Neonatal levels of adiponectin, interleukin-10 and interleukin-12 are associated with the risk of developing type 1 diabetes in childhood and adolescence: A nationwide Danish case-control study

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ABSTRACT

Background/aim: An in-depth understanding of the early phase of type 1 diabetes (T1D) pathogenesis is important for targeting primary prevention. We examined if 14 preselected mediators of immune responses differed in neonates that later developed T1D compared to control neonates.

Methods: The study is a case-control study with a 1:2 matching. The individuals were born between 1981 through 2002. Cases were validated using the National Patient Register and the Danish Childhood Diabetes Register. Interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, interferon gamma, tumor necrosis factor alpha, transforming growth factor beta 1 (active form), leptin, adiponectin, c-reactive protein, mannose-binding lectin and soluble triggering receptor expressed on myeloid cells-1 were measured by using a flowmetric Luminex xMAP® technology. We tested two models both including a number of possible confounders. In the first model (model 1) we also adjusted for HLA-DQB1 genotype. A total of 1930 groups of assay-matched cases and controls (4746 individuals) were included in the statistical analyses.

Results: Adiponectin was negatively associated with later risk of T1D in both models (relative change (RC), model 1: 0.95, P = 0.046 and model 2: 0.95, P = 0.006). IL-10 and IL-12 were both positively associated with T1D risk in the first model (RC, 1.19, P = 0.006 and 1.07, P = 0.02, respectively)—these results were borderline significant in model 1, but showed the same direction as the results from model 2.

Conclusions: Our results indicate that specific immunological signatures are already present at time of birth in children developing T1D before the age of 18 years.

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1. Introduction

Type 1 diabetes (T1D) is a T-cell-mediated autoimmune disease, where loss of self-tolerance towards the insulin-producing pancreatic β-cells leads to their destruction [1,2]. This breach in self-tolerance is believed to develop in the interface of environmental factors, genetic susceptibility, and an imbalanced microbiome, and may be initiated already in early-life [3–7]. An increase in incidence of T1D is observed worldwide—including Denmark—especially in children diagnosed before the age of 5 years (early-onset) [8–11]. Early-onset suggests a more aggressive autoimmune response, though it remains to be examined at which time-point the autoimmune response is initiated.

Immune system maturation is a dynamic physiological process, which is initiated in utero and complete maturation is gained within the first 5 years of life [12], the autoimmune response may already be triggered in utero or around time of birth [13]. A successful self-tolerant maturation is dependent on a balanced interaction between different immune cells either by cell-to-cell contact or by cross-talking via signaling proteins, i.e. cytokines [14]. Twin studies have shown that genetic factors influence an individual’s cytokine production, but environmental factors are the main reason for differences in the cytokine signatures seen between individuals [15,16]. Such cytokine signatures may already be present at birth as a result of gene-environmental interactions in the neonate, and distinct signatures may mirror subtle immunological imbalances, which may result in an altered risk of developing autoimmune diseases, e.g. T1D, later in life.

Certain cytokines and other mediators of immune responses—hereafter referred to as immune mediators—have been associated with
T1D pathogenesis [17–21]. Most of the studies are in vitro or have been conducted after T1D onset whereas the pre-diabetic and neonatal periods largely remain unexplored, though activation of specific cytokine pathways have been shown to precede autoantibody development in children genetically predisposed to T1D [22,23].

To our knowledge, no studies have examined if neonatal circulating levels of immune mediators are associated with later risk of developing T1D. Identification of such associations would provide new information to the understanding of the earliest T1D etiopathogenesis, and open up for optimal timing of selective blockade of immune mediator pathways as a component of preventive T1D immunotherapy [24,25].

We investigated if circulating levels of immune mediators in neonates that later developed T1D compared to healthy controls differed. The Danish Neonatal Screening Biobank offers a unique opportunity to examine this question by using neonatal dried blood spots (DBS) – taken by a heel prick within one week after birth [26]. A priori, we selected 14 immune mediators associated with T1D and/or the innate immune system to be quantified on the DBS.

