen in T1DM development [205], although this hypothesis has not been verified in epidemiological studies. Other environmental factors, including chemicals, can in principal change exogenous and endogenous proteins, leading to mimicry pathways of T1DM triggering.

Improper activation of the immune system may lead to allergy development or trigger autoimmunity, and exposure to PFAS, arsenic, BPA, phthalates, air pollution, ozone, nitric oxide, particulate matter, triclosan, PAHs, tobacco smoke, dioxin, and PCBs have all been reported to be associated with asthma and/or allergy in several epidemiological studies [42, 149, 150, 165, 206–215].

BPA and PDBEs have been shown to reduce cytokine secretion from macrophages, and BPA and arsenic seem to impair phagocytic activity in macrophages, possibly leading to a reduced clearance of apoptotic cells in pancreatic islets which can result in an induced insulitis in the NOD mouse [50, 91, 216].

4.3. Epigenetics. Epigenetic alterations, via histone modifications, DNA methylation and microRNA dysregulation leading to altered gene expression, represent one way in which chemicals can induce effects early in life that manifest disease later in life. Emerging data suggest that prenatal exposures, like for instance to arsenic, may induce epigenetic alterations, already measurable in umbilical cord blood [84, 217]. Prenatal exposure to phthalates and postnatal exposure to BPA have been suggested to work together in a "two-hit model" on hormonal alterations leading to epigenetic regulation of gene expression [149]. Phthalate exposure has been shown to induce DNA methylation of the estrogen receptor alpha in a breast cancer cell line [218]. Environmental factors such as pharmaceuticals, pesticides, air pollutants, industrial chemicals, heavy metals, hormones, nutrition, as well as behavior have been suggested to change gene expression with demonstrated changes in epigenetic markers [214, 218, 219]. Alterations in micro-RNA levels might influence beta-cell functions and overexpression of microRNA miR375 has been shown to be associated with suppressed glucose induced insulin secretion by reduced levels of PDK1 leading to reducing beta-cell viability and cell number [220]. IL-1α and TNFα induce miR21, miR34a, and miR146a in human and NOD mouse pancreatic islets and in the mouse MIN6 beta-cell line and are involved in cytokine-induced cell death [221]. The miR21 as well as miR34a reduces beta-cell apoptosis and protects against T1DM development [222, 223], while overexpression of miR29a/b/c was reported to promote beta-cell apoptosis [224].

4.4. Microbiota. The microbiota composition in the gut has been shown to be crucial for developing a healthy immune system in animals. The right composition is suggested to support oral tolerance and protect against enteral virus infections, and microbial colonization of *Bifidobacterium* has been shown to be lower in patients with T1DM [6, 225–230]. Transfer of microbiota from Myd88-/-NOD mice, which are protected from diabetes, has been shown to reduce insulitis and delay T1DM development in the normal diabetes prone NOD recipient [231]. Furthermore, alterations in microbiota composition results in altered hormone levels in the NOD mouse [232]. Nutritional and chemical constituents in our diet and drinking water have been shown to alter the microbiota composition in animals [233–235] and future studies are needed to clarify the importance of such interactions between environment, microbial flora and autoimmunity. On the other hand, probiotics could possibly interfere with T1DM development and examples hereof are animal studies with probiotics given to the T1DM prone NOD mouse showing protective effects against T1DM development via Th17 induction [236–239]. In an epidemiological context, the ongoing PRODIA study will elucidate if introduction to probiotics during the first 6 months of life decreases the appearance of T1DM-associated autoantibodies in children with genetic risk for T1DM [240].

4.5. Intestinal Permeability. Increased intestinal permeability is an early feature of diabetes before the onset of the disease in the Bio Breeding T1DM rat model, and blocking of the tight junction modulator zonulin has been shown to inhibit the disease in this model [241, 242]. Increased intestinal permeability has also been shown to be an early event in T1DM patients with upregulation of zonulin prior to the onset of the disease [229, 243–247].

Chemicals, like heavy metals and organochloride pesticides, can possibly affect intestinal permeability, as well as

impairing the osmoregulation and calcium transport [248, 249]. *Lactobacillus* has been shown to reduce the intestinal permeability via relocation of occludin and ZO-1 into the tight junction area between duodenal epithelial cells after short term administration to healthy volunteers [250] and this mechanism together with alterations in hormone levels could possibly explain a beneficial effect of probiotics in the NOD mouse model [236–239].

5. Summary

We have presented literature supporting a possible role of environmental chemicals to act as triggers or accelerators for T1DM development. Chemicals may have direct toxic effects on insulin producing beta-cells or have immune modulatory effects, alter hormone levels, affect the microbiota, or alter intestinal permeability. Chemical-induced epigenetic alterations leading to altered gene expression are probably involved, in particular in relation to *in utero* effects.

Whether the doses of environmental chemicals to which humans are exposed are sufficient to impact the risk of T1DM remains largely unexplored. Due to lack of strong evidence for a single factor as the major trigger for T1DM development it is tempting to propose that several factors have additive or synergistic effects, acting via several mechanisms and/or at different stages in the disease development. Human exposure to environmental chemicals is complex. While some chemicals may have beneficial effects, others may have detrimental effects in individuals with autoimmune predisposition, and the adverse consequences of this sum of exposures cannot be elucidated with the information available. Further observational T1DM cohort studies with determination of several biomarkers of chemical exposure in serum and urine, together with animal and cellular experiments using single and combined chemical exposures are encouraged.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 382165, 9 pages
http://dx.doi.org/10.1155/2015/382165

Review Article

Role of Nutritional Factors at the Early Life Stages in the Pathogenesis and Clinical Course of Type 1 Diabetes

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Received 26 June 2014; Revised 2 November 2014; Accepted 3 November 2014

Academic Editor: Kjersti S. Rønningen

Nutrition has been suggested as an important environmental factor other than viruses and chemicals in the pathogenesis of type 1 diabetes (T1D). Whereas various maternal dietary nutritional elements have been suggested and examined in T1D of both humans and experimental animals, the results largely remain controversial. In a series of studies using T1D model nonobese diabetic (NOD) mice, maternal dietary n-6/n-3 essential fatty acid ratio during pregnancy and lactation period, that is, early life stages of the offspring, has been shown to affect pathogenesis of insulitis and strongly prevent overt T1D of the offspring, which is consistent with its preventive effects on other allergic diseases.

1. Introduction

Type 1 diabetes (T1D) results from insulin deficiency mostly due to the autoimmune-mediated destruction of the insulin-producing pancreatic islet β cells (insulitis) and arises from incompletely understood interactions between β cells, the immune system, and the environment in genetically susceptible individuals [1–4] (Figure 1). Concordance of T1D between the monozygotic twin pairs has been reported as approximately 30%, and therefore significant involvement of environmental factors has been implicated in the pathogenesis and clinical course of T1D [5]. Possible mechanisms of environmental factors to destroy islet β cells are (1) direct attacking to kill β cells, (2) destroying β cells to present autoantigens and induce autoimmunity against β cells, (3) inducing autoimmunity by molecular homology between the environmental factor and β cell antigens, (4) perturbing the immune system to induce autoimmunity against β cells, and (5) combinations of the above mechanisms [6]. Environmental factors include viruses, chemicals, and nutrition elements. Fulminant T1D, which constitutes 15–20% of T1D cases with ketosis-onset or ketoacidosis-onset in Japan, is associated with symptoms of common cold such as fever and cough [7], and viral infection has been suggested as the causative factor. A number of viruses have been suggested as environmental factors related to T1D [8]. It has been reported that, in infant T1D patients, Th1 type immune response against coxackie B4 virus, that is, interferon γ production and *Tbet* gene expression, is decreased [9]. T-bet is a type-1 transcription factor that regulates the development of type-1 T cell and type-1 antitumor immunity. T-bet expression in dendritic cells is required for the ability of these antigen-presenting cells to prime type-1-polarized T cell responses. Further, RNA of enterovirus has been detected during the acute phase of T1D occurrence in some reports [10]. Whereas many candidate viruses have been detected as infected at the onset of T1D and existence of the virus have been repeatedly proved in the islets, they have not yet been evidenced as the genuine causes of T1D. Among chemicals, cyclosporin [11], an immunosuppressant, and atorvastatin [12], a hydroxymethylglutaryl-CoA reductase inhibitor, are known to be toxic to pancreatic islets and to induce diabetes. Streptozotocin, an alkylating anticancer agent, has been frequently used to cause diabetes in experimental animals [13–15].

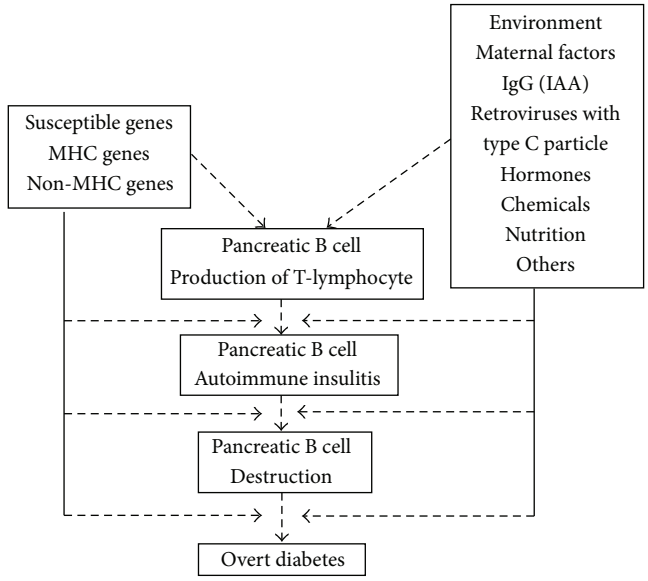

Figure 1: Mechanism on the development of type 1 diabetes (modified from [38, 63]).

A number of nutrients such as cow's milk protein, activated vitamin D, vitamin C, and polyunsaturated fatty acids have also been suggested as environmental factors that affect or are involved in the pathogenesis of T1D which is recently increasing in the incidence. In humans, breast feeding has been reported to reduce the incidence of T1D, while other reports did not find significant difference in the ratio of breast feeding between T1D children and control children [16]. Bovine serum albumin has been suggested as the environmental factor involved in the possible relation between cow's milk intake and T1D occurrence to induce autoimmunity against islet cells based on its molecular homology with pancreatic autoantigens [17]; however, the causative relation still remains controversial [16–19]. Vitamin C is expected to protect islet cells from cytotoxic effects of free radicals. [20]. Multiple chemical compounds such as oxidizers, antioxidants, and synthetic coloring agents are involved in foods, and they may be separately or in combination cytotoxic to islet cells or predispose islet cells to be seriously damaged by other factors [20]. In this review, we describe maternal factors, especially nutritional factors, that are suggested to affect development of insulitis and clinical course of T1D.

2. Maternal Factors during Early Life Stages in the Pathogenesis of T1D

"Maternal factors" have been suggested as environmental factors in the pathogenesis of T1D before and after birth of the offspring in T1D model nonobese diabetic (NOD) mice [21–23] (Figure 1). The inbred NOD mouse strain originated as a hyperglycaemic substrain of the CTS (cataract-prone mouse) at the Shionogi Laboratories, Fukushima-ku, Japan. At the time of weaning, NOD mice develop insulitis that progresses at approximately 100 days of age to invasive insulitis and complete β-cell destruction. Although NOD mice have an increased genetic susceptibility to T1D, the penetrance of disease can be modulated by various environmental factors. Hence not all NOD mice in a colony will develop T1D. A number of T1D-susceptibility genes identified in NOD mice (designated *Idd*) have been found to contribute to T1D susceptibility in humans (designated *IDDM*) [24]. Among "maternal factors" are vertical viral infection from mother to offspring and nutrients via placenta and milk, as well as hormones, and insulin autoantibodies which are transmitted from mothers to offspring [21, 25]. Regarding viral infection, viral particles have been observed in islet cells and suggested to be related with inflammation by autoimmune response, and further involvement of hormones from mother has been suggested in the viral induction and associated progression of insulitis [22, 23, 26, 27]. Insulin autoantibody (IAA) is a potent predictive marker of T1D and has been frequently examined in large scale studies and reported to become positive early after birth both in humans and mice [21, 25]. Greeley et al. [21] reported that IAA can be transmitted from IAA-positive pregnant female T1D model NOD mice to the offspring to cause T1D. However, we and other groups have obtained contradictory results and thus maternal IAA transmission to the offspring and its involvement in the pathogenesis of T1D still remain controversial [21–23, 25].

Regarding effects of nutrition via mother, relation between breast feeding or cow's milk protein intake during lactation period and incidence of T1D has been suggested both in humans and experimental animals [28, 29]. Type 1 Diabetes Prediction and Prevention Project (DIPP) performed HLA typing using the cord blood of newborn babies and examined autoantibodies in the high-risk babies of T1D [29]. They reported that early exposure to formula tended to increase both the ratio of positive autoantibodies and incidence of T1D [29]. Relation between fish oil and T1D has been examined in large scale studies; however, it still

remains controversial. While vitamin D, or the n-3 fatty acids, that is, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been suggested to be related to IAA level and to decrease incidence of T1D, results appear to vary depending on the period and amount of nutrients [30–35]. In addition to "exogenous" maternal factors such as nutrition in the maternal diet, "endogenous" maternal factors based on the genetic constitution have to be considered in the pathogenesis of T1D in the offspring. We previously showed using mutual transfer of embryos into uterus between NOD and two other genetically different nondiabetic mice that "maternal factors" as a whole strongly affect the onsets and ratios of insulitis and overt diabetes [22]. Since NOD and the other two strain mice were maintained and used in the study under the same conditions including foods and water [22], genome-based maternal factors seem to play important role in the observed difference in the onsets and ratios of insulitis and overt diabetes.

3. Nutrition as Environmental Factors in the Early Life Stages

Nutrients in foods are essential to our life through all the life stages. However, required amounts and kinds of nutrients vary depending on each life stage from prenatal to old age periods and may also be different to effectively prevent diseases including T1D depending on the life stages as suggested in reports [36–39]. Several dietary manipulations have been tested as interventions, including infant formulas free of either cow's milk or of bovine insulin, infant formula supplemented with DHA, delayed introduction of gluten-containing foods, and vitamin D supplementation. Secondary prevention studies have been conducted in both children and adults with diabetes autoantibodies. Interventions tested include nicotinamide, insulin injections, oral insulin, nasal insulin, glutamic acid decarboxylase, and cyclosporine [39].

Peppa et al. [36] reported that T1D incidence in NOD mice was evidently increased when advanced glycation end-products (AGEs) were administered during the prenatal and newborn period. Fu et al. [37] showed that epigallocatechin when administered after weaning decreased the occurrence of T1D in NOD mice. We also previously showed that n-6/n-3 ratio of unsaturated fatty acids in the maternal diet during gestation and lactation rather than that of offspring after weaning significantly modified incidence of overt diabetes in NOD mice [38].

4. Effects of Dietary Essential Fatty Acid on the Inflammation and Immune Response

Since mammals require but cannot synthesize fatty acids with double bonds distal to the ninth carbon atom, long chain polyunsaturated fatty acids are essential to their diet [40]. Linoleic acid (18 : 2 n-6) is a major essential fatty acid found in oils derived from plant seeds such as corn or safflower. Linoleic acid can be elongated and desaturated to yield arachidonic acid (20 : 4 n-6; AA). The action of Δ15-desaturase in plants converts linoleic acid to α-linolenic acid (18 : 3 n-3) which can be elongated to EPA (20 : 5 n-3) and DHA (22 : 6 n-3). These latter conversions to EPA and DHA occur slowly in mammals but are carried out readily by marine algae. Transfer of EPA and DHA from these algae through the food chain to fish makes fish oil the primary source of highly unsaturated n-3 polyunsaturated fatty acids in the human diet as well as dietary supplements [41].

The capacity of n-3 essential fatty acid to modulate immune function and suppress inflammatory responses has been reviewed extensively [42–45]. N-3 essential fatty acids suppress proinflammatory cytokine production, lymphocyte proliferation, cytotoxic T cell activity, natural killer cell activity, macrophage-mediated cytotoxicity, neutrophil/monocyte chemotaxis, MHCII expression, and antigen presentation. Evidence that these cellular effects indeed impact immune function *in vivo* is reflected in n-3 essential fatty acid attenuation of mediator production, leukocyte homing, delayed-type hypersensitivity, allograft rejection, and acute inflammatory responses in experimental animals in which human inflammation and autoimmune diseases are modeled. N-3 essential fatty acids appear to mediate these pleiotropic effects via both eicosanoid-dependent and eicosanoid-independent pathways.

Animal studies have demonstrated the potential for n-3 essential fatty acid to suppress onset (e.g., aberrant Ig production and glomerular deposition) and progression (e.g., inflammation, glomerular injury, and proteinuria) of disease in several animal models of autoimmune glomerulonephritis. However, major gaps still exist in our understanding of the precise molecular mechanisms of the effects of n-3 fatty acids as well as identities of their cellular targets [46].

Maternal intake of fatty acids during pregnancy was associated with childhood asthma. Maternal α-linolenic acid, total n-3 essential fatty acid, and palmitic acid intake may decrease, while arachidonic acid intake may increase the risk of asthma in the offspring [47].

An observational study suggested that dietary intake of n-3 fatty acids is associated with reduced risk of islet autoimmunity in children at increased genetic risk for type 1 diabetes [28]. The Trial Net Nutritional Intervention to Prevent (NIP) type 1 Diabetes Pilot Trial is assessing the feasibility of implementing a full-scale study to determine if nutritional supplements with DHA during the last trimester of pregnancy and the first few years of life will prevent the development of islet cell autoimmunity in children at high risk for type 1 diabetes [48].

The data from the Finnish type 1 Diabetes Prediction and Prevention Nutrition Study suggest that maternal consumption of butter, the ratio of n-6 : n-3 fatty acid, and intake of polyunsaturated fatty acid and α-linolenic acid during pregnancy may be potential determinants of allergic rhinitis in the offspring [49].

In the next section, we review the relation between maternal factors, especially maternal and offspring dietary n-6/n-3 ratio of unsaturated fatty acids, and pathogenesis and clinical course of T1D in NOD mouse offspring.

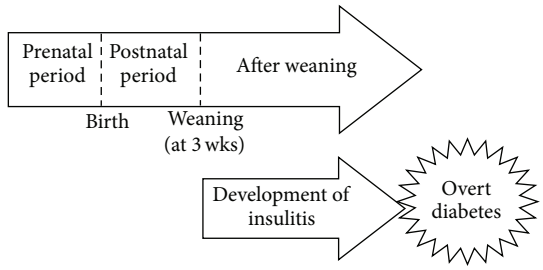

Figure 2: Life stages of NOD mice Type 1 diabetes results from insulin deficiency, mostly due to the autoimmune-mediated destruction of the insulin-producing pancreatic islet β cells (insulitis) (modified from [64]).

5. Effects of Maternal Dietary Essential Fatty Acid Ratio on the Pathogenesis and Clinical Course of T1D of Offspring

Nutrients including essential fatty acids are not only transmitted to offspring via mother but also may affect maternal immune and endocrine systems and thus may affect cytokines, antibodies, and hormones that are transmitted to the offspring [39–41]. n-6/n-3 essential fatty acid ratio in diet has been reported to increase in Europe and USA, and the average in USA is reported from 12 to 15 [50]. While n-6/n-3 ratio remains from 2 to 3 among Japanese who frequently eat fish, it is increasing to 8 or 9 among young Japanese whose diet is being westernized [50]. The increase in n-6/n-3 ratio has been suspected as being involved in the recent increase in allergic, vascular, and immunologic diseases [51]. Experimental animal studies showed that fat intake even before pregnancy affects its involvement in the fetal tissue and that n-6/n-3 ratio plays important roles in histogenesis, growth, and development of immune system during fetal and neonatal periods [52, 53].

NOD female mice mostly start to show insulitis after weaning, and 100% of individuals have insulitis by 12 weeks of age (Figure 2). Among chemicals and nutrients studied using NOD are cyclosporin and streptozotocin whose effects on the development of insulitis were examined after 8 weeks of age, whereas barley protein's effects on the induction and suppression of insulitis differ depending on the timing of exposure [54]. We have performed series of studies to investigate the effect of maternal or postweaning offspring's nutrition in particular n-6/n-3 ratio on the development of insulitis and T1D [38, 55, 56]. In these studies, NOD mothers were fed with the randomly assigned chow for the gestation period for longer than 4 weeks before being mated and becoming pregnant. We prepared two kinds of chows with different n-6/n-3 ratios, "low-n-3" chow (L: n-6/n-3 = 14.5) or "n-3" chow (n: n-6/n-3 = 3.0), and provided them to NOD female mice (1) four weeks before pregnancy through gestation, (2) during lactation after birth of their offspring, and (3) to the offspring after weaning until the end of the experiment [38, 55, 56]. We showed that different n-6/n-3 ratios modified the incidences of insulitis and overt diabetes and suggested that DHA and EPA have to be included in the diet to cause the effect [38, 55, 56].

We further used L chow that induced the highest incidence of overt diabetes and n chow that prevented overt diabetes to the lowest incidence to examine in detail the effects of different n-6/n-3 ratios in nutrition on T1D depending on the different life stages (prenatal, lactation, and postweaning periods) [38]. In the n-chow-fed offspring from n-chow-fed mother (nnn), levels of insulitis were higher than those in the L-chow-fed offspring from L-chow-fed mother (LLL) at 4 weeks of age, while the levels in the nnn offspring became lower than those in the LLL after 6 weeks (Figure 3(a)). Early IAA expressions were observed from 2 to 6 weeks in the LLL offspring but not in the nnn (Figure 3(b)). The nnn offspring (Figure 4(a)) exhibited strong suppression of overt diabetes development in regard to the onset and accumulated incidence of diabetes compared to the LLL (Figure 4(e)). The study with different combination of n and L chows during gestation and lactation in mother and in postweaning offspring revealed that the only nnL-chow in which n chow was provided through prenatal to lactation periods (Figure 4(b)) significantly suppressed the development of diabetes with similar kinetics to nnn-chow, whereas the other combinations may delay the onset of diabetes (Figures 4(f), 4(c), 4(d), 4(g), and 4(h)). These results indicated that n-6/n-3 ratio in the maternal diet during pregnancy and lactation period affects the development of insulitis and overall incidence of overt diabetes [38] and thus strongly suggest that diets with appropriate n-6/n-3 ratios should be provided during the appropriate life stages to prevent T1D.

T helper type 17(Th17) cells have been shown to play important roles in mouse models of several autoimmune diseases that had previously been thought to be Th1-dominant. In the NOD mouse, however, the relevance of IL-17/Th17 is still controversial, because of their inherent plasticity. Th17 cells derived from BDC2.5 NOD mice transfer diabetes through conversion to Th1 cells *in vivo* [57]. Allen et al. reported that n-3 essential fatty acids reduce CD4(+) T-cell activation and differentiation into pathogenic Th17 cells by 25–30% [58]. Their data suggested that n-3 essential fatty acids suppress Th17 cell differentiation in part by reducing membrane raft-dependent responsiveness to IL-6, an essential polarizing cytokine [58]. Kuriya et al. studied the impact of IL-17 single-deficiency or IL-17/IFN-γ receptor double-deficiency on the development of insulitis/diabetes in NOD mice [57]. IL-17 single-deficiency significantly delayed

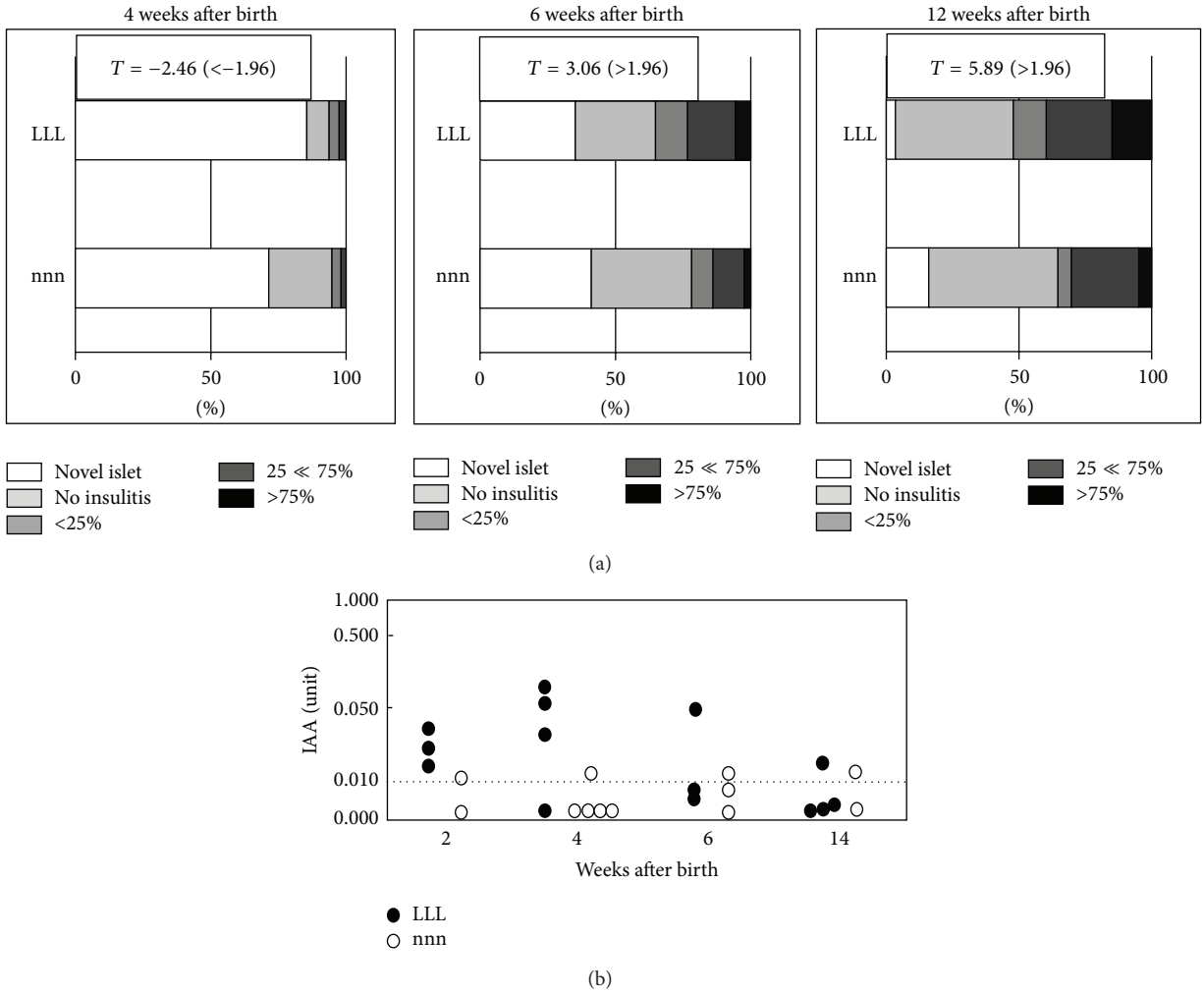

Figure 3: Levels of insulitis (a) and serum insulin autoantibody (IAA) levels (b) in the offspring at different ages (modified from [38]).

the onset of diabetes and attenuated the severity of insulitis, but the cumulative incidence of diabetes until 50 weeks of age in IL-17 deficient mice was similar to that in wild-type (wt) mice. Adoptive transfer study with CD4+CD25−T cells from young nondiabetic IL-17 single-deficient NOD mice, but not that from older mice, showed also significantly delayed disease in the recipient NOD-Scid mice as compared with those from the corresponding wt mice. On the other hand, IL-17/IFN-γ receptor double-deficiency significantly suppressed the development of diabetes, although the levels of insulitis were similar between single-deficient and double-deficient mice. Kuriya et al. reported that IL-17/Th17 plays a significant role in the development of insulitis in prediabetic NOD mice and the interaction of Th1-Th17 cells contributes to diabetes development [57].

Taken together with these reports, data from our NOD mouse studies suggest that low n-6/n-3 ratio diet (a higher ratio of n-3 essential fatty acid) fed during prenatal period and postnatal period before weaning affected Th17 cell differentiation and interaction between Th1 and Th17 cells and eventually prevented development of overt diabetes. Delayed onset of overt diabetes in the groups fed with low n-6/n-3 ratio diet after weaning may also be related with a similar mechanism. We have no clear explanation at present for the earlier onset of insulitis albeit the eventual prevention of overt TID in the nnn group. Norris et al. showed based on the Diabetes Autoimmunity Study in the Young (DAISY) cohort that low erythrocyte membrane docosapentaenoic acid (DPA) levels are associates with an increased risk of developing IAA [59]. Fatty acid desaturase activity varies depending on the life stages and may complicate the relation between n-3 essential fatty acids and development of T1D. Data from our studies also suggest that appropriate dietary n-6/n-3 ratio may differ at each life stage. During the early life stages, that is, prenatal and early postnatal periods, fatty acid desaturase activity is low, and therefore dietary requirement of n-3 essential fatty acids such as DHA and EPA may be higher to prevent development of autoimmune responses.

6. Maternal Prepregnant Environment as Another Life Stage for Offspring

Maternal diet and nutrition are expected to have different influences on the offspring depending on the different life

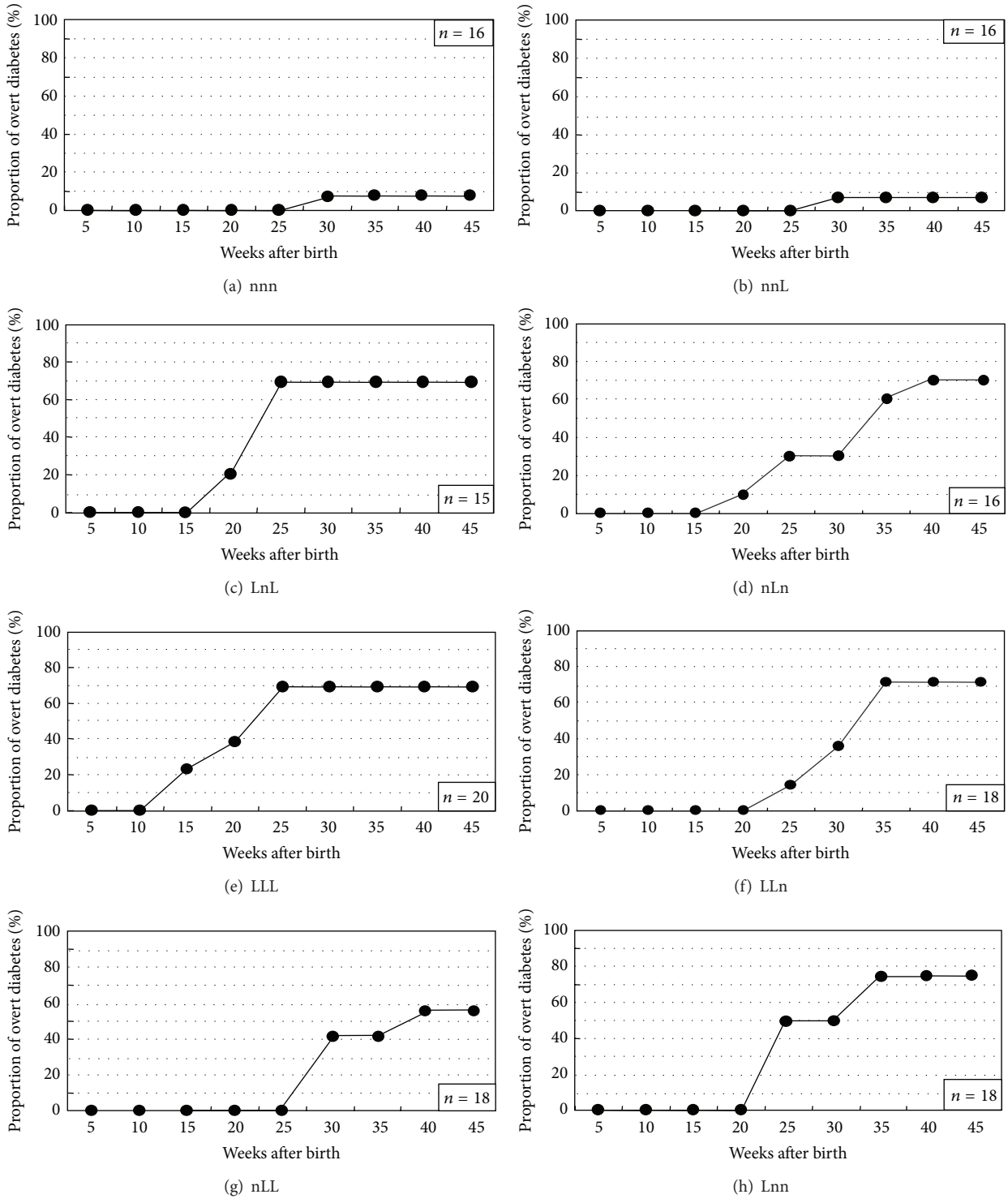

Figure 4: Effects of combination of n-3(n) and low n-3(L) chows on the onset and incidence of overt diabetes in NOD mice [38, 63].

stages for the offspring. Amount and quality of maternal nutrition before pregnancy should have a close relation with maternal endocrine and immune conditions, albeit different from that after pregnancy. Our preliminary study showed that changes in n-6/n-3 ratio in the maternal diet before and after pregnancy affected the incidence and onset time of T1D [60].

As Developmental Origins of Health and Disease (DOHaD) hypothesis provoked by Barker and Osmond [61] and Silveira et al. [62] has been extensively examined, the effects of human maternal environment for the development of disease in different life stages are increasingly reported. Maternal environment including neural, endocrine, and immune systems

during pregnancy is based on that before the pregnancy, maternal environment even before the pregnancy has to be considered as another important life stage, in addition to maternal environment by direct relation between mother and offspring via placenta and milk, for the health and diseases of the offspring.

7. Conclusion

Among maternal factors, dietary n-6/n-3 EFA ratio during pregnancy and lactation period may affect pathogenesis of insulitis and clinical course of T1D in the offspring.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 321656, 10 pages
http://dx.doi.org/10.1155/2015/321656

Review Article

Environmental Trigger(s) of Type 1 Diabetes: Why So Difficult to Identify?

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Received 22 August 2014; Revised 15 October 2014; Accepted 16 October 2014

Academic Editor: Jill M. Norris

Type 1 diabetes (T1D) is one of the most common chronic diseases with childhood onset, and the disease has increased two- to fivefold over the past half century by as yet unknown means. T1D occurs when the body's immune system turns against itself so that, in a very specific and targeted way, it destroys the pancreatic β-cells. T1D results from poorly defined interactions between susceptibility genes and environmental determinants. In contrast to the rapid progress in finding T1D genes, identification and confirmation of environmental determinants remain a formidable challenge. This review article will focus on factors which have to be evaluated and decision to take before starting a new prospective cohort study. Considering all the large ongoing prospective studies, new and more conclusive data than that obtained so far should instead come from international collaboration on the ongoing cohort studies.

1. Introduction

Type 1 diabetes (T1D) is one of the most common chronic diseases with childhood onset, and the disease has increased two- to fivefold over the past half century by as yet unknown means [1, 2]. It was interpreted that if present trend continues, the prevalence of children with the disease in Europe will increase with 50% within year 2020 and with 70% for those less than 5 years of age [3]. Most recently Europe was compared for the periods 1989–1998 and 1999–2008, and it was shown that the increase in T1D is not necessary uniform, showing periods of less rapid and more rapid increase in incidence for different countries [4]. This pattern of change suggests that important risk exposure differs over time in different European countries.

T1D occurs when the body's immune system turns against itself so that, in a very specific and targeted way, it destroys the pancreatic islet β-cells, the only cells in the body that produce the vital hormone insulin [5, 6]. This autoimmune destruction is irreversible and the disease is incurable. If pancreas or islets are transplanted they too are destroyed, unless heavy immunosuppression is applied [7].

T1D results from poorly defined interactions between susceptibility genes and environmental determinants. T1D susceptibility is primary defined by genetic factors within the human leukocyte antigen (HLA) on chromosome 6. The main disease factors are the HLA-DQ molecule encoded by DQA1 and DQB1 genes and the HLA-DR molecule defined by DRB1 alleles [8–12]. In addition, recent genome-wide association studies have identified more than 40 other intervals that may harbour T1D susceptibility genes [13].

In contrast to the rapid progress in finding T1D genes, identification and confirmation of environmental determinants remain a formidable challenge [14]. The reason underlying the lack of progress is multifaceted. First, different categories and large numbers of environmental determinants could contribute to the triggering or protection of T1D [15–38]. Although many candidates have been suggested in the past, few have been definitively proven beyond reasonable doubt. Second, exposures may occur any time before the onset of disease, from in utero to T1D onset [39–43]. Third, environmental determinants may differ in different populations, partly depending on the genetic architecture. Fourth, the individual risk of developing T1D in the general population is not very high and quite variable in different populations. Therefore, large study populations with elevated T1D risk must be identified.

Table 1: HLA typing and time points for follow-up with blood samples in MIDIA.

(a) Eligible HLA type	DRB1*03-DQA1*05-DQB1*02/DRB1*0401-DQA1*03-DQB1*0302
(b) Time points for blood samples	3, 6, 9, and 12 months, thereafter annually If positivity for one autoantibody, a new blood sample after 6 months. If positivity for two or three autoantibodies, a new samples after 3 months
(c) Questionnaires	Collected at 3, 6, 9, and 12 months and thereafter annually
(d) Stool samples	Collected each month from 3 months to 3 years of age

Islet autoantibodies precede the development of T1D and can appear throughout childhood [44]. In prospective studies of offspring of parents with T1D, a peak incidence of islet autoantibodies appearance at around 1 year of age has been observed, followed by a decline through 2–5 years and a subsequent rise in incidence towards puberty [45]. Children with increased HLA-associated risk for T1D followed up from the general population get positive for autoantibodies at all ages. Early seroconversion occurs in children who progress fast to T1D [46]. It has been observed that, in children who progress to T1D during a follow-up of 13 years, 64% became autoantibody positive before the age of 2 years and 82% before the age of 3 years [46]. Early antibody positivity frequently starts with insulin autoantibodies (IAA) followed by glutamic acid decarboxylase antibodies (GADA). Usually insulinoma-associated protein 2 (IA-2) and zinc transporter 8 (ZnT8) antibodies develop closer to onset of T1D [45].

Identification of environmental determinants requires frequent follow-up for autoantibody testing and a large number of individuals from early in life until disease onset for studying a variety of exposures using epidemiological and laboratory methodologies. To accomplish such ambitious goals, long-term prospective studies on cohorts of children at increased risk of developing the disease are necessary. To design the study as good as possible is of most importance to achieve the identification of important environmental factor before recruitment starts. The MIDIA study will be used for the discussion of my own and my group experiences.

2. The MIDIA Study

2.1. Which HLA Class II Genes to Type for? Based on the hypothesis that it should be most easy to identify the environmental trigger(s) among children with the highest genetic risk and that this limited number of children for follow-up would make it possible to recruit all over Norway with a centralized laboratory and working staff in Oslo, the decision for how to recruit and run the MIDIA (Norwegian abbreviation for Environmental trigger(s) of type 1 diabetes) study was taken. It is approximately 60,000 births in Norway per year, and only 2.2% of all babies carry the highest genetic risk for T1D (e.g., the HLA-DR3-DQ2/DR4-8 (DRB1*03-DQA1*05-DQB1*02/DRB1*04:01-DQA1*03-DQBI*03:02/03:04) genotype).