2. Methods

2.1. Data sources

The Danish Civil Registration System, established in 1968, registers all persons in Denmark alive from April 2, 1968 and born thereafter [27]. Unique personal identification numbers (CPR number) from the Danish Civil Registration System can be used for linking individual information from large clinical databases in the country. Through Statistics Denmark, which maintains a large number of national registers, we gained access to variables in the Medical Birth Registry, the National Patient Register and the Danish Civil Registration System using the CPR number as the key variable [28].

2.2. Study design, sample population, data sources and variables

Our study is a case-control study with a 1:2 matching. Cases born from January 1, 1981 through December 31, 2002 and diagnosed with T1D before May 1, 2004 were identified from the National Patient Register. Cases were furthermore validated against the Danish Childhood Diabetes Register. Controls were selected by pulling out the neighbouring DBS cards when cases were identified in the Danish Newborn Screening Biobank [26]. Cases and controls were therefore individually matched on date of birth. Since 1981 the DBS have been stored at −20 °C to −4 °F in the Danish Newborn Screening Biobank and this biobank covers close to 100% of the Danish population born since 1982 [29].

We considered the following variables as either basic or as possible confounders: gender, birth weight (BW) (coding: ≤2499 g, 2500–4499 g and ≥4500 g), gestational age (GA) (weeks), mother’s age at delivery (years), season (spring (March through May), summer (June through August), autumn (September through November) and winter (December through February)) and HLA-DQB1 genotype (HLA-risk) (genotyping has been described elsewhere [31]). In all assays, matched pairs were run together to avoid batch effects/interassay variation [32]. Biomarker analyses are described in detail elsewhere [31].

Quality control of the analysis were made using mouse IL-6 as an internal analyte added to the extraction buffer to detect pipetting errors, and biotinylated beads to detect signal errors (more thoroughly described in Skogstrand [33]). Calibration curves were used on each plate together with one high and two low controls. Samples, calibrators, and controls were analyzed in duplicates.

2.3. Assessment of biomarkers on DBS

By using a multiplexed sandwich immunoassays, based on flowmetric Luminex xMAP® technology, which can measure up to 25 inflammatory markers simultaneously on DBS (3.2 mm diameter), we were able to quantify the following cytokines: interleukin(IL)-1β, IL-4, IL-6, IL-8, IL-10, IL-12(p70), interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), transforming growth factor beta 1 (active form) (TGFβ1), leptin, and adiponectin. Furthermore, we also quantified c-reactive protein (CRP), mannose-binding lectin (MBL) and soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) [31]. In all assays, matched pairs were run together to avoid batch effects/interassay variation [32]. Biomarker analyses are described in detail elsewhere [31].

2.4. Assessment of autoantibodies on DBS

Autoantibodies against glutamic acid decarboxylase-65 (GAD65Ab) and insulinoma-associated protein 2 (IA-2Ab) were determined in a standard radioligand binding assay, details are described elsewhere.

| Table 1 |
| Descriptive characteristics of the sample population. |
| Variables | Case-control study |
| | (n = 1600) | (n = 3146) | P-value |
| **Basic characteristics** | | | |
| Gender | 758/47.4 | 1504/47.8 | 0.8 |
|  Male, n/% of total | 842/52.6 | 1642/52.2 | 0.8 |
| Age at onset | 7.8/4.8–11.3 | – | – |
| Median/Q1–Q3, years | 28/25–31 | 28/25–31 | 0.6 |
| Mother’s age at child’s birth | | | |
| Median/Q1–Q3, years | 28/25–31 | 28/25–31 | 0.6 |
| Pregnancy and birth | | | |
| Gestational age | | | |
| Median/Q1–Q3, weeks | 40/39–40 | 40/39–40.4 | <0.0001 |
| Birth weight (g), n/% of total | 74/4.6 | 145/4.6 | 0.9 |
| <2500 | 1489/93.1 | 2933/93.2 | 0.7 |
| 2500–4500 | 37/2.3 | 68/2.2 | 0.7 |
| >4500 | | | |
| Season at birth, n/% of total | | | |
| Winter | 339/21.2 | 623/19.8 | 0.3 |
| Spring | 394/24.6 | 741/23.6 | 0.3 |
| Summer | 426/26.6 | 917/29.2 | 0.3 |
| Autumn | 441/27.6 | 865/27.5 | 0.3 |
| HLA-risk groups | | | |
| High*, n/% of total | 1011/63.2 | 447/14.2 | <0.0001 |
| Moderate* n/% of total | 331/20.7 | 756/24.0 | 0.9 |
| Low/protective* n/% of total | 258/16.1 | 1943/61.8 | 0.9 |
| Diabetes status during pregnancy* | | | |
| Yes | 84/5.2 | 55/1.7 | <0.0001 |
| No | 1516/94.8 | 3091/98.3 | 0.9 |
| Autoantibody status at birth* | | | |
| Child’s autoantibody status, n/% of total | | | |
| Yes | 101/6.3 | 16/0.5 | <0.0001 |
| No | 1409/93.7 | 3130/99.5 | 0.9 |