2.2. Follow-Up in MIDIA. The aim of MIDIA was to genotype 100,000 babies to achieve 2000 children for follow-up for 15 years. Such babies have 7% risk for getting T1D before 15 years of age and a life long risk at 20%, compared to 0.4% risk before 15 years of age and lifelong at 1% for newborns of the general population. A special MIDIA computer program was made before the start of recruitment and covered recruitment (reports were made and sent out twice per year for the status of the 638 communities and 19 counties in Norway); and a certain incoming sample had a well-defined place in the laboratory tracking system. In addition the MIDIA program could analyze real-time PCR results and conclude for a certain HLA gene. One list showed letters to parents not having a child at the highest risk for T1D, and another list showed other parents that should be called because the genotyping had shown that they had a child with the highest genetic risk. Such children did also come on lists for getting a second call (how the families were dealing with the high-risk information, and if they had more questions). The program was and is still responsible for follow-up of high-risk children. The time point for sending out all follow-up packages, the content for a certain package, and the time for sending it out are told. The program also follows data for autoantistoff positivity, and with positivity for one autoantibody it is told by the program to send out a package 6 months after the incoming result and for two or three autoantibodies to send out a new package for blood drawing after 3 months; see Table 1.

With the aim of performing HLA class II typing to identify the highest risk genotype—DQB1*04:01-DQB1*03:02/04/DR3-DQA1*05-DQB1*02—as easy and robust as possible, it was decided to use a four-step strategy [47]. Albumin with monomorphic primers was used in the polymerase chain reaction (PCR) to show if a sample contained enough DNA to be genotyped successfully. Sequence-specific reactions for all samples were performed for DQA1*03 and -*05. When both DQA1*05 and DQA1*03 were identified for a certain sample, the next step was DQB1 sequence-specific typing. The identification of DQB1*03:02/04 and DQB1*02 brought the sample to the third step in the typing protocol. DRB1*4 subtyping was performed for all samples positive for the DQA1*03-DQB1*03:02/04 haplotype. All different DR4 subtypes were positively identified, but only the DRB1*04:01 gene is in Norwegian conferring the highest risk [11, 12]. All samples found positive for the highest risk genotype (DRB1*04:01-DQA1*03-DQB1*03:02/4/DRB1*03-DQA1*05-DQB1*02) went to a confirmatory step (e.g., step 4) through all 3 steps in the typing protocol once more together with the most recent incoming DNA samples for typing. This strategy of confirmation detected only two errors during the genotyping in MIDIA (2001–2007). The parents of the babies

where this happened had already received a nonrisk letter, since such letters were sent out daily for samples not fulfilling the demands to go further in the MIDIA genotyping. Luckily both two parent pairs understood the explanation given to them, and they joined the follow-up for high-risk children in MIDIA.

2.3. Inclusion Criteria. Mothers of preterm babies as well as those who had got a child with malformations did not receive invitation to MIDIA participation. For being eligible for participation at least one of the parents needed to be of Caucasian origin. Asian and African people do not carry the DRB1*0401-DQB1*03-DQB1*03:02/4 haplotype [9, 48]. Responsible for recruitment, and in most cases for taken a buccal sample of the baby, was a public health care nurse that in advance had got updated knowledge on T1D and learned how to inform about genetic risk for T1D by the Principal Investigator (PI) of the study and a study coordinator (a public health care nurse working closely together with the PI of MIDIA at the Norwegian Institute of Public Health).

2.4. All over Norway. All contact with the participating families had to take place by phone calls since there was never enough funding to bring participating families to the Norwegian Institute of Public Health. The distance from Kirkenes in the North-Eastern Norway is the same as the distance from Oslo to Rome, Italy. From the beginning of the study, there were less than 5% of parents informed about genetic high-risk for their baby that did not want to take part in the follow-up. Stool samples were asked for from the baby for 3–35 months of age and blood samples at 3, 6, and 9 and 12 months, and thereafter annually. Questionnaires were asked for at the same time point as blood samples. Until 2006 we got 94% of all stool samples, 83% of all questionnaires, and 86% of all blood samples that we asked for [49].

2.5. Stopping of an Ongoing T1D Study Based on the Norwegian Biotechnology Law. The MIDIA study had the needed approvals for research studies in Norway (from the Regional Ethic Committee and the Data Inspectorate) before recruitment started in the summer of 2001. Since all recruitment was based on special teaching of Norwegian public health care nurses given by the PI and a study coordinator (a pubic health care nurse working together with the PI), the recruitment started in small scale. Most of the public health care nurses in Norway started after they had got the needed information and education to voluntary recruit to MIDIA as well as being responsible for most of the blood samples taken. From 2006 the recruitment covered the whole country. In June 2007, one of the mothers of a participating baby was, however, interviewed in the biggest newspaper in Norway. She here complained about not having received good enough information about MIDIA before she and her husband had consented to participate [50, 51]. The Directorate for Health and Social Affairs then immediately decided that recruitment to MIDIA had to be stopped. Some days later it was, however, decided that new evaluation of the project had to take place according to the Norwegian Biotechnology Law, which tells that genotyping of children under the age of 18 years can only take place if there are a clear health benefit for a certain disease. During the fall of 2007, the Biotechnology Board, the Ethical Committee for the Norwegian Medical Association, the National Committee for Medical Ethics, and several experts contacted by the Directorate of Social and Health Affairs evaluated the MIDIA project. All these boards had earlier evaluated the MIDIA study (e.g., during the time of recruitment to the study). In addition, the Health Department had clearly told that children who also had developed autoantibodies in MIDIA could get health insurance. The last aspect was based on the Biotechnology Law, which Norway has had since 1994, where it is clearly told that genetic risk for a disease cannot be used by the health insurance companies. The Directorate of Social and Health Affairs found, however, genotyping in MIDIA illegal on December 10, 2007. A few days later, the Norwegian Data Inspectorate said in newspapers that all data already collected from participants in MIDIA had to be thrown away. All ended good by the Norwegian Parliament voting in June 2008. As long as the Medical Regional Committee and the Norwegian Data Inspectorate approved the MIDIA study once more, and all parents of children who already had been identified as high-risk children gave a new informed consent, research in MIDIA could continue. Close to 47,000 babies had been genotyped before December 10, 2007, and 1,047 had been identified with high-risk genotype. The parents of 706 children gave new informed consent starting from the fall of 2008 until early in 2009, Figure 1.

Norway is different from Sweden, Finland, Germany and five states in USA were no similar Biotechnology Law has given problems with genotyping of 420,000 children for The Environmental Determinants of Diabetes in the Young (TEDDY) study.

2.6. Ethics and Data Protection in Human Biomarker Studies. The Norwegian Biotechnology Law states the following: "genetic testing of a child under the age of 18 years is not allowed if circumstances cannot be detected that can reduce or prevent health disadvantages for the child." Since the law came in 1994, it had only counted for clinical practice, and the MIDIA project had been run for 6.5 year before the study was stopped. Both Approval from the Regional Ethic Committee and the Norwegian Data Inspectorate were given before recruitment to the study started. The reason for a new understanding of the Biotechnology law started in the biggest newspaper in Norway. The public debate got the Notional Ethic Committe, The Etic Commitee of the Norwegian Association for Physicans, the Norwegian Biotechnology Committee and The Norwegian Directorate of Helath to evaluate the MIDIA project once more. When the Directorate of Health (directly under the Department of Health, The Norwegian Government) stopped the MIDIA project, important questions did come up.

(1) Do important scientific T1D projects involving genotyping of children have to be performed elsewhere in the world? Should not Norway as one of the riches countries in the world have a certain responsibility?

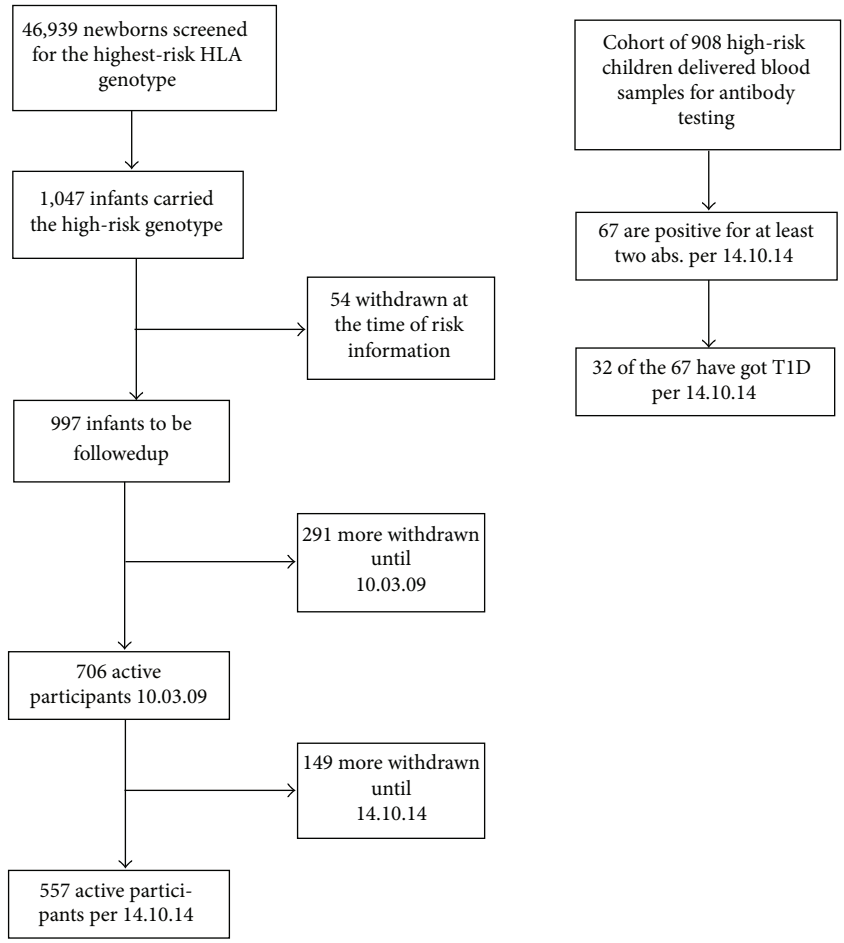

Figure 1: The first box to the left shows total number of children genotyped for the high-risk genotype in MIDIA. Of the 997 children who had parents participating in MIDIA, 908 delivered blood samples more than once for autoantibody testing. The rest of participants delivered stool samples and questionnaires. 706 children had parents who had given a new informed consent in 2008/2009. Abs.: autoantibodies.

(2) Are not the parents able to give informed consent on behalf of their child?

(3) How should health benefit be defined?

(4) Is it not so that if clear health benefit has been shown, it is no longer research but part of general recommendation for public health or part of the health care system?

3. Results from MIDIA

3.1. Viral Infections

3.1.1. Enterovirus. With the aim to test whether the frequency of human enterovirus RNA in faecal samples collected monthly from early infancy was associated with development of multiple islet autoantibodies in children with the highest risk HLA genotype faecal samples from 911 children that were used, 27 had developed positivity for two or more islet autoantibodies in two or more consecutive samples (case subjects) [52]. Two control subjects per case subject were matched by follow-up time, date of birth, and county of residence. The frequency of human enterovirus RNA in stool samples from case subjects before seroconversion (12.7%) did not differ from the frequency in control subjects (13.6%). There was no support for the hypothesis that faecal shedding of enteroviral RNA is a major predictor of advanced islet autoimmunity [53].

Since no association was found between children carrying the high-risk genotype and enterovirus, our aim was to assess whether genetic polymorphisms could play a role. There was no statistically significant association between other T1D associated HLA genotypes and the occurrence of human enterovirus gut infections [54]. Polymorphisms in the IFIH1 (common rs1990760 and four rare rs35667974, rs35337543, rs35744605, and rs35732034) have been convincingly associated with T1D. We therefore investigated whether the polymorphisms are associated with differences in the frequency of enterovirus RNA in blood. The genotypes of IFIH1 rs1990760 were associated with different frequencies of enterovirus RNA in blood (7.0%, 14.4%, and 9.5% bloods were enterovirus positive among children carrying the Ala/Ala, Ala/Thr, and Thr/Thr genotypes, respectively, $P = 0.012$) [55]. The common IFIH1 SNP may modify the frequency of

enterovirus RNA in blood of healthy children. This effect can help explain the association of IFIH1 with T1D [56].

Since an association between T1D and enterovirus so far only had been found in Finland, we investigated enterovirus RNA in blood and islet autoimmunity. We analyzed serial blood samples collected at age of 3, 6, and 9 months and then annually from 45 children who developed confirmed positivity for at least two autoantibodies (insulin, GAD65, and/or IA-2) and 92 matched controls in the Norwegian MIDIA study. Of 807 blood samples, 72 (8.9%) were positive for enterovirus. Positivity for enterovirus RNA in blood did not predict the later induction of islet autoantibodies, but enterovirus tended to be detected more often at the islet autoantibody conversion state [57]. There was no support for the hypothesis that faecal shedding of enteroviral RNA is a major predictor of advanced islet autoimmunity.

3.1.2. Parechovirus. The objective of this study was to investigate a possible association between human parechovirus infections in early infancy, diagnosed in faecal samples, and the development of islet autoimmunity in the MIDIA study [58]. A nested case-control study, including 27 children who developed islet autoimmunity (repeatedly positive for two or three autoantibodies) and 53 children matched for age and community of residence, was used. Monthly stool samples from these children were analyzed for human parechovirus. There was no significant difference in the prevalence of human parechovirus in stool samples when cases and controls were compared: 13.0 and 11.1%, respectively [59]. There was not also any difference in the number of infection episodes. In analyses restricted to samples collected 3, 6, or 12 months prior to seroconversion for islet autoantibodies, there was a suggestive association in the shortest time window of 3 months (20.8 versus 8.8%, odds ratio (OR) = 3.2, 95% CI 1.2–8.5, uncorrected $P = 0.022$). Neither was there found any Ljungan virus in the large dataset studied [60].

3.1.3. Saffold Virus. We could not detect any significant associations between Saffold virus and development of islet autoimmunity (estimated OR = 2.06 (0.59–7.20)). SAFV virus genotypes 2 and 3 seem to be dominant. However, only 2.6% of samples were positive for Saffold virus, indicating that this virus is rarely present in stool (Tapia and Bøås, unpublished data).

3.2. Dietary Factors and T1D Risk. Breastfeeding protected against enterovirus [61], and breastfeeding for a period for more than 12 months delayed disease progression from autoimmunity to clinical T1D [62]. No differences were found in the MIDIA study for the time point of introducing solid food—but it seemed important that the mother still was breast feeding.

In the MIDIA study, a cohort design was used for assessing whether body mass index (BMI) before pregnancy and weight gain during pregnancy predicted the risk of islet autoimmunity in 885 children. 36 of the children developed autoimmunity, of who 10 developed T1D. Both maternal body mass index (BMI) before pregnancy and weight gain > or = 15 kg predicted increased risk for islet autoimmunity with significant hazard ratio (HR) at 2.5 for both situations [63].

3.3. Wheezing in Early Infancy. When a cohort of 42 cases and 843 noncases in MIDIA was studied, self-reported "pneumonia, bronchitis, or RS-virus" had HR at 3.5, $P = 0.001$, for development of autoimmunity before 4 years of age [64]. Also a Swedish study with data collected at the age of 2.5 years found that wheezing during the first year of life was significantly associated with islet autoimmunity [65].

3.4. Enterobius vermicularis. Enterobius vermicularis still seems to be common during childhood. However, pinworm infections seem to be uncommon in children younger than 2 years and have the highest prevalence in children older than 5 years of age (34%). Increased number of siblings was linked to more infections, and there were fewer infections in the children with the high-risk genotype [66]. A possible association between current pinworm infections and food allergy was found (OR = 2.9 (1.1–8.0)) and needs to be studied in a larger material [67].

3.5. The Diversity of the Data Obtained in the Different National Cohort Studies. As appearing from what is found above, different factors have been reported to confer T1D susceptibility in the different national prospective cohorts, leaving a number of holes and a troubling lack of consistency in the findings to date. It is likely that the results have been confounded by imprecise assessment of dietary exposure, recall bias, failure to assess dietary exposures at very early ages, different definitions of exposure, and small sample sizes. To solve these issues was the background for the large collaborative study TEDDY [68]. 420,000 newborns were screened for human leukocyte- (HLA-) conferred genetic risk for T1D; 21,589 were HLA eligible, and 8,668 joined the TEDDY study (40% participation rate). As of September 2014, 2613 families have withdrawn (28% participation rate of the eligible) [69, Personal communication, the TEDDY group]. Although so much efforts and funding have been given to the TEDDY study, probably also here that many reports will show too little power to conclude for a specific environmental factor.

3.6. A New Collaborative Effort. Most probably there are already enough prospective cohort data collected or under collection to identify the environmental trigger(s) of T1D. New valuable information about factors and their contribution associated with the development of β-cell autoimmunity and progression to T1D could be achieved by a hurge international collaborative effort. It would then be possible to integrate demographic, genetic, autoimmune, and exposure data from the existing cohorts in Finland, Norway, Sweden, Germany, and Denver, Colorado.

4. The DIPP Study

The Type 1 Diabetes Prediction and Prevention (DIPP) study in Finland is a population-based long-term clinical follow-up

Table 2: HLA typing and time points for follow-up in DIPP.

(a) Eligible HLA types	DQB1*02/DQB1*0302 DQB1*0302/X (X # DQB1*02, 0301, 0602/0603)
(b) Time points for blood samples	Autoantibodies are measured in Tampere and Oulu at 3, 6, 18, and 24 months and then annually. In Turku every 3 months until 2 years, and then every 6 months until the age of 14 years
(c) Questionnaires	Filled out at the clinical visits at 3, 6, 18, and 24 months, and then annually
(d) Stool samples	Parents are asked to send it from 3 months to 3 years

Table 3: HLA typing and time points for follow-up in DiPiS.

(a) Eligible HLA types	DQB1*02/DQB1*0302 DQB1*0302/X (X # DQB1*02, 0301, 0602/0603)
(b) Time points for blood samples	Autoantibodies are measured in every second year
(c) Questionnaires	Filled just after the children were born, gestational and perinatal health
(d) Stool samples	Not asked for

study established in 1994, 1995, and 1997 in three university hospitals in Finland (Turku, Oulu, and Tampere, respectively) to understand the pathogenesis of T1D, predict the disease, and find preventive treatment [70, 71]. Both recruitment and follow-up of children in this study have since then been constantly ongoing; 186,000 cord blood samples have been genotyped so far. Families with a newborn baby carrying a DR-DQ genotype associated with increased risk for T1D (approximately 10% of all infants) are invited to participate in regular follow-up at the age of 3, 6, 9, 12, 18, and 24 months and thereafter once a year until the age of 15 years or until T1D is diagnosed, Table 2. Clinical details including maternal diet during pregnancy and lactation and child's diet starting from the age of 3 months are recorded, blood samples are collected, and serum autoantibodies associated with development of T1D are measured. In the DIPP study about 750 children have developed multiple islet autoantibodies, and more than 300 of these have progressed to clinical T1D.

5. The DiPiS Study

The Diabetes Prediction in Skåne (DiPiS) study is a population-based long-term follow-up study in Skåne, the southernmost region in Sweden representing 1.2 million inhabitants, 12,000 newborns per year, and nearly 100 children below 18 years of age diagnosed with T1D every year. In 2000–2004, more than 35,000 (70% of all newborns) were screened at birth for T1D high risk HLA, and 25,000 filled out a questionnaire on gestational and perinatal health [72], Table 3. Nearly 6,000 children at increased risk for T1D were offered follow-up and 4,200 are followed since two years of age, 82 have developed two or more islet autoantibodies, and 33 (40%) have gone on to a clinical diagnosis of T1D. The DiPiS children will be followed until 15 years of age.

6. The BABYDIAB Study

The BABYDIAB is a study from birth in 1,650 children born to a mother or father with T1D. Recruitment began in 1989 and ended in 2000. All children (840 boys, 810 girls) were recruited in Germany [73–76], Table 4. The population is not population based, and 97% of the families are of German or of European descent. Islet autoantibodies directed against insulin (IAA), glutamic acid decarboxylase (GAD), insulinoma-associated protein 2 (IA-2), and Zinc transporter 8 (ZnT8) are tested at all scheduled visits and every 6 months in children positive for islet autoantibodies. The median follow-up from birth to last sample in BABYDIAB is 11.7 years [45]. HLA genotyping has not been any inclusion criteria but has been performed later on for scientific purposes.

Table 4: HLA typing and time points for follow-up in BABYDIAB.

(a) No HLA typing required for recruitment and to be found eligible for follow-up
(b) Time points for blood samples: 9 months, 2 years, 5 years, 8 years, 11 years, 14 years, 17 years, and 20 years
(c) Questionnaires were asked for at 9 months and at 2 years with respect to breastfeeding
(d) Stool samples, not asked for

7. The DAISY Study

Between December 1993 and October 2004. The Diabetes Autoimmunity Study In the Young (DAISY) screened for T1D susceptibility HLA-DR, DQ genotypes and tested over 33,000 newborns from the general population of Denver, Colorado. The study population was representative of the general population of the Denver Metropolitan Area and included children classified by their mothers as non-Hispanic white (58%), Hispanic (28%), African American (7%), Asian American (2%), or biracial/others (5%). Newborns were categorized into four risk groups: (1) high T1D risk, 20-times higher than in the general population (HLA-DRB1*03/04,DQB1*0201/0302 genotype and negative for DRB1*0403); (2) moderate T1D risk, 3–7-times higher than in the general population (HLA-DR,DQ 4/4, 1/4, 8/4, and 9/4 (the DR4 haplotype carrying

Table 5: HLA typing and time points for follow-up in DAISY (general population).

(a) Eligible HLA types[†]	DRB1*03-DQB1*02/DRB1*04-DQB1*0302, and negative for DRB1*0403 DRB1*04-DQB1*0302/DRB1*04-DQB1*0302, and negative for DRB1*04034 DRB1*03-DQB1*02/DRB1*03-DQB1*02 DRB1*04-DQB1*0302/DRB1*01-DQB1*0501, and negative for DRB1*0403 DRB1*04-DQB1*0302/DRB1*08-DQB1*0402 DRB1*04-DQB1*0302/DRB1*09-DQB1*0303
(b) Time points for blood samples	9, 15, and 24 and thereafter annually
(c) Questionnaires	Filled out at the clinical visits at 9, 15, and 24 months of age, and thereafter annually
(d) Rectal swabs and salvia:	At 9, 15, and 24 months of age, and annually thereafter

[†]No HLA typing for siblings of a child with T1D.

DQB1*0302), and DR3/3); (3) average T1D risk, similar to that for the general population (HLA-DRB1*03/x or *04/x), and (4) low diabetes risk—all others. The combination of high- (2.1%) and moderate-risk genotypes (7.5%) was present in 9.6% of the general population. All high-risk children and selection of those at moderate or low risk were invited to participate in the follow-up [77–85], Table 5. Families with a member with T1D were identified using The Barbara Davis Centre for Childhood Diabetes in Denver, other diabetes care clinics, the Colorado T1D Registry, and newspaper publicity.

7.1. How to Find the Environmental Triggers of T1D without New Prospective Cohorts? The synthesis of multiple data sources to increase our understanding of a research field is an essential part of the scientific method. In the case of T1D, a close collaboration between the PI and close coworkers of existing cohorts (MIDIA, DIPP, DiPiS, BABYDIAB, and DAISY) would be a very vulnerable recourse in identification of the environmental trigger(s) of T1D. Given the diversity of systems and formats in which the data are currently stored, it is very difficult to create association between the results from the different studies. Several manual steps would be necessary to collect the data from various sources, transform them into a common format, and prepare them for a single kind of analysis. Such an ad hoc process would be not only complex and time consuming, but also hard to reuse and benefit from further studies of different analysis. To solve this interoperability issues, it would be much better to create a combined database that acts as a central repository, providing methods to store and retrieve data quickly and efficiently. Having a unified view on all the available information will allow the application of advanced analysis methods. It will also make it much easier to add further results, as it will only be necessary to adapt them to the common data format. The database would hold information, relative to all kinds of risk factors, for more than 20 000 children at various grades of increased HLA-conferred risk for T1D, followed from their early infancy (3 months) until seroconversion for β-cell specific autoantibodies and, in many cases, to clinical onset of the disease. To create a harmonized representation of the data, the start would be collecting and studying the results provided by each of the partners in this huge cohort project. Detailed documentation will have to be created on the factors presenting each of the cohort studies: how they are stored and how they relate to each other. The next step would be to design a database solution that aggregates those factors and that satisfies the requirements of the data analysis tasks to be performed. To incorporate and update the results on a regular basis an automated information integration process for each of the cohort data sources should be incorporated into the database.

In conclusion, based on international experiences with cohort studies, and with the relatively small participation rate in the TEDDY study, it is probably now a better idea with a new international effort to find the environmental trigger(s) of T1D. All the ongoing cohort studies will give a unique resource for collaboration. Performing large-scale integrative analysis on the combined database of available and incoming cohort data will give new insights and unfold complex relationships between the factors that determine the pathogenesis of the T1D.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 708289, 9 pages
http://dx.doi.org/10.1155/2015/708289

Research Article

Assessing Age-Related Etiologic Heterogeneity in the Onset of Islet Autoimmunity

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Received 4 August 2014; Revised 7 October 2014; Accepted 18 October 2014

Academic Editor: Mikael Knip

Type 1 diabetes (T1D), a chronic autoimmune disease, is often preceded by a preclinical phase of islet autoimmunity (IA) where the insulin-producing beta cells of the pancreas are destroyed and circulating autoantibodies can be detected. The goal of this study was to demonstrate methods for identifying exposures that differentially influence the disease process at certain ages by assessing age-related heterogeneity. The Diabetes Autoimmunity Study in the Young (DAISY) has followed 2,547 children at increased genetic risk for T1D from birth since 1993 in Denver, Colorado, 188 of whom developed IA. Using the DAISY population, we evaluated putative determinants of IA, including non-Hispanic white (NHW) ethnicity, maternal age at birth, and erythrocyte membrane n-3 fatty acid (FA) levels, for age-related heterogeneity. A supremum test, weighted Schoenfeld residuals, and restricted cubic splines were used to assess nonproportional hazards, that is, an age-related association of the exposure with IA risk. NHW ethnicity, maternal age, and erythrocyte membrane n-3 FA levels demonstrated a significant age-related association with IA risk. Assessing heterogeneity in disease etiology enables researchers to identify associations that may lead to better understanding of complex chronic diseases.

1. Introduction

Type 1 diabetes (T1D) results from the destruction of the insulin-producing pancreatic beta cells. The incidence of T1D is increasing at an annual rate of about 3% worldwide [1]. The most rapid increase has been in children younger than 5 years old [1–4].

T1D is preceded by a preclinical phase of islet autoimmunity (IA) where the body produces antibodies (IAA, GAD_{65}, or IA-2) against the insulin-producing beta cells of the pancreas, which can be detected as early as 6 months of age [5]. There appears to be two peaks in IA incidence at approximately 1-2 years of age and in adolescence, with distinct characteristics at each peak [5].

IA and T1D development may be subject to age-related etiologic heterogeneity, where exposures influence the disease process more strongly at certain ages. T1D development is more likely to occur earlier in life for those with disease-associated HLA genotypes and a parental history of T1D [6–12]. A recent study found differences in serum metabolite profiles relative to age; an association between lower methionine levels and presence of diabetes autoantibodies in younger onset (≤2 years) but not older onset (≥8 years) autoimmunity was described [13]. Additionally, Virtanen et al. found early introduction of wheat, rye, oats and/or barley cereals, and egg was associated with increased IA risk, but only during the first 3 years of life, suggesting an age-related association [14].

Assessment of age-related heterogeneity allows understanding of *if* and *when* exposures play a role in the disease process. Valuable associations may be overlooked if they are averaged across ages and not evaluated for heterogeneity. Knowing when exposures play a role in the disease process can guide treatment and prevention efforts by creating more accurate risk prediction models and informing the design of targeted interventions.

Prospective cohorts of children at increased T1D risk are often followed from birth to IA and T1D development. Time-to-event analyses, frequently implemented using Cox proportional hazards (PH) regression, are utilized to identify risk factors. A Cox PH model assumes the hazard ratio (HR) is constant over time, meaning the association of a covariate is the same at all time points. If age-related heterogeneity is present for a given variable, the association of that variable changes over time (i.e., age) and the PH assumption is not valid. Therefore, age-related heterogeneity can be assessed by evaluating the PH assumption. We demonstrate the use of three methods for testing and modeling non-PH: a supremum test, evaluation of weighted Schoenfeld residuals, and restricted cubic splines.

1.1. Supremum Test. The supremum test, a regression diagnostic for PH models, plots the path of the observed cumulative sum of martingale residuals for a covariate against time [15]. Rather than a test statistic, it produces a P value which represents the percentage of 1000 simulated paths embodying the PH assumption whose supremum (or largest) values exceed the supremum of the observed path for the covariate of interest [15]. Higher P values (ideally much greater than 0.05) are a stronger indication that the PH assumption holds, suggesting the supremum of the observed path is substantially smaller than a large proportion of the suprema of the 1000 simulated paths that actually follow the PH assumption for that covariate [15]. The test is implemented in SAS PROC PHREG.

1.2. Weighted Schoenfeld Residuals. Weighted Schoenfeld residuals can be plotted as another PH regression diagnostic as described by Grambsch and Therneau [16]. In the R package using the cox.zph function of the *survival* library, these residuals produced separately for each covariate for each individual are visualized through scatterplot smoothing. This effectively shows how the regression coefficient, $\beta(t)$, varies with time [16]. If the assumption of PH is satisfied, the residuals will be independent of time; thus, a non-zero slope indicates a violation of the PH assumption.

1.3. Restricted Cubic Splines. Restricted cubic splines (RCS) are piecewise polynomials smoothly joined at k knot values that can also be used to identify and model non-PH [17, 18]. RCS provide a statistical test as well as a visual assessment of the HR as a function of time and allow for flexible modeling of the HR without a specific functional form, for example, linear or quadratic. The number of knots selected for the splines is chosen based on Akaike information criterion (AIC), where a lower value indicates better fit. The SAS RCS macro, designed to assess PH for fixed covariates, first tests whether the covariate of interest is associated with the event. If the covariate is associated with the event, one can then test whether the association is nonconstant with time (indicating a violation of the PH assumption) and, if so, whether the relationship between the HR and time is linear or not [17, 18].

1.4. Diabetes Autoimmunity Study in the Young (DAISY). DAISY has prospectively collected 20 years of data from birth on children at increased genetic risk for T1D. DAISY data can be used to study prospective exposures across childhood and assess whether the risk associated with determinants of IA and T1D differs by age when the child develops IA and/or T1D. T1D has a complex etiology with numerous identified factors that either increase or decrease disease risk. The goal of this study was to demonstrate the aforementioned three methods of identifying exposures that influence the disease process at varying ages by assessing age-related heterogeneity (or lack of PH) of putative IA determinants, including non-Hispanic white (NHW) ethnicity, maternal age at birth, and erythrocyte membrane n-3 fatty acid (FA) levels. We were interested in assessing age-related heterogeneity of IA because if risk factors are shown to have associations that vary by age, then age-appropriate interventions can be designed to prevent or slow the development of IA and, ultimately, T1D.

2. Materials and Methods

2.1. Study Population. DAISY recruited two groups of children between 1993 and 2004, who are at increased T1D risk and followed prospectively for IA and T1D development. One group consists of first-degree relatives of patients with T1D, identified and recruited between birth and age 8, mainly through the Barbara Davis Center for Childhood Diabetes. The second group consists of infants born at St. Joseph's Hospital in Denver, CO, whose umbilical cord blood was screened for diabetes-susceptibility HLA-DR, DQ genotypes and recruited if they had these genotypes [19, 20]. Details of the newborn screening, sibling and offspring recruitment, and follow-up of both cohorts have been published previously [21]. Cord blood or the first available blood sample (depending on enrollment group) was sent to Roche Molecular Systems, Inc., Alameda, CA, for PCR-based HLA-DR, DQ typing. All study protocols were approved by the Colorado Multiple Institutional Review Board, and informed consent was given by parents of all participating children.

The DAISY cohort is composed of 2,547 children, of whom 188 have developed IA. Nineteen IA cases were positive for autoantibodies on their first clinic visits; these left-censored cases were removed from the analysis. We examined NHW ethnicity and maternal age at birth as fixed covariates for age-related heterogeneity in this cohort. We also assessed the time-varying covariate, erythrocyte membrane n-3 FA levels, for age-related heterogeneity. As described previously [22], erythrocyte membrane n-3 FA levels were investigated in a case-cohort design, for which a representative sample of 380 children (i.e. subcohort) was selected from the main DAISY cohort using stratified sampling based on HLA-DR

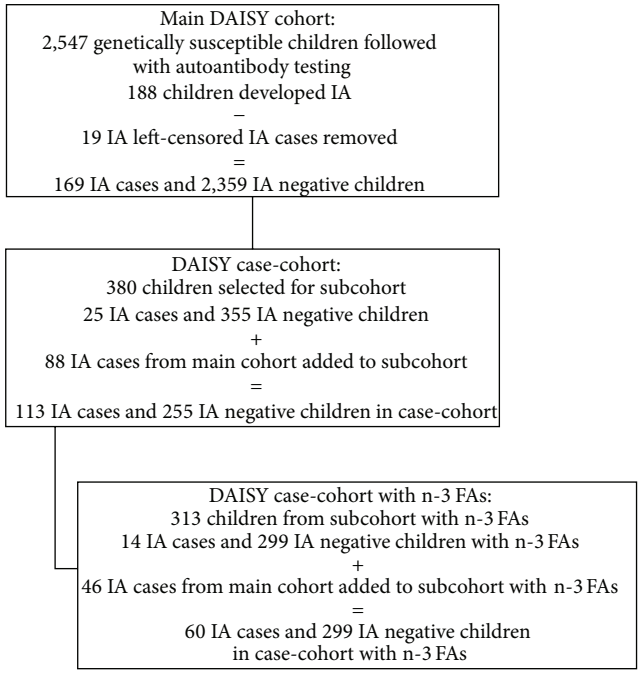

Figure 1: Flow chart illustrating the formation of the cohorts for the investigation of age-related heterogeneity.

genotype and family history of T1D; 313 of these children had erythrocyte n-3 FA measurements. Erythrocyte n-3 FA levels were measured at 9, 15, and 24 months of age and annually thereafter. The median age at which n-3 FA levels were determined was 6.9 years (IQR: 5.2 years). During follow-up, 14 children with erythrocyte membrane n-3 FA levels developed IA within the subcohort. We supplemented these with 46 children who developed IA outside of the subcohort to complete our case-cohort study population. Therefore, 60 children with IA and 299 IA negative children were included in the analysis of erythrocyte membrane n-3 FA levels (see Figure 1 for flow-chart illustrating the formation of the analysis cohorts).

2.2. Measurement of Erythrocyte Membrane n-3 FA Levels. Erythrocytes from blood samples were separated within 30 minutes of blood draw, flash-frozen in liquid nitrogen, and stored at −70°C. Lipids were extracted from erythrocytes following the method developed by Bligh and Dyer [23] and stored at −20°C in sealed cryotubes following flushing with nitrogen gas. The FAs present in the lipid isolates were methylated using the base-catalyzed procedures by Maxwell and Marmer [24] in preparation for analysis by gas chromatography (Hewlett-Packard 6890; Agilent, Santa Clara, CA, USA) with mass spectral detection (Hewlett-Packard 5973; Agilent). The samples, separated across a CP-WAX column (25 m × 0.25 mm internal diameter, 0.2-μm film; Varian, Palo Alto, CA, USA), were identified by comparing the retention times and *m/z* of selected ions from analytes in the samples with those of authentic standards (NuCheckPrep; Elysian, MN, USA; Supelco; St. Louis, MO, USA). Quantification was determined against five-point standard curves and FA percentage is reported as a percent of total lipids (g of FA/100 g erythrocyte lipid). The following n-3 FAs were measured in the membranes and combined to estimate total n-3 FAs in the membrane (as a percent of total lipids): α-linolenic acid (ALA) (18:3n-3), eicosapentaenoic acid (EPA) (20:5n-3), docosahexaenoic acid (DHA) (22:6n-3), and docosapentaenoic acid (DPA) (22:5n-3).

2.3. Measurement of Autoantibodies. Autoantibodies were tested at 9, 15, and 24 months, or at their first visit (if enrolled after the first year of life), and annually thereafter. Radioimmunoassays measured serum autoantibodies to insulin, glutamic acid decarboxylase $(GAD)_{65}$, and insulinoma antigen (IA-2) (also known as BDC512), as previously described [25–27], with confirmation of all positive and a subset of negative results. Cut-off for positivity was established at the 99th percentile of healthy controls. Children testing autoantibody positive were put on an accelerated testing schedule of every 3–6 months. IA cases were defined as children positive for at least one autoantibody (IAA, GAD_{65}, IA-2) on ≥2 consecutive visits within 6 months with the end of follow-up in children who developed IA defined as the first of the antibody positive visits and the last negative sample in children who did not develop IA (median duration of follow-up was 8.1 years (IQR: 6.3 years)).

2.4. Statistical Analyses

2.4.1. Assessment of the PH Assumption. We assessed violation of the PH assumption using three different methods: the supremum test, weighted Schoenfeld residuals, and restricted cubic splines. The supremum test was performed in SAS version 9.3 (SAS Institute, Cary, NC) using the ASSESS statement in the PHREG procedure. Weighted Schoenfeld residuals were plotted using the cox.zph function in R 2.15.2 [28]. A significant supremum test ($P < 0.30$) or a non-zero slope for the loess smoothed curve of the weighted Schoenfeld residuals indicated a violation of PH [16]. Using the weighted Schoenfeld residuals, a global test of PH was assessed first; if the global test P value was not large ($P < 0.30$), the individual covariate tests of PH were used to identify the source(s) of the non-PH. Restricted cubic splines were modeled using the RCS macro in SAS [17, 18]. Due to a limited number of events, the number of knots for the RCS was selected to be 3, placed at the 5th, 50th, and 95th percentiles of age of the IA cases; this minimizes the number of coefficients to fit the RCS models [17, 18]. The RCS macro provides the three statistical tests described above which should be performed hierarchically. The first test has 3 df for a 3-knot spline model and tests whether the covariate of interest is associated with the event. If the null hypothesis is rejected ($P < 0.05$), the second statistical test with 2 df for a 3-knot spline model can be performed to determine whether the association is nonconstant with time ($P < 0.05$ indicating a violation of PH). Finally, if the null hypothesis is rejected for both the first and second statistical tests, the third statistical test with 1 df for a 3-knot spline model can be performed to

determine whether the relationship between the HR and time is linear ($P < 0.05$ indicates nonlinearity) [17, 18].

2.4.2. Fixed Covariates. The three methods (supremum test, weighted Schoenfeld residuals, and RCS) were evaluated for the fixed covariates: NHW ethnicity and maternal age at birth. Statistical analyses were conducted using SAS software, Version 9.3 of the SAS System for Windows. Copyright © 2002–2010 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA, except for the weighted Schoenfeld residuals, which were generated and plotted in R [29].

2.4.3. Time-Varying Covariates. Existing tools are limited with regard to examination of PH with time-varying covariates. The supremum test for violation of the PH assumption can theoretically accommodate time-varying covariates but requires higher dimensional plots for time-varying covariates and is not implemented in standard statistical software. The cox.zph function used to plot the weighted Schoenfeld residuals is valid for time-varying covariates; however, the software assumes the variance of the time-varying covariate is constant over time [16]. If this assumption is violated, results from the weighted Schoenfeld residual tests are not reliable. Motivated by these limitations in assessing PH with time-varying covariates, Kroehl et al. (unpublished) recently adapted RCS for use with time-varying covariates and evaluated their performance in identifying and modeling a nonconstant HR. Using this approach, non-PH was investigated for erythrocyte membrane n-3 FA levels.

3. Results

Table 1 describes the DAISY children by IA status. Of 188 IA-positive children in DAISY, 19 were excluded from analyses of IA because they tested autoantibody positive on their first study visit (i.e., left-censored). Median age at first IA-positive visit was 6.0 years and 9.0 years at last follow-up visit in those without IA. Children who developed IA were more likely to have the HLA-DR3/4, DQB1*0302 genotype and have a first-degree relative with T1D compared to children who did not develop IA (Table 1). Table 2 shows the characteristics of the DAISY subcohort by IA status in which the median age at first IA-positive visit was 5.1 years and 8.6 years at last follow-up visit in those without IA.