**Note:**

a Chi-square test for categorical, and Mann-Whitney U test for numerical variables.

b HLA-DQB1 allele_1/allele_2: 03:02/99:99, 03:02/02, 06:04/03:02, 06:04/03:02, 06:04/99:99, 03:01/02, 06:03/03:01, 06:03/03:01, 06:03/03:01, 06:03/03:02, 06:03/04:02, 06:03/04:02, 06:03/04:02, 06:03/04:02, 99:99:99:99:99 = remaining alleles.

d HLA-DQB1 allele_1/allele_2: 03:02/99:99, 03:02/02, 06:04/03:02, 06:04/99:99, 03:01/99:99, 06:02/99:99, 06:02/03:01, 06:03/03:01, 06:03/03:02, 06:03/04:02, 06:03/04:02, 99:99:99:99:99 = remaining alleles.

e All diabetes diagnoses, excluding gestational diabetes, given in a hospital setting before the individual’s birth.

f Positive for glutamic acid decarboxylase-65 antibodies, islet antigen-2 antibodies, or both.

g Quality control of the analysis were made using mouse IL-6 as an internal analyte added to the extraction buffer to detect pipetting errors, and biotinylated beads to detect signal errors (more thoroughly described in Skogstrand [33]). Calibration curves were used on each plate together with one high and two low controls. Samples, calibrators, and controls were analyzed in duplicates.

h Autoantibodies against glutamic acid decarboxylase-65 (GAD65Ab) and insulinoma-associated protein 2 (IA-2Ab) were determined in a standard radioligand binding assay, details are described elsewhere.
[30]. GAD65Ab above 35 U/ml and IA-2Ab above 6 U/ml were considered positive [34].

2.5. Statistical analysis

The relative change (RC) in estimated mean levels for each immune mediator was modeled by robust log-normal regression taking into account that measurements are potentially both left and right censored and accounting for correlation within assay. To account for correlation within assay inference was based on a working independence generalized estimation equation (GEE) approach. Our variable of main interest was T1D status, and the following variables/possible confounders were also included in model 1: gender, BW, GA, mother’s age at delivery, season and HLA-risk. An additional analysis excluding HLA-risk (model 2) was also conducted. Due to low numbers of individuals with a positive autoimmune status and mothers with a diabetes diagnosis, these variables were not included in the statistical models, but the distributions are shown in Table 1. Complete-case analysis was used meaning only individuals with observations in all variables are included in the statistical analyses. We expect that values are missing completely at random. Simultaneous evaluations of risk factors on all the immune mediators are done using the model stacking approach detailed in Pipper et al. [35]. Subsequent adjustment for multiple testing and familywise 95% confidence limits (95%CI) are calculated using the single step procedure [36]. GEE estimates of mean ratios and accompanying 95%CI are calculated on a log scale and then back-transformed to the original scale. Spearman rank correlation coefficients were calculated on log scale including all immune mediators and a heatmap was also constructed. All analyses are made using the statistical software package R version 3.2.0 (www.r-project.org) and the add-on packages: survival, ggplot2, and multcomp.

3. Results

All the characteristics of the sample population are presented in Table 1.

3.1. Adiponectin

We found that neonatal levels of adiponectin were negatively associated with later risk of T1D: model 1 (RC [95%CI], 0.95 [0.90; 1.00], $P = 0.05$), and model 2 (excl. HLA-risk) (RC [95%CI], 0.95 [0.91; 0.99], $P = 0.006$).