3.1. Fixed Covariates. We first assessed age-related heterogeneity of two fixed covariates: NHW ethnicity and maternal age at birth. The supremum test P value was 0.01 for NHW ethnicity adjusting for HLA-DR3/4, DQB1*0302 genotype and first-degree relative status, indicating a violation of PH (Table 3). The weighted Schoenfeld residuals had a global PH test $P = 0.02$ and an individual PH test $P = 0.01$, indicating a violation of PH (Figure 2(a)). We modeled the RCS to evaluate the HR as a function of time. NHW ethnicity showed an overall significant association with IA risk adjusting for HLA-DR3/4, DQB1*0302 genotype and first-degree relative status (Association $P = 0.03$) (Table 3). Based on rejection of the null hypothesis, the nonconstant association was tested, producing a $P = 0.01$, indicating non-PH (Table 3). Finally, a nonlinear association was tested based on rejection of the null hypothesis for the nonconstant association, which was not significant (Nonlinear $P = 0.62$), indicating a linear decrease in IA risk associated with NHW ethnicity over time (Table 3). The restricted cubic spline demonstrated an elevated risk in early childhood (age 2 HR: 1.74, 95% CI: 0.90, 3.36) diminishing with increasing age (age 11 HR: 0.64, 95% CI: 0.38, 1.06) (Figure 3(a)).

The supremum test P value for maternal age was 0.01 adjusting for HLA-DR3/4, DQB1*0302 genotype and first-degree relative status, indicating a violation of PH (Table 3). The weighted Schoenfeld residuals for maternal age also had a significant global PH test $P = 0.01$ and an individual PH test $P = 0.003$, another indication of a PH violation (Figure 2(b)). The modeled RCS resulted in a significant overall association of maternal age with IA risk adjusting for HLA-DR3/4, DQB1*0302 genotype and first-degree relative status (Association $P = 0.003$) (Table 3). Based on rejection of this null hypothesis, the nonconstant association of maternal age was tested with a resulting $P = 0.001$, demonstrating age-related heterogeneity (Table 3). Finally, based on rejection of the null hypothesis for the nonconstant association test, a nonlinear association was tested. In contrast with the restricted cubic spline result for NHW ethnicity, the nonconstant association of maternal age for a five-year difference was also nonlinear (Nonlinear $P = 0.03$) with greater maternal age at birth associated with increased risk in early childhood (age 2 HR: 1.14, 95% CI: 0.92, 1.42), which became protective later in adolescence (age 11 HR: 0.83, 95% CI: 0.68, 1.02) (Figure 3(b)).

3.2. Time-Varying Covariates. We then assessed age-related heterogeneity for the time-varying covariate, erythrocyte membrane n-3 FA levels, by fitting a restricted cubic spline model (Figure 4). Erythrocyte membrane n-3 FA levels had an overall significant association with IA risk (Association $P = 0.001$) adjusting for HLA-DR3/4, DQB1*0302 genotype and first-degree relative status, previously established in DAISY by Norris et al. [21]. With rejection of this null hypothesis, the nonconstant association of erythrocyte membrane n-3 FA levels was tested and resulted in a $P = 0.001$, indicating non-PH (Figure 4). Finally, a nonlinear association was tested based on rejection of the null hypothesis for the nonconstant association, which was not significant ($P = 0.17$), indicating a linear decrease in IA risk associated with erythrocyte membrane n-3 FA levels over time (age 2 HR: 1.08, 95% CI: 0.75, 1.56 and age 11 HR: 0.30, 95% CI: 0.14, 0.64) (Table 3 and Figure 4).

4. Discussion

This study elucidates potential challenges in identifying triggers of T1D, which are associations that change with age. We demonstrate three age-related associations of putative IA determinants that would be masked if averaged across age

Table 1: Characteristics of main DAISY cohort by IA status (n = 2,528).

Characteristic	Number (%)		P value
	Developed IA (n = 169)	Did not develop IA (n = 2,359)	
Age, median (IQR), y	6.0 (6.6)[1]	9.0 (10.9)[2]	<0.0001
HLA-DR3/4, DQB1*0302 genotype	63 (37%)	435 (18%)	<0.0001
First-degree relative with T1D	100 (59%)	1019 (43%)	<0.0001
Female	88 (52%)	1126 (48%)	0.28
Race/ethnicity, non-Hispanic white	132 (78%)	1690 (72%)	0.10
Maternal age, mean (SD), y	30.3 (5.8)	29.8 (5.7)	0.25

DAISY, Diabetes Autoimmunity Study in the Young; HLA, human leukocyte antigen; IA, islet autoimmunity; T1D, type 1 diabetes.
[1]Age at first IA positive visit.
[2]Age at last follow-up.

Table 2: Characteristics of DAISY subcohort by IA status (n = 359).

Characteristic	Number (%)		P value
	Developed IA (n = 60)	Did not develop IA (n = 299)	
Age, median (IQR), y	5.1 (6.3)[1]	8.6 (6.3)[2]	<0.0001
HLA-DR3/4, DQB1*0302 genotype	21 (35%)	83 (28%)	0.26
First-degree relative with T1D	31 (52%)	118 (39%)	0.08
Female	35 (58%)	148 (50%)	0.21
Race/ethnicity, non-Hispanic white	47 (78%)	222 (74%)	0.51
Maternal age, mean (SD), y	30.5 (5.5)	30.0 (5.4)	0.47

DAISY, Diabetes Autoimmunity Study in the Young; HLA, human leukocyte antigen; IA, islet autoimmunity; T1D, type 1 diabetes.
[1]Age at first IA positive visit.
[2]Age at last follow-up.

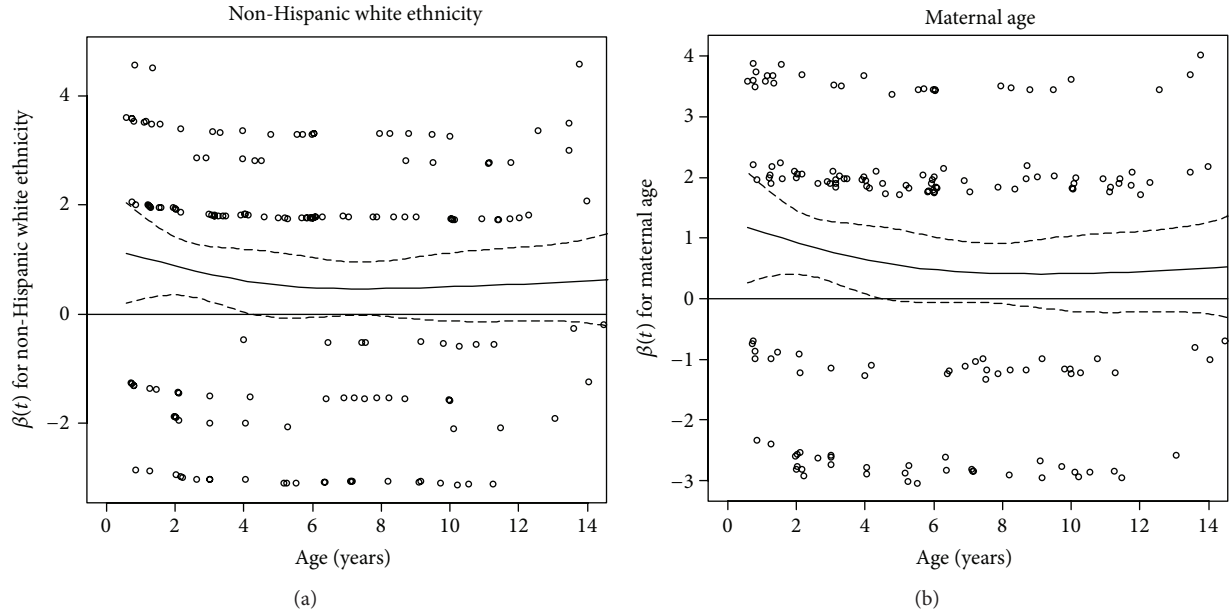

Figure 2: The weighted Schoenfeld residual plots are displayed for non-Hispanic white ethnicity (NHW) (a) and a 5-year difference in maternal age (b) in the prospective DAISY cohort. The x-axis represents age in years and the y-axis represents the coefficient estimate for non-Hispanic white ethnicity in (a) and coefficient for maternal age in (b). The dots represent the residuals for each individual. The solid line is a smoothing-spline fit to the plot, with the dashed lines representing the 95% confidence interval. The global PH test P values based on the Schoenfeld residuals are 0.02 and 0.01 for NHW ethnicity and a 5-year difference in maternal age, respectively, indicating a violation of the PH assumption.

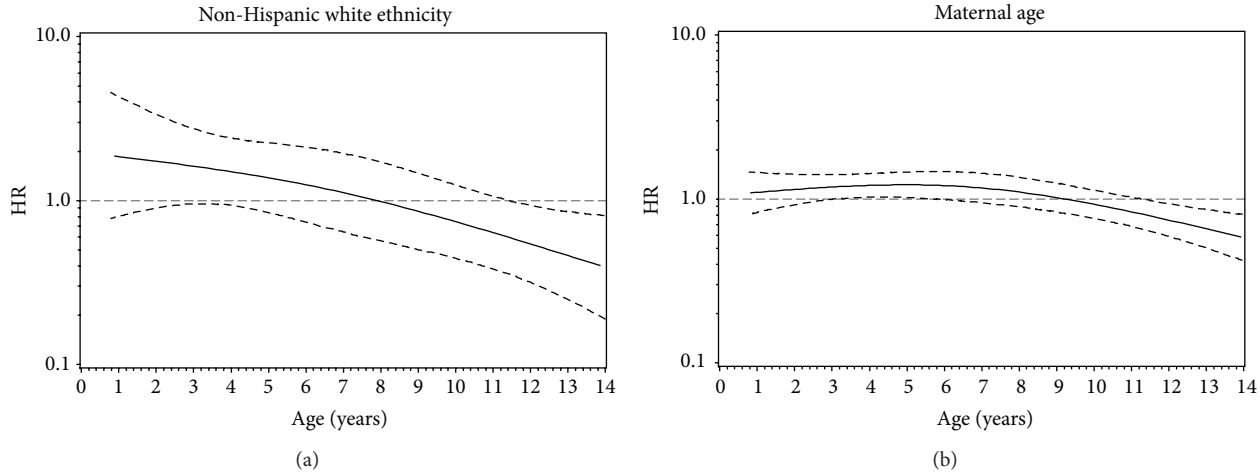

Figure 3: A restricted cubic spline model was used to estimate the hazard ratio as a function of age. The restricted cubic spline for non-Hispanic white ethnicity exhibits an increased risk of islet autoimmunity (IA) early on that then becomes protective in the older ages (a). The restricted cubic spline for a 5-year difference in maternal age exhibits a slightly elevated risk of IA in early childhood that becomes protective in adolescence (b). The x-axis represents age in years and the y-axis represents the hazard ratio on the log scale. The solid line represents the hazard ratio and the dashed lines represent the pointwise 95% confidence bands.

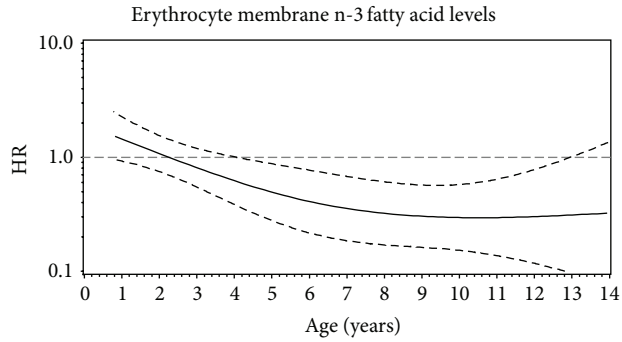

Figure 4: The restricted cubic spline function for erythrocyte membrane n-3 fatty acid levels and islet autoimmunity development in the prospective DAISY cohort displays a linear decrease in risk across childhood. The x-axis represents age in years and the y-axis represents the hazard ratio on the log scale. The solid line represents the hazard ratio and the dashed lines represent the pointwise 95% confidence bands.

and not evaluated for heterogeneity. Knowing when exposures play a role in the disease process is important in better understanding complex diseases. RCS aid in visualizing risk over time and provide statistical tests to determine whether risk is nonconstant with respect to time or age.

The assumption of PH is central to Cox PH analysis, and if violated, inferences made from an analysis may be incorrect. For example, the association of an age-sensitive risk factor could be missed if the association, averaged over time, is determined to be nonsignificant. All three of the variables presented here violated the PH assumption, indicating it may be somewhat common for risk factors to be subject to age-related heterogeneity. It is important to recognize even if a covariate does not have a statistically significant P value for the nonconstant association when assessing non-PH with RCS, marginal associations may be clinically interesting or meaningful. RCS also provide HRs and 95% CIs for any time point or age of interest. Attributable risks can then be calculated to determine whether the number of cases attributed to the disease at a certain age is meaningfully different from other ages [30–32].

We were interested in assessing age-related heterogeneity of IA, because if IA risk factors can be identified, preventive efforts may be designed to slow or even prevent progression to T1D. NHW ethnicity, maternal age, and erythrocyte membrane n-3 FA levels that demonstrated significant age-related heterogeneity with statistically significant P values for the nonconstant association have not been previously shown to demonstrate etiologic heterogeneity in T1D.

The linear decrease in IA risk associated with NHW ethnicity may reflect the fact that the age of IA development in DAISY is younger in NHW children compared to other racial/ethnic groups (mean: 6.0 years in NHW children versus 7.9 years in children of other racial/ethnic groups). Another study in DAISY found that children who developed IA after age 7 years were more likely than those who developed IA before age 7 years to be ethnic minorities [33]. A study using the Colorado IDDM Registry found that, on average, NHW children developed T1D six months earlier than Hispanic children (mean: 9.5 years versus 10.0 years) [34]. This may also reflect a higher genetic load of non-HLA T1D genotypes in NHW children resulting in a younger disease onset in NHW children. Children of other racial/ethnic groups may develop T1D later, reflecting a different disease that may initially appear to be type 2 diabetes but is, in fact, T1D.

Our finding with maternal age may be interesting for hypothesis generation. The slight increased IA risk associated with increased maternal age at birth in younger children may

Table 3: Assessment of the proportional hazards assumption in DAISY cohort.

Characteristic	Supremum test P	Schoenfeld residuals global P	Schoenfeld residuals individual covariate P	Restricted cubic splines association P	Restricted cubic splines nonconstant P	Restricted cubic splines nonlinear P
Non-Hispanic white ethnicity[1]	0.01	0.02	0.01[2]	0.03[3]	0.01[3]	0.62[3]
Maternal age[1]	0.01	0.01	0.003[4]	0.003[5]	0.001[5]	0.03[5]
Erythrocyte membrane *n*-3 fatty acid levels[1]	N/A	N/A	N/A	0.001[6]	0.001[6]	0.17

DAISY, Diabetes Autoimmunity Study in the Young; HLA, human leukocyte antigen; T1D, type 1 diabetes.
N/A: statistical tests were not performed based on the inability to reject the null hypothesis of the prior test in the hierarchical structure.
[1]Adjusted for HLA-DR3/4, DQB1*0302 genotype and first-degree relative with T1D.
[2]Figure 2(a).
[3]Figure 3(a).
[4]Figure 2(b).
[5]Figure 3(b).
[6]Figure 4.

be due to a number of perinatal exposures. Older mothers may be more likely to have complicated pregnancies that result in cesarean or complicated vaginal deliveries (i.e., breech or use of forceps or vacuum), increasing their child's risk of IA and T1D development [35–38]. We have also observed that older mothers are more likely to delay the introduction of solid foods, including cereals, which has been associated with an increased risk of IA and T1D [37, 39]. Another explanation may be that older mothers have more accumulated exposures throughout their lifetime, which may influence biological programming in the child [35, 40]. These early risk factors for T1D may only exert influence in the early years and once these susceptible children develop IA, maternal age may no longer be associated with increased IA risk. Younger mothers are more likely to have lower SES and educational attainment, which could start to have an influence as children get older by resulting in fewer opportunities for extracurricular physical activity or healthy food choices for their children in adolescence [41], possibly increasing IA risk. It is also possible that NHW ethnicity and maternal age are measuring similar things, as mothers of NHW children are significantly older than mothers of children of other racial/ethnic groups in our population (mean: 30.6 years versus 27.8 years, resp.).

The inverse association between n-3 FA levels and IA risk after age 4.25 may provide an important age for the efficacy of n-3 FAs and future n-3 FA intervention studies. In our population, increased n-3 FA levels were not significantly associated with IA risk before age 4.25 years. It is possible that only a small portion of young children consume fatty fish, the primary dietary source of n-3 FA, resulting in limited variability in n-3 FA levels and making it difficult to see an effect of n-3 FA levels in early childhood. The variability in n-3 FA intake in children younger than 4.25 years in the DAISY population is significantly lower than the variability in n-3 FA intake in children 4.25 years and older (mean ± SD: 1.09±0.49 versus 1.19 ± 0.57, resp.).

Assessing age-related heterogeneity is an important step in understanding etiologic heterogeneity of complex diseases and ensuring key associations are not overlooked. We encourage researchers using Cox PH regression analyses to assess covariates for violation of the PH assumption when using them in a Cox PH model. As a preliminary step preceding assessment of PH, continuous covariates should be examined for correct functional form with all important covariates and confounders included in the model [42]. If the variable is not linearly associated with the HR, a transformation may be appropriate (such as a log transform) prior to assessing for PH. Martingale residuals are a common diagnostic tool for evaluating functional form. It should also be recognized that missing covariates can also erroneously induce nonproportionality [42].

Regarding the three methods for testing the PH assumption presented in this paper, we suggest the supremum test or the weighted Schoenfeld residuals for diagnostic testing of the PH assumption to determine if a fixed covariate can be used in a Cox PH model. However, if one is interested in examining the nature of age-related heterogeneity or evaluating the PH assumption with a time-varying covariate, we recommend modeling RCS and performing the hierarchical testing of the PH assumption by first determining whether the covariate of interest is associated with the event. If the null hypothesis is rejected and the covariate is associated with the event, a statistical test can be performed to determine whether the association is nonconstant with time, violating the assumption of PH. Using this approach, DAISY recently detected a single nucleotide polymorphism, rs10517086, that demonstrated age-related heterogeneity with IA risk, with increased risk before 2 years of age (age 2 HR: 1.67, 95% CI: 1.08, 2.56), but not older ages (age 4 HR: 0.84, 95% CI: 0.43, 1.62) [43].

5. Conclusions

RCS are a powerful way of visualizing the true form of an exposure with time with the ability to test whether this association exhibits a nonconstant or nonlinear association.

They have the added advantage of being applicable to time-varying exposures. This method may aid in identifying or confirming environmental determinants that play a role in the etiology of T1D.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by National Institutes of Health Grant nos. R01-DK49654 and R01-DK32493 and the Diabetes Endocrine Research Center, Clinical Investigation & Bioinformatics Core P30 DK 57516. The authors thank the dedicated and talented staff of the DAISY study for their clinical, data, and laboratory support and all the DAISY children and families who generously volunteered their time and knowledge.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 203947, 5 pages
http://dx.doi.org/10.1155/2015/203947

Research Article

Daycare Attendance, Breastfeeding, and the Development of Type 1 Diabetes: The Diabetes Autoimmunity Study in the Young

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Received 11 July 2014; Accepted 8 September 2014

Academic Editor: Kjersti S. Rønningen

Background. The hygiene hypothesis attributes the increased incidence of type 1 diabetes (T1D) to a decrease of immune system stimuli from infections. We evaluated this prospectively in the Diabetes Autoimmunity Study in the Young (DAISY) by examining daycare attendance during the first two years of life (as a proxy for infections) and the risk of T1D. *Methods.* DAISY is a prospective cohort of children at increased T1D risk. Analyses were limited to 1783 children with complete daycare and breastfeeding data from birth to 2 years of age; 58 children developed T1D. Daycare was defined as supervised time with at least one other child at least 3 times a week. Breastfeeding duration was evaluated as a modifier of the effect of daycare. Cox proportional hazards regression was used for analyses. *Results.* Attending daycare before the age of 2 years was not associated with T1D risk (HR: 0.89; CI: 0.54–1.47) after adjusting for HLA, first degree relative with T1D, ethnicity, and breastfeeding duration. Breastfeeding duration modified this association, where daycare attendance was associated with increased T1D risk in nonbreastfed children and a decreasing T1D risk with increasing breastfeeding duration (interaction P value = 0.02). *Conclusions.* These preliminary data suggest breastfeeding may modify the effect of daycare on T1D risk.