3.2. IL-10

In model 1, with adjustment for HLA-risk, we found a positive association between neonatal levels of IL-10 and T1D risk, which was borderline significant: (RC [95%CI], 1.20 [0.99; 1.44], $P = 0.07$), but in model 2 this positive association was statistical significant: (RC [95%CI], 1.19 [1.03; 1.38], $P = 0.006$).

3.3. IL-12

In model 1, with adjustment for HLA-risk, we found a positive association between neonatal levels of IL-12 and T1D risk, which was borderline significant: (RC [95%CI], 1.08 [1.00; 1.18], $P = 0.06$), but in model 2 this positive association was statistical significant: (RC [95%CI], 1.07 [1.01; 1.14], $P = 0.02$).

3.4. The remaining 11 mediators of immune responses

These were not found to be statistically significant associated with T1D (Table 2).

3.5. Correlations between mediators of immune responses

We created a heatmap to illustrate the correlations between the different immune mediators. The strongest positive correlations were found between the pro-inflammatory cytokine IL-6 and the two pro-inflammatory cytokines TNFα and IFNγ (Fig. 1). Noteworthy, we found no sign of strong correlations between anti- and pro-inflammatory immune mediators e.g. between IL-10 and IL-12 (Fig. 1), even though the majority of the correlations were found to be highly statistical significant (data not showed).

4. Discussion

We found three circulating immune mediators in neonates significantly associated with later risk of T1D. The anti-inflammatory adiponectin showed a negative association whereas, the anti-inflammatory IL-10 and the pro-inflammatory IL-12 positively associated with later risk of T1D.

Neonates that later developed T1D had a 5% lower mean level of adiponectin than controls. Mean levels of IL-10 and IL-12 were 19–20% and 7–8% higher in neonates that later developed T1D compared to controls, respectively. These effect sizes were borderline significant in model 1, but were statistical significant in model 2, suggesting this can be explained by loss of power when adjusting for HLA-risk. The physiological significance of e.g. a 5% difference in mean levels of adiponectin between cases and controls is uncertain, but may be a clue of a skewed immunological/physiological homeostasis that predisposes the neonate to developing T1D.

Adiponectin is a protein secreted from adipose tissue and may influence insulin sensitivity and other immune mediators [37]. Adiponectin may have anti-inflammatory properties by inducing the production of IL-10 and IL-1RA and by impairing the production of IFNγ in human leukocytes [38]. In addition, several studies have found a negative association between β-cell function and adiponectin levels after onset of T1D and concludes that this may be due to a compensatory mechanism to boost insulin secretion and reduce insulin resistance [21,39–41]. Our results indicate that neonatal levels of adiponectin could have a protective role against T1D development, but we also find an increase in levels of IL-10 and no sign of higher levels of IFNγ. A study of 422 first-degree relatives of patients with T1D found no predictive effect of adiponectin levels in regards to autoantibody (Ab) status or T1D risk. Only 37 of the 211 Ab-positive relatives developed T1D, which limits this study from detecting small effects of adiponectin on later T1D risk [42]. Discrepancy between the studies may also be a result of different effects of adiponectin on different time-windows in regards to T1D pathogenesis.

IL-10 is generally regarded as an anti-inflammatory cytokine secreted mainly by immune cells. SNP in the IL-10 gene have been associated with T1D risk [43]. Murine models of T1D have suggested that early-life exposure to IL-10 and overexpression of IL-10 in the pancreatic islets accelerate T1D pathogenesis. IL-10 acceleration of T1D may be mediated through an intercellular adhesion molecule 1 (ICAM-1). ICAM-1 is situated on the vascular endothelium and is important for extravasation of auto-reactive lymphocytes e.g. to the pancreatic tissue, hereby fueling the auto-reactive process. ICAM-1 may be up-regulated by IL-10 and ICAM-1-deficient mice were protected against T1D [18,44]. The higher levels of IL-10 in neonates that later develop T1D may be caused by the immune system trying to counterbalance a more generalized pro-inflammatory profile reflected by elevated levels of IL-12, but as seen in Fig. 1 we only find a weak correlation between IL-10 and IL-12, which does not support the phenomena named “IFNγ to IL-10 switching” [45].