1. Background

Type 1 diabetes (T1D) is an autoimmune disease where the body's immune system destroys the pancreatic beta cells that produce insulin. The incidence of T1D is increasing at roughly 3% globally, with the greatest increase of incidence in children younger than 4 years of age [1]. It is likely that an individual with the genetic makeup for diabetes will not develop T1D without an immunologic trigger that initiates the autoimmune response [2]. While the autoimmune pathophysiology of T1D has been established, a deeper understanding of this trigger has remained elusive.

The hygiene hypothesis proposes that the recent increase in incidence of T1D is due to increased hygiene and low pathogen burden environments [3]. Exposures to infectious agents early in life are hypothesized to activate regulatory pathways in our immune system that suppress development of autoimmunity and thus T1D [4]. Social mixing is a variable used to encompass the numerous exposures to infectious agents that individuals experience when sharing space together. Social mixing captures asymptomatic or minor infections that would otherwise not be reported or recalled. Previous studies used social mixing as a proxy for infections to test the hygiene hypothesis and have observed lower risk of T1D in high social mixing environments [5, 6]. Parslow et al. observed a significant association with higher incidence of T1D for children 0–14 years of age in areas with low levels of social mixing [7]. In Scotland, Patterson and Waugh examined social mixing socioeconomically and geographically and found that incidence of T1D was lower in deprived urban areas compared with affluent rural areas [8]. In Austria, Schober et al. examined social mixing through population density and observed protection from T1D in areas with high percentages of children less than 15 years of age [5].

Daycare offers social mixing during critical immune development stages early in life. Like social mixing, attending daycare can be used as a proxy for measuring asymptomatic or minor infections to test the hygiene hypothesis. McKinney et al. found evidence that social mixing through daycare attendance early in life protected against the development of T1D [6]. A meta-analysis of several case-control studies showed a statistically significant protective effect of daycare on the risk of T1D [9]. The previous studies examining daycare attendance and the risk of developing T1D have been retrospective; and the authors have recommended that future studies analyze this association prospectively. This study will attempt to close the gap on the lack of prospective analysis by examining daycare attendance and the risk of developing T1D prospectively using the Diabetes Autoimmunity Study in the Young (DAISY) cohort.

Breastfeeding has also been shown to be protective in the risk of developing T1D, albeit inconsistently [10, 11]. It is believed that breastfeeding provides immune support through immunoglobulin A antibodies and increased β-cell proliferation [12] to protect against infections and thus reduce the risk of T1D.

We hypothesized that daycare attendance is associated with a decreased risk of developing T1D in children in DAISY. We further hypothesized that the effect of daycare attendance is modified by breastfeeding.

2. Methods

2.1. Study Population. DAISY is a prospective study of children in Colorado who are at increased risk of developing T1D. It includes children born at St. Joseph's Hospital in Denver that were screened by umbilical cord blood for diabetes-susceptibility alleles in the human leukocyte antigen (HLA) region. It also includes unaffected children recruited between birth and 8 years of age with a first degree relative that has T1D. For these analyses, we included only the DAISY children who had a clinic visit before 1.35 years of age and who had prospective daycare exposure data from birth until two years of age and complete breastfeeding duration data. Interviews collecting diet and daycare data were completed at 3, 6, 9, 12, 15, and 24 months and then annually thereafter. Clinic visits occurred at 9, 15, and 24 months and annually thereafter for the tracking of autoimmunity and T1D.

The following descriptive factors were examined: HLA genotype (HLA-DR3/4, DQB1*0302 versus others), first degree relative with T1D (mother versus father or sibling versus none), birth order (first/only child versus second child or more), sex (female versus male), race/ethnicity (non-Hispanic white versus other race/ethnicity), maternal age at child's birth, maternal education (>12 years versus ≤12 years), crowding (≥1 person/room versus <1 person/room at 6 months of age), and breastfeeding duration (in months). Crowding was calculated by taking the reported number of persons living in a household and dividing this by the number of rooms in the household, not including bathrooms, when the child was six months of age.

2.2. Daycare Measure. Daycare information was collected by parent interview with the following query, "Does ___ attend daycare (family daycare home or daycare center) or preschool on a regular basis?" Follow-up questions regarding the size of the daycare/preschool class and the frequency of attendance were asked. The daycare variable used in this study was defined as supervised time with at least one other child, not including a sibling, at least three times a week.

2.3. Breastfeeding Duration Measure. Breastfeeding duration was defined as the length of time, in months, that the child was breastfed, either partially or exclusively.

2.4. Diagnosis of Type 1 Diabetes. T1D was diagnosed by a physician based on symptoms of excessive urination and/or excessive thirst with at least a glucose level greater than 200 mg/dL, a fasting plasma glucose level at or above 126 mg/dL, or an oral glucose tolerance test with a 2-hour glucose level at or above 200 mg/dL.

2.5. Analysis Population. Of the 2,632 children followed by DAISY, 1,856 children were followed from birth; that is, they had a clinic visit before 1.35 years of age. Of these, 1,799 children had prospective daycare exposure data. From these, 16 were excluded due to missing breastfeeding duration or ethnicity information, leaving 1,783 children in the analysis cohort. The analysis cohort included 58 children who developed T1D during follow-up of an average of 8.5 years (range 0.9–17.4 years). Three children developed type 1 diabetes before 2 years of age (at ages 0.9, 1.8, and 1.9 years). In these instances, only the information regarding daycare attendance prior to the development of diabetes was used to determine their daycare exposure variable.

2.6. Statistical Analysis. The SAS version 9.3 (SAS Institute Inc.) statistical software package was used for all statistical analyses. Hazard ratios (HR) and 95% confidence intervals (CI) were estimated using Cox regression, to account for right-censored data. Follow-up time began at birth. A clustered time to event analysis was performed treating siblings from the same family as clusters, and robust sandwich variance estimates were used for statistical inference [13]. Based on our *a priori* hypothesis, we tested the significance of an interaction between the dichotomous daycare attendance variable and continuous breastfeeding duration variable; interaction models contained the base terms and the interaction term. The significance of the interaction term was determined by improvement in model fit as indicated by the chi-squared statistic from the likelihood ratio test.

3. Results

Children who developed T1D in the analysis cohort were more likely to have the HLA-DR3/4, DQB1*0302 genotype and a father or sibling with T1D (Table 1). Being non-Hispanic white was marginally associated with an increased T1D risk. Univariately, daycare attendance and breastfeeding duration were not associated with T1D risk (Table 1). After

Table 1: Characteristics of the analysis cohort by T1D status (the Diabetes Autoimmunity Study in the Young).

	Number (%)			
	Developed T1D (n = 58)	Did not develop T1D (n = 1725)	Univariate HR (95% CI)	P value
Age (mean (SD) at T1D diagnosis or at last followup, years)	8.5 (3.8)	8.8 (5.6)	N.A.	N.A.
HLA-DR3/4, DQB1*0302	32 (55.2)	412 (23.9)	3.35 (1.99–5.64)	<0.0001
First degree relative with T1D				
Mother	4 (6.9)	174 (10.1)	1.08 (0.37–3.14)	0.88
Father or sibling	32 (55.2)	390 (22.6)	3.50 (1.99–6.17)	<0.0001
Birth order (first/only child)	20 (36.4)	712 (43.3)	0.78 (0.45–1.38)	0.40
Female	27 (46.5)	836 (48.5)	0.93 (0.54–1.60)	0.80
Race/ethnicity (non-Hispanic white)	50 (86.2)	1202 (69.7)	2.10 (0.92–4.81)	0.08
Maternal age, mean (SD), years	30.6 (5.9)	30.0 (5.7)	1.00 (0.95–1.05)	0.97
Maternal education (>12 years)	41 (70.7)	1255 (75.6)	0.62 (0.35–1.12)	0.11
Crowding (≥1 people/room at 6 mo.)	6 (10.3)	227 (13.2)	1.05 (0.45–2.47)	0.91
Ever breastfed	54 (93.1)	1483 (86.0)	1.84 (0.67–5.03)	0.23
Breastfeeding duration, mean (SD), months	5.6 (6.9)	6.4 (7.0)	0.96 (0.91–1.02)	0.16
Daycare attendance in the first 2 years of life	27 (46.5)	803 (46.5)	0.89 (0.53–1.49)	0.65

Table 2: Association between daycare attendance and risk of developing T1D (the Diabetes Autoimmunity Study in the Young).

Variable	Adjusted HR	95% CI	P value
HLA-DR3/4, DQB1*0302	5.06	(2.95–8.69)	<0.0001
First degree relative with T1D			
Mother	1.80	(0.64–5.06)	0.27
Father or sibling	4.79	(2.60–8.84)	<0.0001
Race/ethnicity (non-Hispanic white)	1.95	(0.78–4.85)	0.15
Breastfeeding duration, months	*	*	0.38
Daycare attendance in the first 2 years	*	*	0.21
Breastfeeding duration * daycare attendance in the first 2 years of of life	*	*	0.02

*The HRs and CIs of the breastfeeding duration and day care attendance in first 2 years variables were not calculated as these variables were components of the significant interaction term. The interaction between these variables is depicted in Figure 1.

adjusting for HLA, first degree relative with T1D, ethnicity, and breastfeeding duration, attending daycare during the first two years of life was not associated with the risk of developing T1D (HR: 0.89; CI: 0.54–1.47, P value = 0.64), while each additional month of breastfeeding duration was associated with a 5% decreased risk of developing T1D (HR: 0.95; CI: 0.90–1.00, P value = 0.05).

We *a priori* hypothesized that breastfeeding would modify the effect of attending daycare on the risk of developing T1D. Our analyses showed that breastfeeding duration interacted with daycare attendance, where daycare attendance was associated with increased risk of T1D in nonbreastfed children and a decreasing risk of T1D with increasing breastfeeding duration (interaction P value = 0.02) (Table 2). To demonstrate this relationship, we calculated HR estimates and 95% CI for daycare attendance for 0, 3, 6, 9, and 12 months of breastfeeding duration (Figure 1). The highest risk of developing T1D was observed in children who attended daycare and were not breastfed (HR: 1.56; CI: 0.77–3.16), and the lowest risk of T1D was observed in children who attended daycare and were breastfed for 12 months (HR: 0.37; CI: 0.13–1.06).

4. Discussion

We found that breastfeeding modified the effect of daycare, where daycare attendance was associated with increased risk of T1D in nonbreastfed children and a decreasing risk of T1D with increasing breastfeeding duration. These findings lend support to both the trigger-booster hypothesis and the hygiene hypothesis. The trigger-booster hypothesis argues that the immunologic trigger in the natural history of T1D is an infection, such as an enterovirus infection. This infection then triggers the autoimmune response that progresses towards overt diabetes [14]. The Eurodiab Substudy 2 showed that reported infections early in a child's life, noted in the hospital record, were found to be associated with an increased risk of T1D (i.e., evidence for the trigger-booster hypothesis); however, preschool/daycare attendance used as a proxy to

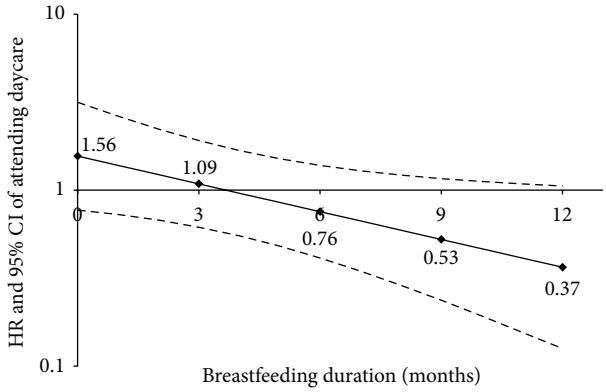

Figure 1: The association between attending daycare in the first 2 years of life and risk of developing T1D in children who were breastfed for 0 months (i.e., not breastfed), 3 months, 6 months, 9 months, and 12 months. The HRs and 95% CIs are calculated from the interaction term, daycare ∗ breastfeeding duration (as a continuous variable), in the model adjusting for HLA, first degree relative with T1D, and ethnicity (interaction P value = 0.02).

measure total infections in early childhood was found to be inversely associated with diabetes [15], suggestive of the hygiene hypothesis. Our findings of an increased risk of T1D for attending daycare in the absence of breastfeeding support the trigger-booster hypothesis that daycare may be increasing exposure to diabetogenic infections that are triggering the development of autoimmunity. The decreased risk associated with daycare attendance in breastfed children supports the hygiene hypothesis, suggesting that breastfeeding is providing immunological support to fight off diabetogenic infections while daycare provides an environment that stimulates the immune system with nonspecific infections preventing immune responses against self-antigens. These findings suggest that breastfeeding may be required to glean the benefits of the daycare environment. In sum, breastfeeding may provide the immune support to fight off diabetogenic infections, while allowing the low immune stimulation found in daycare environments to prevent the development of autoimmunity and T1D.

One limitation to using daycare as a proxy for infections is that it does not account for the effects of specific infections, as some infections have been associated with increased risk of T1D development and this detail is lost in using daycare as a proxy for all infections [16]. Furthermore, our questionnaire data lacked the level of detail to calculate duration or intensity of daycare exposure; therefore, this study could not evaluate a dose-response relationship between amount of time in daycare and risk of developing T1D. A strength of the study is that the data were collected prospectively, increasing the accuracy. However, the small number of children with T1D may limit the inference.

The presence of the interaction between daycare attendance and breastfeeding duration suggests a complex interplay between exposures in the etiology of T1D and may explain, in part, the difficulty in identifying environmental risk factors for the disease. Due to the small number of children with T1D in our analysis cohort, our findings should be confirmed in other populations. Future analyses examining environmental exposures in the risk of T1D should hypothesize and test biologically plausible effect modifications such as the one identified here, in order to more clearly elucidate the etiology of the T1D.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by National Institutes of Health Grant nos. R01-DK49654, R01-DK32493, R01-DK302083, and R01-DK050979 and the Diabetes Endocrine Research Center, Clinical Investigation & Bioinformatics Core P30 DK 57516. The authors thank the dedicated and talented staff of the DAISY study for their clinical, data, and laboratory support and all the DAISY children and families who generously volunteered their time and knowledge.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 326918, 7 pages
http://dx.doi.org/10.1155/2015/326918

Review Article

Luminex and Other Multiplex High Throughput Technologies for the Identification of, and Host Response to, Environmental Triggers of Type 1 Diabetes

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Received 4 August 2014; Accepted 7 September 2014

Academic Editor: Jill M. Norris

Complex interactions between a series of environmental factors and genes result in progression to clinical type 1 diabetes in genetically susceptible individuals. Despite several decades of research in the area, these interactions remain poorly understood. Several studies have yielded associations of certain foods, infections, and immunizations with the onset and progression of diabetes autoimmunity, but most findings are still inconclusive. Environmental triggers are difficult to identify mainly due to (i) large number and complex nature of environmental exposures, including bacteria, viruses, dietary factors, and environmental pollutants, (ii) reliance on low throughput technology, (iii) less efforts in quantifying host response, (iv) long silent period between the exposure and clinical onset of T1D which may lead to loss of the exposure fingerprints, and (v) limited sample sets. Recent development in multiplex technologies has enabled systematic evaluation of different classes of molecules or macroparticles in a high throughput manner. However, the use of multiplex assays in type 1 diabetes research is limited to cytokine assays. In this review, we will discuss the potential use of multiplex high throughput technologies in identification of environmental triggers and host response in type 1 diabetes.

1. Introduction

Type 1 diabetes (T1D) results from complex yet poorly defined interactions between environmental agents, the immune system, and genetic factors (Figure 1). T1D is a chronic T-cell mediated disease, characterized by selective loss of insulin-producing β-cells in the pancreatic islets [1]. There is an annual average of 3% increase in T1D incidence worldwide and the incidence rates are also increasing in the countries with no previous record of having T1D [2, 3]. It is believed that genetic susceptibility is a prerequisite for the development of T1D; however, not all genetically predisposed individuals develop clinical disease and subjects with low risk or protective genes also have been found to develop T1D. These observations suggest that apart from genetic susceptibility additional factors trigger the process of β-cell autoimmunity and subsequent clinical disease.

If these environmental triggers are known, change in life style is likely to offer the most powerful strategy for effective prevention of T1D. If successful, this approach can target the whole population or at least the population with increased genetic susceptibility. In pilot studies, dietary interventions have been successfully tested to manipulate appearance of β-cell autoimmunity in high risk children [4, 5]. However, there has been little progress in this area partly due to nonavailability of technologies to measure different types of environmental exposures and host response in large sample sets. The recently developed multiplex technologies have enabled the measurement of greater number of analytes in a high throughput manner.

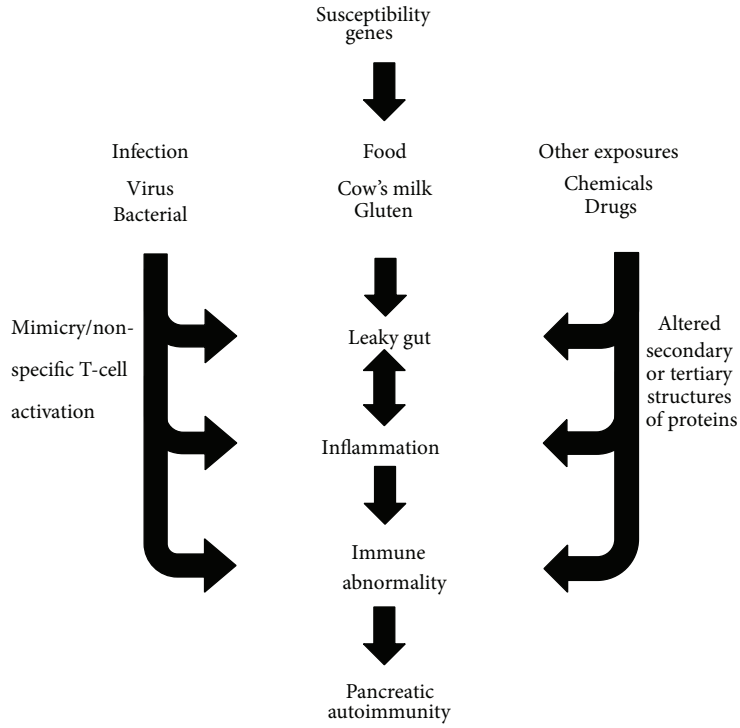

Figure 1: Susceptibility genes and environmental triggers in development and progression of type 1 diabetes. In genetically susceptible individuals, different classes of environmental exposures such as diet, infection, and pollutants lead to increase in peripheral and mucosal inflammation causing leaky gut and aberrant immune reaction towards pancreatic β-cells.

2. Environmental Triggers of T1D

Several studies have observed seasonal patterns in the presence of serum antibody titers (incidence being more common during cold), in part due to the role of recent infections in the development of β-cell auto-antibodies [6]. Viral infections have been suggested to be responsible for T1D autoimmunity for a century, but recent studies have provided stronger data [7]. A number of viruses have been shown to be associated with T1D autoimmunity including *Enterovirus*, rubella, mumps, and rotavirus [8]. Taking into account the timing and profiles of the autoantibody peaks observed in several studies, *Enterovirus* infections appear to be the most probable trigger of β-cell autoimmunity [9]. Despite significant amount of evidence, the role of viruses in the development of T1D autoimmunity is not conclusive. The long silent period between the infection and clinical onset of T1D may lead to loss of the viral signatures in serum [9, 10]. Some authors believe that infections may protect from development of T1D autoimmunity. The proponents of the "hygiene hypothesis" suggest that children experiencing more infection in childhood are more protected; however there has been no consensus among researchers [11–13]. On the other hand the "trigger-booster hypothesis" claims that progression to clinical type 1 diabetes typically requires the unfortunate combination of genetic disease susceptibility, a diabetogenic trigger, and a high exposure to a driving antigen [14].

A number of dietary factors have also been found to be associated with development of T1D, including cow's milk, wheat gluten, and vitamin D deficiency [15, 16]. Some studies have shown the protective role of breast feeding and other nutrients [17, 18]. Several other studies have shown positive association of β-cell autoantibodies with introduction of milk based or wheat based formula early in life [19]. The results of these studies have always been mixed with no consensus on specific dietary factor or nutrient being conclusively responsible for development of T1D [20].

Recent studies suggest that β-cell autoantibodies are preceded by active inflammation [21]. Viral infections, dietary factors, and changes in gut microbiome lead to intestinal inflammation and may contribute to the increased permeability of the gut [16]. Vaarala et al. showed that the complex interactions between gut microbiome, intestinal permeability, and mucosal immunity contribute to the pathogenesis of T1D [22, 23]. These authors suggested that leaky gut allows entry to certain proteins present in cow's milk and wheat and as such leads to T1D autoimmunity in at risk subjects [22].

3. Measurement of Environmental Exposures and Host Response in T1D

Elucidation of the environmental exposure in T1D has been a highly contentious issue. Although studies have postulated a role of several environmental agents in T1D, progress in this area has been slow. This at least in part is attributed to the complex nature of the environmental exposures. A large number of environmental exposures need to be explored

Table 1: Characteristics of currently available array based high throughput technologies.

Technology/manufacturer	Maximum number of analytes	Maximum number of samples	Volume of sample	Dynamic range
Luminex/Luminexcorp	500	96 or 384	1–5 μL	>4.5 logs
SimOa/Quanterix	10	384	1–10 μL	>4 logs
Flow Cytomix/Afffymetrix	20	96	25 μL	—
Cytometric Bead assay/BD biosciences	30	96	25–50 μL	—
Barcoded Magnetic Beads/Applied Biocode	128	96	1–5 μL	5 logs
Antibody arrays/Quansys Bioscience*	25	96	30–50 μL	—
Antibody arrays/Meso Scale discovery*	10	96 or 384	30–50 μL	>4 logs
Antigen Arrays/Thermo Scientific	10	96	40 μL	—

*Detection is based on chemiluminescence or electrochemiluminescence.

from different classes, including bacteria, viruses, dietary factors, and pollutants and the measurement involves different classes of molecules including DNA, RNA, proteins, metabolites, small molecules, and antibodies.

Also, due to a huge lag time between the time of exposure and the onset of disease, sometimes it is difficult to identify the environmental trigger itself. However, such an exposure may leave a signature or fingerprint which may be present for a longer time (host response). Thus measuring this host response may provide additional information correlated with the environmental triggers. For example, circulating levels of IgG and IgM against viruses have been shown in T1D patients [9, 24–26]. Similarly, measurement of cytokines, chemokines, and other plasma proteins could provide us a hint on the class of environmental exposures [27]. Measuring the host response may provide us unique fingerprints which may be used as additional markers for disease progression.

4. Current Approaches

Currently, many available "omics" technologies are being used to study the environmental exposures and host response in T1D. To identify molecular and cellular signatures, we have measured several classes of biomolecules in our laboratory [28–32]. ELISA assays are being used for detection of *Saccharomyces cerevisiae* [33], wheat protein Glb-3 [34, 35], gluten [36], gliadin, cow milk proteins in T1D, and celiac disease [37]. Giongo et al. used pyrosequencing approach to show that the intestinal microbiome of the children progressing to clinical disease was less diverse than healthy children [38]. PCR based typing was utilized to identify the *Enterovirus* DNA circulating in the serum of newly diagnosed T1D patients [16, 39]. However, the studies focusing on measurement of single environmental agent provide a skewed view on the environmental exposure. This can be remedied by measuring several types of environmental agents and host response to obtain a fingerprint of overall environmental exposure in a high throughput manner.

5. Multiplex Technologies

To measure several hundreds of the environmental triggers and the host response in larger sample sets economically, high throughput technologies are needed [28–32]. ELISAs or radioimmunoassays have been the preferred technologies for the measurement of low abundance agents in the serum. Recently, multiplex assays have been developed from traditional ELISA assays with the purpose of measuring multiple analytes in the same sample at the same time. Multiplex assays are available in several different formats based on the utilization of flow cytometry, chemiluminescence, and array technology (Table 1). Compared with traditional ELISA, multiplex arrays have a number of advantages including (i) high throughput multiplex analysis, (ii) less sample volume requirements, (iii) efficiency in terms of time and cost, (iv) ability to evaluate the levels of given analyte in the context of multiple others, (v) ability to perform repeated measures of the multiplex panels in the same experimental assay conditions, and (vi) ability to reliably detect analytes across a broad dynamic range of concentrations [40].

Bead-based multiplex assays represent probably the most commonly used format developed by several companies. Multianalyte profiling (xMAP) technology from Luminex (http://www.luminexcorp.com/) and several other companies employ proprietary bead sets which are distinguishable under flow cytometry (Figure 2). The platform is a suspension array where capture moieties are covalently coupled with internally dyed microspheres, and phycoerythrin-labeled antihuman antibodies bind to the specific antigen-antibody complex on the bead set. Response is thus recognized and measured by the differences in both bead sets, with fluorogenic emissions detected using red (bead set) and green

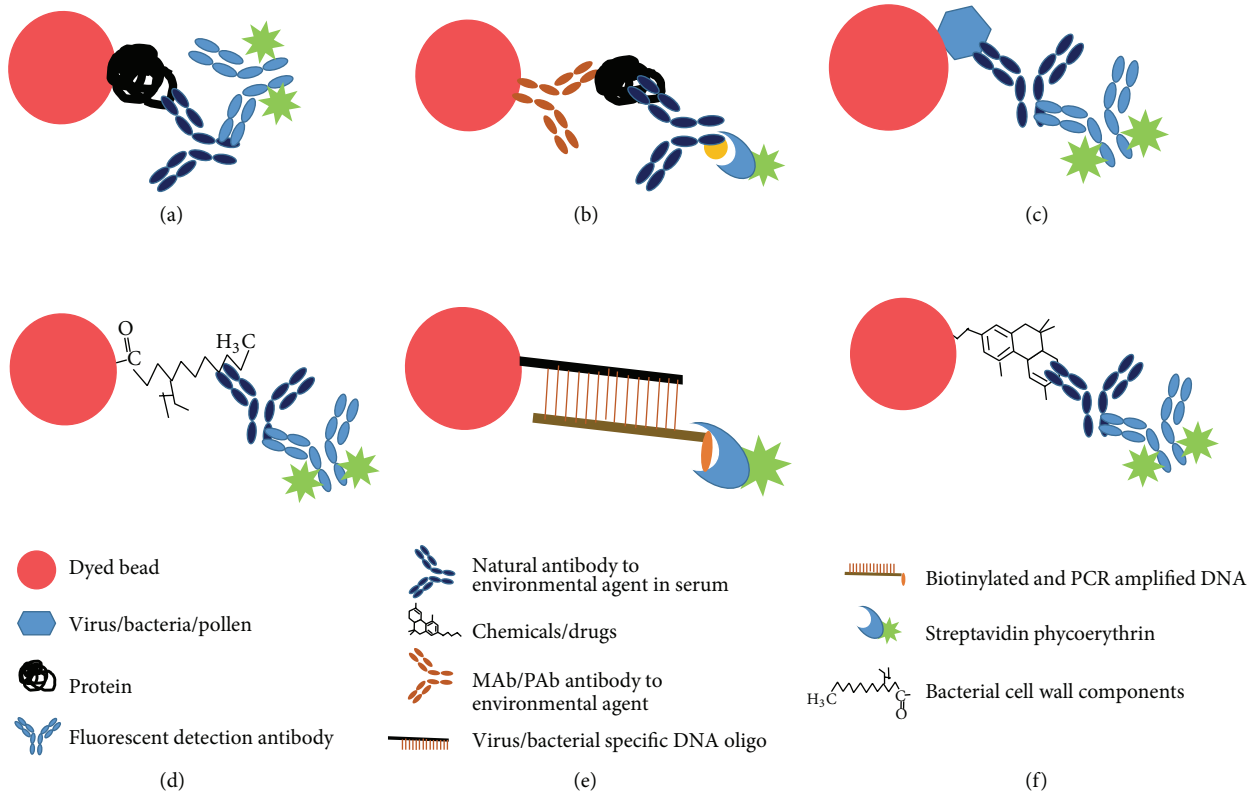

FIGURE 2: Luminex bead arrays could be used to detect different classes of environmental triggers. (a) Protein(s), (b) monoclonal (MAb) or polyclonal (PAb) antibodies, (c) viruses, (d) bacterial cell wall components, (e) DNA from virus/bacteria, and (f) chemicals/drugs can be covalently coupled to the beads. Coupled entities can be detected using fluorescently labeled appropriate detection agents.

(detection of entities) lasers. The flexibility of the system allows covalent coupling and detection of several different classes of molecules or macroparticles.

6. Use of Multiplex Assays in Other Research Areas

Multiplex assays have been at the forefront for epidemic monitoring by health agencies in USA and abroad. Developments in the PCR technology and discrimination methods combined with the multiplex assays have improved the detection of coinfections with reduced cost and sample volumes required for analysis [40]. The most active research areas using the multiplex immunoassays are allergy, asthma, infectious disease, autoimmunity, and toxicology (Table 2). Extensive research efforts have been taken to test the feasibility of the Luminex xMAP technology to detect the autoantibodies to autoantigens, IgE response to grass and tree pollen, virus and bacterial serotypes, and weaponized microbial agents. Researchers in the fields of vaccine development and epidemiology have extensively documented the use of multiplexed assays to identify targets using antibody-based capture or DNA fragments specific to each serotype of bacteria or viruses. Using monoclonal antibodies to the individual serotypes, Yu et al. used Luminex technology to detect 26 different serotypes for *Streptococcus pneumoniae* in serum [41]. Other investigators have used antibody-based multiplex assays to characterize microbial pathogens and agents [42–44]. Conventional and real-time RT-PCR, combined with Luminex bead array, were used in detection of multiple viruses to identity the microbial agents in disease individuals [45, 46]. All these reports provide sufficient feasibility regarding the development of multiplex assays to identify the environmental triggers in T1D.

7. Technological Considerations for Developing Multiplex Assays for Environmental Triggers of T1D

Although multiplex technologies offer several advantages over ELISA approaches, caution must be exercised for developing assays. In this section some of the critical issues are discussed and possible solutions are offered for development of these assays.

(1) Multiplex bead assays, by their very nature, involve measurement of several potential analytes in a single well. Therefore, cross-interactions between different capture antibodies and antigens in the sample/assay solution are inherently possible. Cross-reactivity of antibodies should be tested first and the lowest amount should be used to minimize such cross-reactions. The individual panels should be designed using

Table 2: Research areas and available assays for Luminex platform.

Research Areas	Manufacturers	Species
Immunology/inflammation/apoptosis/tissue remodeling markers	Millipore, RnD Systems, Life Technologies, Luminex, Biorad	Hu/Ms/Rt/Ca/Mo
Phosphoproteins, signal transduction proteins	Millipore, Life Technologies, Biorad	Hu/MS
Cancer markers	Millipore, Biorad	Hu/Ms
Metabolic markers	Millipore, Life Technologies	Hu
Cardiovascular markers	Millipore, Life Technologies, Biorad	Hu
Toxicity markers	Millipore	Hu
Neuroscience	Millipore, Biorad	Hu
Antibody isotyping	Biorad	Hu/Ms
Auto-antibody measurement	One Lambda, Origene	Hu
Genotyping, epigenetics, and gene expression profiling	Affymetrix, One Lambda, Origene, Active Motiff	Hu
HLA typing	One Lambda	Hu
Environmental agents/allergens/food	Thermo Scientific	Hu
Bacterial/virus serotyping	Biovet/Luminex	Hu
Drugs/chemical	Under development in research laboratories	Hu
Vaccine testing	Luminex	Hu

Ca: cat, Hu: human, Mo: monkey, Ms: mouse, Rt: rat.

the analytes with minimal cross-reactivity among the analytes and detection reagents. Secondly, multiplex assays are performed using a common binding and wash buffers, and these may not be the optimum conditions for all the analytes. This can be solved by creating custom multiplex assays having similar binding and washing conditions.

(2) In the bead-based multiplex arrays, the reactions take place among capture entities and analytes which are freely mobile in solution, providing more sensitivity to measure circulating levels of analytes. However, abundant proteins present in bodily fluids, such as serum, may affect multiplex results. The abundant proteins serve both as reservoir and as carrier of small molecules such as cytokines and metabolites. The bound complexes may not get captured in the solution phase or the detecting reagent may not be able to access the required binding site. This interference from abundant proteins may require development of additional processing steps prior to multiplex assay.

(3) The commercially available Luminex kits can measure up to 60 cytokines, chemokines, and ligands. However the linear range of the standard curve and the levels in the samples limits the number of analytes which can be measured simultaneously. This issue can be solved by performing pilot experiments to select the analytes having similar dynamic range (customized panels). For a particular value of sample dilution, the analyses will be selected in the same panel if their median fluorescence intensities fall in the linear range of the standard curve.

(4) A defined set of principles are required to establish good laboratory practices and must be followed in the planning, performing, monitoring, recording, reporting, and archiving of all laboratory measurements. To prevent quality problems a good quality assurance policy must be established. The variations in high throughput measurements emerge from many sources. To reduce plate to plate variation and to produce consistent results over time, a dilution series of pooled control serum on each plate should be included for the normalization.

(5) The bulk of our knowledge about T1D pathogenesis comes from studies of animal models. Data from human subjects are scarce and difficult to replicate for many reasons including, but not limited to, large variations of the studied phenotypes at the individual and population levels and differences in study design (insufficient sample size, poor matching of patients and controls, case/control versus prospective, and so forth). Important considerations in the proper design of human studies include prospective studies, which minimize many of the drawbacks associated with cross sectional comparisons that are commonly used in human studies.

8. Conclusions

Multiplex technologies offer opportunities to examine the different "classes" of environmental triggers of T1D in a time- and cost-efficient high throughput manner. While the use of such technologies is still at early stage, recent reports from other research areas highlight their usefulness and feasibility to evaluate the environmental exposure and host response in T1D pathogenesis. Also, multiplex technologies offer substantial sample savings over traditional ELISA measurements.

Despite potential advantages of this new technology, expertise and experience are required for new assay development. We have used this technology in examining several classes of serum proteins in T1D [47–49]. In our view, multiplex technology could be successfully used for the evaluation of different classes of environmental exposures and host responses in T1D pathogenesis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work is partly supported by NIH Grants to Jin-Xiong She (U01DK063865 and RO1HD37800). Sharad Purohit was supported by JDRF Grants (10-2006-792 and 2-2011-153).

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Volume 2014, Article ID 176564, 8 pages
http://dx.doi.org/10.1155/2014/176564

Clinical Study

Investigation on Carbohydrate Counting Method in Type 1 Diabetic Patients

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Received 21 May 2014; Revised 16 July 2014; Accepted 19 July 2014; Published 17 August 2014

Academic Editor: Kjersti S. Rønningen

Objective. The results from Diabetes Control and Complications Trial (DCCT) have propounded the importance of the approach of treatment by medical nutrition when treating diabetes mellitus (DM). During this study, we tried to inquire carbohydrate (Kh) count method's positive effects on the type 1 DM treatment's success as well as on the life quality of the patients. *Methods*. 22 of 37 type 1 DM patients who applied to Eskişehir Osmangazi University, Faculty of Medicine Hospital, Department of Endocrinology and Metabolism, had been treated by Kh count method and 15 of them are treated by multiple dosage intensive insulin treatment with applying standard diabetic diet as a control group and both of groups were under close follow-up for 6 months. Required approval was taken from the Ethical Committee of Eskişehir Osmangazi University, Medical Faculty, as well as informed consent from the patients. The body weight of patients who are treated by carbohydrate count method and multiple dosage intensive insulin treatment during the study beginning and after 6-month term, body mass index, and body compositions are analyzed. A short life quality and medical research survey applied. At statistical analysis, t-test, chi-squared test, and Mann-Whitney U test were used. *Results*. There had been no significant change determined at glycemic control indicators between the Kh counting group and the standard diabetic diet and multiple dosage insulin treatment group in our study. *Conclusion*. As a result, Kh counting method which offers a flexible nutrition plan to diabetic individuals is a functional method.

1. Introduction

The importance of maintaining a strict glycemic control in diabetics is well-established. To achieve the desired targets, medical diet therapy, exercise, and the medical strategies should be administered accurately and regularly. The results from the DCCT have demonstrated the importance of medical diet therapy in the treatment of diabetes mellitus (DM). One of the diet strategies recommended by the DCCT is the carbohydrate counting method, which has started to draw attention in the recent years [1–7]. As the diabetes mellitus is a chronic disease which effects quality life and the mental status of patients, it is important to improve the quality life of such patients and to pay attention to the life style changes [8, 9].

Counting carbohydrates leans upon 3 basic facts.

(1) Clinical studies have shown that carbohydrates are the main factor that effects the postprandial blood glucose level and determines the need of insulin.

(2) Carbohydrates are transformed into glucose in 2 hours after the ingestion and they get into systemic circulation from the first 15 min.

(3) Postprandial glycemic response and need of insulin levels are determined by total carbohydrate amount that is ingested rather than the kind of carbohydrate [3–7].

The present study is designed to investigate the effects of the carbohydrate counting method, a medical diet strategy, on the quality of life as well as the success of the treatment in type 1 diabetic patients.

2. Materials and Methods

Thirty-seven patients with DM type 1 who were under surveillance in Eskişehir Osmangazi University, Medical Faculty, Department of Internal Diseases, Field of Endocrinology and Metabolic Diseases, Diabetes Outpatient Department, were included in the study. Kh counting method was applied on 22 patients who contented to experience this method and the other 15 cases had multiple dose intensive insulin treatment and standard diabetic diet as control group, and these two groups were closely followed up. Required approval was taken from the Ethical Committee of Eskişehir Osmangazi University, Medical Faculty, as well as informed consent from the patients. Required approval was taken from the Ethical Committee of Eskişehir Osmangazi University, Medical Faculty, as well as informed consent from the patients. Twenty-two patients were given Kh counting method training by a dietitian in three steps mentioned below. At Level 1, Kh counting method, its pros, and cons were explained to the patients. In the second step, the patients were asked about their opinions about Kh counting method and their questions were answered. At Level 2, the patients were informed about how much carbohydrate exists in which food, the effects of the changes in food preparation, and the effects of protein, fiber, and fat on carbohydrate absorption. Moreover, in this period we tried to find out the carbohydrate amount in various food portions using measuring cups. At Level 3, the patients who were familiar with Kh counting method were admitted to the hospital and first application was started accompanied by a dietitian. In this period, other than various food groups the patients were made to practice the method according to their food preferences. Besides, conformity of the method practiced with the insulin used was assessed. The patients were made to stay at the hospital until they could apply the method on their own. This period was found as 7 days according to the learning capacities of the patients. Furthermore, their glycemic indices in their normal life routine were evaluated once every 3 days. The patients were monitored in continuous communication with the doctor and the dietitian from whom they learned the Kh counting method. Diabetes years of the patients involved in the study were recorded from their diabetes files. In the first examination and in the examination after 6 months of the 22 patients practicing carbohydrate counting and 15 patients defined as control group all with Type 1 DM, the following data were reported. Height and weight of the cases were recorded with the same standard measurement device. Systolic and diastolic blood pressure (mmHg) of the patients were recorded with the same tension gauge while being seated after 15 minutes of rest. Waist and hip circumference of the patients were recorded in centimeters. Waist/hip ratio was calculated by dividing waist circumference (cm) into hip circumference (cm). Moreover, BMI (body mass index), FAT% (fat ratio), fat mass, and FFM (fatless mass) ve TBW (total body water) of the patients were measured by using Body Composition Analyzer (TBF-300 M) device. Preprandial morning venous blood samples of the patients after around 10 hours were taken and HbA1c and fructosamine levels were measured using Roche/911 Hitachi device and proper (modular p) kit. Average of three-day preprandial and postprandial blood glucose (mg/dL) values, total cholesterol (mg/dL), triglyceride (mg/dL), and HDL cholesterol (mg/dL) were measured using immunometric chemiluminescence method and Immulite\1000 device. LDL cholesterol value was calculated with Friedewald formula. LDL cholesterol = total cholesterol – (HDL + triglyceride/5). Total cholesterol/HDL ratio was calculated. The patients were assessed for diabetic retinopathy and nephropathy at the beginning of the study and in the 6th month of the study by means of fundoscopy and GFR (glomerular filtration rate) and of their microalbuminuria levels. Daily total insulin doses of the patients in the carbohydrate counting group and in the control group were recorded before and 6 months after the study. Major hypoglycemia attack frequency (symptomatic and/or blood glycose value below 50 mg/dL) doses of the patients in the carbohydrate counting group and in the control group were recorded before and 6 months after the study of the living quality of the patients in the carbohydrate counting group and in the control group were assessed before and 6 months after the study using Turkish version of a 36-item short health survey and living quality scale *(Medical Outcomes Study 36-Item Short Form (MOS SF-36))*. With this scale living quality was assessed taking 9 functions into account. These are general state of health, change in health over the last one year, physical function, mental function, social function, pain, mental health, and energy [10, 11]. Results were given as mean ± standard error using parametric tests for the variables showing a homogenous distribution. For the variables not showing a homogenous distribution it was defined as median using nonparametric tests. The difference between the values at the beginning of the study and in the 6th month of the study was calculated with t-test and the difference between nonparametric values with chi-squared test and Mann-Whitney U test. A total of 37 type 1 diabetic patients under follow-up at the Eskisehir Osmangazi Medical Faculty, Internal Diseases Department, Endocrinology and Metabolism Section, Diabetes Polyclinic, were included in the trial. 22 patients volunteering to apply the carbohydrate counting method were administered with this method while the remaining 15 patients received multiple dose intensive insulin treatment and standard diabetic diet and kept under close monitoring for 6 months.