IL-12 is a cytokine that is produced by e.g. dendritic cells and macrophages in response to antigenic stimulation. IL-12 promotes differentiation of Th1 cells from naive T cells, hereby shifting the immune system towards T-cell mediated immunity [46]. A higher IL-12 gene expression
encoding the 35p subunit) in peripheral blood mononuclear cells (PBMCs) and circulating levels of IL-12 have also been observed in patients with T1D compared to controls [19,47,48], though results are contradictory. Inconsistency in findings can be due to sample size issues, duration from onset and so forth. Furthermore, IL-12 is structurally a heterodimer composed of a 35 kD α-chain (p35 subunit) and a 40 kD β-chain (p40 subunit), which are linked by disulphide-bonds. The p35 subunit and p40 subunit are also involved in the formation of IL-35 and IL-23, respectively. In addition, the p40 subunit can also form a homodimer, which has been shown to antagonize IL-12p70. Therefore, when comparing studies one should keep in mind which part of the "IL-12 family" is examined—we measured IL-12p70 [49].

Interestingly, no differences between cases and controls in mean levels of the pro-inflammatory cytokines IL-1β, IL-6 and IFNγ or the anti-inflammatory cytokine IL-4 shown to be involved in β-cell destruction in vitro were found [50,51]. A reason for this can be that no ongoing β-cell damage is taking place during the first week of life. Additionally, local doses of these cytokines in the islets may not be reflected in the circulation.

Previously we have found decreasing levels of immune mediators with age—levels decline as the immune system develops [19,52]. The heatmap (Fig. 1), which includes all measured immune mediators at birth, shows no clear sign of correlations between pro- or anti-inflammatory immune mediators. The definition of an immune

![Fig. 1. Correlations between the 14 measured immune mediators illustrated in a heatmap. Pro-inflammatory immune mediators are first mentioned from left-to-right and from bottom-to-top followed by the four anti-inflammatory immune mediators. Adipo, adiponectin; IL12, IL-12(p70); TGFβ, transforming growth factor beta 1 (active form).](image)
mediator as either pro- or anti-inflammatory may be less strict. Alternatively, immunological events during early childhood may be more important for the direction of the immune system later in life than the orientation at birth. Though a study emanating from the DIABIMMUNE study found differences in the cord blood whole-genome transcriptome, between newborns with a similar genetic background, but from different socio-economic areas. Their findings support the hygiene hypothesis, according to which environmental factors, such as microbial and parasite infections, encountered during fetal and early life suppresses autoimmune responses later in life. Interestingly, newborns from Russian Karelia, which both have the lowest incidence of T1D and the highest frequency of exposure to microbial infections [53, 54] of the three countries included, had, on a gene-expression level, the most mature innate immune system. On the other hand, newborns from Russian Karelia also displayed inhibited or homeostasis mechanisms which could potentially inhibit autoimmune responses [35]. In line with this study, our results could mirror environmental differences between cases and controls e.g. diet, exposure to microbial infections in utero, caesarean section and so forth. 

With regards to the statistical method, principal component analysis cannot accommodate left and right censoring and assay variation. In essence this means that results/patterns of immune mediators would be very design specific and not necessarily generalizable. We used an alternative robust method, which takes censoring of values and correlations into account. Further, the results were adjusted for multiple testing. 

Our study is large and population-based, where cases were thoroughly validated using two registers. The DBS utilized in this study were collected within one week after birth and we believe that the majority of the neonates were healthy e.g. no infections [26]. Due to short half-lives of the measured immune mediators and the time frame of one week our results should reflect the neonates own basal secretion and not transplacental transmission from the mother [56]. Furthermore, the DBS is a very stable way of storing blood e.g. IL-10 and IL-12 measured on DBS stored at −24 °C/−11.2 °F for 23 years show no significant sign of degradation. If degradation should occur it would be the same for both cases and controls, due to same storage length [31]. Moreover, we have measured the immune mediators at one time-point only. This makes us unable to evaluate longitudinal changes during early-life and does not capture the complexity of the pleotropic effects of these signaling proteins. Immune mediators in the current context are most likely to be immunomodulatory and not necessarily β-cell damaging.

5. Conclusion
In conclusion, our results from a large-scale population-based study suggest that there are differences in levels of adiponectin, IL-10 and IL-12 between neonates that later develop T1D compared to their healthy controls.

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Conflicts of interest
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