Table 1: Patients characteristics.

	Kh counting group	Control group
Number of patients (n)	22	15
Gender (male/female)	(8/14)	(7/8)
Age (year)	29.18 ± 2.06	29.67 ± 2.32
DM duration (years)	11.00 ± 1.34	6.80 ± 1.74

3. Results

22 patients were trained on the carbohydrate counting method by the dietician. Patients' characteristics were summarized in Table 1.

Table 2: The progress of the metabolic markers of the patients in the carbohydrate counting group.

	Carbohydrate counting group ($n = 22$)		P values
	Pre-treatment baseline values	Values at 6 months	
TA (mmHg)			$P = 0.065$
Systolic	109.77 ± 5.24	122.04 ± 2.60	($P > 0.05$)
Diastolic	74.09 ± 1.63	79.54 ± 0.72	$P = 0.005$
			(**$P < 0.01^{**}$**)
Fasting blood glucose (mg/dL)	149.63 ± 17.65	160.40 ± 19.00	$P = 0.630$ ($P > 0.05$)
Post-prandial blood glucose (mg/dL)	174.59 ± 15.46	169.27 ± 8.05	$P = 0.710$ ($P > 0.05$)
HbA1c (%)	8.14 ± 0.48	8.00 ± 0.38	$P = 0.699$ ($P > 0.05$)
Fructosamine (mg/dL)	417.77 ± 22.56	404.90 ± 23.59	$P = 0.525$ ($P > 0.05$)
Uri acid (mg/dL)	3.43 ± 0.26	3.94 ± 0.30	$P = 0.003$ (**$P < 0.01^{**}$**)
Total cholesterol (mg/dL)	174.50 ± 8.24	170.77 ± 6.41	$P = 0.534$ ($P > 0.05$)
Triglyceride (mg/dL)	79.04 ± 6.83	75.90 ± 5.87	$P = 0.587$ ($P > 0.05$)
HDL cholesterol (mg/dL)	71.18 ± 4.19	67.40 ± 3.44	$P = 0.112$ ($P > 0.05$)
LDL cholesterol (mg/dL)	84.49 ± 7.01	92.86 ± 6.01	$P = 0.120$ ($P > 0.05$)
Total cholesterol/HDL cholesterol ratio	2.52 ± 0.18	2.71 ± 0.18	$P = 0.174$ ($P > 0.05$)

Patients were monitored for 6 months. While one of the patients using the carbohydrate counting method continuously used aspart insulin via insulin infusion pump, all the other patients were administered with multiple dose insulin injection. Prior to treatment, patients were using NPH or insulin glargine as basal insulin and short acting (regular) or fast-acting (aspart insulin) insulin as bolus. In patients using the carbohydrate counting method, insulin glargine was initiated as the basal insulin on the day they shifted to this method (initiated as 40% of the pretreatment total insulin dose and dose adjusted according to patient's requirements) and fast-acting aspart insulin as the bolus while patients in the control group maintained their current pretreatment insulin.

The group of patients applying the carbohydrate counting method and the control group were observed not exhibiting a statistically significant difference in age and diabetes duration ($P > 0.05$) and having similar characteristics.

At study baseline, there was no statistically significant difference between the carbohydrate counting group and the control group with respect to systolic and diastolic blood pressure, fasting and postprandial blood glucose, HbA1c, fructosamine, uric acid, triglyceride, total cholesterol, LDL cholesterol, and total cholesterol/HDL ratio ($P > 0.05$). The baseline HDL level was higher in the carbohydrate counting group ($P < 0.05^{*}$).

At 6 months of the trial, an increase was detected in both systolic and diastolic blood pressure in the carbohydrate counting group. Although these increases remained within the normal limits, there was no statistical significance for systolic blood pressure while the increase in the diastolic blood pressure was considerable ($P < 0.01^{**}$).

In the carbohydrate counting group, there was no statistically significant difference in fasting and postprandial blood glucose, HbA1c, and fructosamine levels at 6 months of treatment compared to the study baseline ($P > 0.05$).

In the carbohydrate counting group, total cholesterol, triglyceride, and HDL cholesterol values were decreased and a statistically nonsignificant increase was detected in LDL cholesterol and total cholesterol/HDL cholesterol ratios at the end of the 6th month. In the 6-month period, a statistically considerable increase was detected in the uric acid values ($P < 0.01^{**}$) (Table 2).

In the control group, there was no statistically significant difference in systolic and diastolic blood pressure, fasting and postprandial blood glucose, HbA1c, fructosamine, uric acid, triglyceride, total cholesterol, LDL cholesterol, and total cholesterol/HDL ratio at the end of 6 months compared to study baseline ($P > 0.05$).

No statistically significant difference was detected between the carbohydrate counting group and the control group with respect to diabetic retinopathy findings, microalbuminuria, GFR, and hypoglycemia frequency markers ($P > 0.05$) at study baseline. There were no findings of macrovascular complications in either group.

There was no change detected in findings of diabetic retinopathy in the carbohydrate counting group and the control group. Despite the reduction observed in the frequency of microalbuminuria and hypoglycemia in the carbohydrate counting group, this did not reach a level of statistical significance ($P > 0.05$).

In the control group, values for microalbuminuria, GFR, and hypoglycemia frequency showed a statistically nonsignificant increase at the end of 6 months compared to study baseline ($P > 0.05$).

At 6 months of treatment, hypoglycemia frequency was detected to be decreased in the carbohydrate counting group while it increased in the control group. However these values were not statistically significant ($P > 0.05$). In addition, upon comparison of the change in the hypoglycemia frequency between the two groups, the difference was not detected to be statistically significant (Mann-Whitney U test i: 0.138 $P > 0.05$).

At study baseline, no marked difference was detected in the total insulin doses between the carbohydrate counting group and the control group ($P > 0.05$). In the carbohydrate counting group, there was a statistically significant reduction in the total insulin dose at 6 months compared to baseline (55.22 ± 4.70 IU versus 43.77 ± 3.05, $P < 0.004$). In the control group**,** total insulin doses were also detected to be decreased at the end of 6 months compared to study baseline; however this reduction did not reach statistical significance ($P > 0.05$) (Table 3).

Comparison of the body composition findings in the carbohydrate counting group prior to trial and at 6 months of the trial revealed the following results: there was no statistically significant difference in waist circumference, body weight, waist/hip ratio, and BMI values at 6 months compared to baseline ($P > 0.05$). While a statistically significant reduction was detected in the fat mass and %FAT values, a significant increase was detected in the FFM and TBW values ($P < 0.05^{*}$).

In the carbohydrate counting group, comparison of the baseline quality of life data with the 6-month data revealed a favorable trend in all variables indicating an increased quality of life except for pain. The favorable changes in overall health and the change in health status within the last one year ($P < 0.01^{**}$), physical function, mental function, social function and energy ($P < 0.05^{*}$) were detected to be statistically significant (Table 4).

In the control group, the comparison of the baseline quality of life data with the 6-month data revealed a statistically significant increase only in overall health scores (general point of view: 47.66 ± 5.27 versus 56.06 ± 3.97, $P = 0.015$) ($P < 0.05^{*}$). There was no statistically significant change in the other values ($P > 0.05$) (Table 5).

As for the assessments of the physical function, physical functionality, mental function, and social function, the statistically significant favorable change is more marked in the carbohydrate counting group compared to the control group ($P < 0.05^{*}$). There was no statistically significant difference between the two groups in pain, mental health, and energy level ($P > 0.05$).

4. Discussion

The primary targets of the diabetes treatment include maintenance of life, reduction of symptoms, and increase of the quality of life. Secondarily, the treatment is aimed at the prevention of the long-term chronic complications and early mortality [1–7, 12].

Although both systolic and diastolic blood pressure were at the recommended values in our study, diastolic blood pressure levels were higher in the carbohydrate counting group at the controls performed 6 months later relative to study baseline. This may be attributed to the excessive salt intake resulting from the lack of constraint in diet although salt intake was not recorded in the patients.

Since diabetes mellitus is a disease that affects the carbohydrate, protein, and fat metabolism, nutrition should always be included in the diabetes treatment and training programs [3–9]. Recently, the interest in medical diet therapy has increased in the treatment of DM because medical diet therapy in diabetic individuals was demonstrated to indicate an improved glycemic control as confirmed by an approximate reduction of 1-2 units in HbA1c [3–9, 12, 13].

The trials have suggested that the total amount of carbohydrate intake is more important than the type and source of carbohydrates taken during the main and intermediate meals in type 1 and type 2 DM [3–13].

In type 1 diabetic patients, the lipid profile observed along with the high blood glucose level includes hypertriglyceridemia and HDL lowness, which may be corrected by active insulin treatment [2, 14].

In our study, HDL and triglyceride values were decreased to some extent while LDL cholesterol, total cholesterol/HDL ratio, and the uric acid level were increased. As for the control group, there was no significant change detected. Carbohydrate counting method is beneficial for motivated patients in whom this method can be administered by dieticians. However while applying this method that provides flexibility in eating, patients focus on a macronutrient. Some patients may deviate from their normal diet regimen along with excessive daily energy intake. Therefore, the importance of protein and fat intake should also be adequately explained to the patients applying the carbohydrate counting methods as well as the carbohydrate values. Carbohydrate counting method should be considered in the context of fundamental healthy nutrition plan [2–15].

The association of type 2 DM with macrovascular complications is well-established. However in type 1 diabetic patients, the incidence of macrovascular complications will increase along with the duration of diabetes. In this study, no findings of macrovascular complication were detected in the carbohydrate counting group or the control group. This situation may be related to the young age and the short duration of diabetes observed in our patients.

In patients with a long history of diabetes, the risk of developing both microvascular and macrovascular disease is high. In the UKPDS trial, the importance of a strict blood pressure control as well as a strict blood glucose control in prevention of diabetic complications in type 2 diabetes has been demonstrated while the DCCT study showed the

Table 3: Values for total insulin dose in the carbohydrate counting group and the control group.

	Carbohydrate counting group ($n = 22$)	Control group ($n = 15$)	P values
Total insulin doses Pre-treatment baseline	55.22 ± 4.70	53.53 ± 5.73	$P = 0.820$ ($P > 0.05$)
Total insulin doses at 6 months of the trial	43.77 ± 3.05	46.20 ± 5.06	$P = 0.666$ ($P > 0.05$)
P value	$P = 0.004$ ($P < 0.01^{**}$)	$P = 0.326$ ($P > 0.05$)	

Table 4: Quality of life results in the carbohydrate counting group.

	Carbohydrate counting group ($n = 22$)		P values
	Pre-treatment baseline values	Values at 6 months	
Overall health (general point of view)	55.13 ± 3.90	68.31 ± 2.42	$P = 0.002$ (**$P < 0.01^{**}$**)
Change in health status within the last one year	63.63 ± 5.13	89.77 ± 2.68	$P = 0.000$ (**$P < 0.001^{***}$**)
Physical function	89.77 ± 2.64	92.72 ± 1.96	$P = 0.238$ ($P > 0.05$)
Physical functionality	82.95 ± 5.03	94.31 ± 2.28	$P = 0.047$ (**$P < 0.05^{*}$**)
Mental function	77.00 ± 5.58	92.77 ± 3.10	$P = 0.022$ (**$P < 0.05^{*}$**)
Social function	84.40 ± 3.37	93.18 ± 2.55	$P = 0.018$ (**$P < 0.05^{*}$**)
Pain	82.31 ± 3.45	80.18 ± 3.22	$P = 0.629$ ($P > 0.05$)
Mental health	59.09 ± 3.82	65.09 ± 2.59	$P = 0.169$ ($P > 0.05$)
Energy	53.63 ± 3.08	63.40 ± 2.72	$P = 0.003$ (**$P < 0.01^{**}$**)

Table 5: Quality of life findings in the control group.

	Control group ($n = 15$)		P values
	Pre-treatment baseline values	Values at 6 months	
Overall health (general point of view)	47.66 ± 5.27	56.06 ± 3.97	$P = 0.015$ (**$P < 0.05^{*}$**)
Change in health status within the last one year	53.33 ± 6.39	61.66 ±4.13	$P = 0.238$ ($P > 0.05$)
Physical function	85.33 ± 4.09	79.33 ± 6.01	$P = 0.098$ ($P > 0.05$)
Physical functionality	75.00 ± 7.71	73.33 ± 7.89	$P = 0.865$ ($P > 0.05$)
Mental function	70.80 ± 7.21	73.00 ± 6.71	$P = 0.809$ ($P > 0.05$)
Social function	67.70 ± 6.98	79.53 ± 5.78	$P = 0.083$ ($P > 0.05$)
Pain	71.33 ± 5.53	66.20 ± 6.51	$P = 0.470$ ($P > 0.05$)
Mental health	57.60 ± 4.37	57.33 ± 4.39	$P = 0.953$ ($P > 0.05$)
Energy	49.66 ± 4.81	54.33 ± 5.13	$P = 0.416$ ($P > 0.05$)

importance of a strict blood glucose control in type 1 diabetes [16–19].

In type 1 diabetic patients, intensive treatment delays the onset of clinically important retinopathy, nephropathy, and neuropathy and slows down the progression by 30–75% [17].

In our study, microalbuminuria values were observed to be decreased to some extent in the carbohydrate counting group while they were detected to be increased in the control group. However these changes did not reach a level of significance. In the DCCT trial, intensive insulin treatment decreased the risk of albuminuria and microalbuminuria by 54% and 39%, respectively [16–18].

If not hindered by hypoglycemia, the HbA1c levels of all diabetic patients would be normal throughout life [10, 20–24]. Hypoglycemia limits the long-term benefits of glycemic control in type 1 diabetes.

In studies where the prandial (bolus) insulin dose is adjusted according to the total carbohydrate content of the meal (or the intermediate meal), the HbA1c level is reported to be decreased by 1–1.5 units [23].

Hypoglycemia frequency was detected to decrease in the carbohydrate counting group while it increased in the intensive treatment control group compared to baseline. However these changes were not statistically significant. At 6 months, there was a favorable difference in the carbohydrate counting group with respect to hypoglycemia frequency; however this difference was not statistically significant compared to the control group.

One of the potential problems that may be experienced by patients applying the carbohydrate counting method is the increased food consumption due to the lack of restriction applied in patients and thus the increase in daily insulin amount administered. Among the patients participating in our trial, the total insulin doses statistically significantly decreased 6 months later compared to baseline in the carbohydrate counting group contrary to what is feared. As for the patients in the control group, the total daily insulin dose decreased; however this reduction did not reach a level of significance.

Weight gain represents a major issue in diabetic patients. In type 1 diabetes, weight gain may result from the imbalance in nutritional factors, physical inactivity, or increased food consumption due to frequent hypoglycemic attacks.

When the carbohydrate counting method is to be applied, the other macronutrients such as fat and protein and their intake amount should be taken into consideration [6–10, 12, 13, 24].

In the carbohydrate counting method, focus is given on only one macronutrient. Patients only count the carbohydrate in their food. In addition, there is no fixed calorie limitation in their diet. Accordingly, patients may deviate from their regular nutritional regimen. In addition, these patients have the tendency to maintain their habits of intermediate meals. Therefore, patients should also pay attention to their daily energy, fat, and protein intake as well as the carbohydrate intake [25].

In our study, there was no difference in body weight, BMI, waist circumference, or waist/hip ratio at the 6-month evaluation in the carbohydrate counting group. However contrary to what is feared, a statistically significant reduction was detected in fat mass and % FAT values in patients applying this method. As for the patients in the control group, there was no significant difference in these parameters at 6 months compared to baseline. These results suggest that, when applied to motivated patients, carbohydrate counting method may prevent excessive food consumption via prevention of frequent hypoglycemic attacks, thereby contributing also to weight loss.

Throughout life, it is very important to detect the quality of life in individuals in cases of disease [26]. Despite the presence of active medical therapies in diabetic patients, most of them do not have a good health and life quality. Most of the diabetic patients worry about their life continuously getting worse. Therefore, while presenting a new offer to a diabetic patient on his/her disease, empathy and active communication should be established with the patients and participation of the patient in this organization should be ensured [27].

Diabetes is a disease that progresses primarily with physical and psychological problems and impairs the associated quality of life significantly. The underlying acute and chronic complications affect the quality of life. Social status, level of education, perception of the disease, diabetes-related diet, exercise, and treatment protocol affect the quality of life as well as the glycemic control in diabetic patients.

In our study, the baseline parameter of social function was detected to be higher in the carbohydrate counting method compared to the control group on quality of life scales. This has demonstrated the importance of the social status of patients in perceiving their disease and the carbohydrate counting method. Therefore, this method is only appropriate for a selected motivated group of patients with a high perception [28–34].

Carbohydrate counting is a method that provides flexibility and increases quality of life within the nutritional regimen in voluntary patients. The improvement observed in the short term is reported to be maintained also in the long term. In the trials performed, the strategy based on adjustment of the insulin dose according to the carbohydrate content of the meal is suggested to be more successful compared to low glycemic index diet in type 1 diabetics. This approach enables lack of constraint in eating and selection of food for diabetic individuals while maintaining glycemic control [35–39].

In type 1 diabetes, achievement of success was demonstrated by teaching of the way to adjust blood glucose as well as the lack of constraint in eating offered to patients. While a significant improvement was demonstrated in HbA1c level in this patient group, there was no significant increase in severe hypoglycemia. What is more, the quality of life was increased by this method; tolerability of treatment and psychological well-being were observed despite the increase in the number of injections and blood glucose monitorization.

In our study, the quality of life was detected to be increased in the carbohydrate counting group as compared to the control group and the pretrial period.

In conclusion, the carbohydrate counting method is not a new approach. There are data indicating that this method has been applied in the nutritional plan of the diabetic

patients since 1921 when insulin was discovered. However recently, the interest in this method has increased along with the use of insulin pumps and insulin analogues extensively in the clinics. The success of this method that provides eating flexibility and increases quality of life in diabetics is dependent on how much the diabetic individual perceives the method. The first of the two major factors that affect this perception is the level of information of the health staff to teach the method and the time spared for the patient. The other factor is patient motivation. The carbohydrate counting method that offers a flexible eating regimen to diabetic individuals is a functional method to render the patient more conscious and active about his/her disease and treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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