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Association between age, IL-10, IFNγ, stimulated C-peptide and disease progression in children with newly diagnosed Type 1 diabetes

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Abstract

Aims The relation of disease progression and age, serum interleukin 10 (IL-10) and interferon gamma (IFNγ) and their genetic correlates were studied in paediatric patients with newly diagnosed Type 1 diabetes.

Methods Two hundred and twenty-seven patients from the Hvidoere Study Group were classified in four different progression groups as assessed by change in stimulated C-peptide from 1 to 6 months. CA repeat variants of the IL-10 and IFNγ gene were genotyped and serum levels of IL-10 and IFNγ were measured at 1, 6 and 12 months.

Results IL-10 decreased ($P < 0.001$) by 7.7% (1 month), 10.4% (6 months) and 8.6% (12 months) per year increase in age of child, while a twofold higher C-peptide concentration at 1 month ($p = 0.06$), 6 months ($P = 0.0003$) and 12 months ($P = 0.02$) was associated with 9.7%, 18.6% and 9.7% lower IL-10 levels, independent of each other. IL-10 concentrations did not associate with the disease progression groups. By contrast, IFNγ concentrations differed between the four progression groups at 6 and 12 months ($P = 0.02$ and $P = 0.01$, respectively); patients with rapid progressing disease had the highest levels at both time points. Distribution of IL-10 and IFNγ genotypes was equal among patients from the progression groups.

Conclusion IL-10 serum levels associate inversely with age and C-peptide. As age and C-peptide also associate, a triangular association is proposed. Genetic influence on IL-10 production seems to be masked by distinct disease mechanisms. Increased serum IFNγ concentrations associate with rapid disease progression. Functional genetic variants do not associate with a single progression pattern group, implying that disease processes override genetically predisposed cytokine production.

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Keywords disease progression, interferon gamma, interleukin 10, Type 1 diabetes

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Introduction

Type 1 diabetes is an autoimmune disease [1,2]. After initiation of insulin therapy, approximately 43–56% of the patients develop a temporal remission of the disease, where the need for exogenous insulin declines [3]. Based on changes in stimulated C-peptide 1–6 months after diagnosis with Type 1 diabetes, we have previously shown that newly diagnosed paediatric patients have different patterns of loss in residual β-cell function,

varying from rapid, to slow, to stable, or even to an increases [4]. Several factors may contribute to remission, including partial β-cell recovery [3], resolution of the inflammatory process [5], improved insulin sensitivity and higher glucose uptake in peripheral tissue [6]. Systemic cytokines associated with autoimmunity may be involved in the destruction of the β-cells [1].

Interferon gamma (IFNγ) is a Th1 cytokine and may play a role in the inflammation of the islets [7], while interleukin 10 (IL-10), a cytokine produced by regulatory T-cells, may protect against development of Type 1 diabetes [8]. Several polymorphisms in the genes encoding for IFNγ and IL-10 influence the level of cytokine production. A CA microsatellite in the IFNγ gene has been linked to various autoimmune diseases, such as multiple sclerosis, Grave's disease, coeliac disease and Type 1 diabetes [9–12]. A CA repeat microsatellite in the IL-10 gene has been shown to influence serum levels of IL-10 [13]. Previously, we found that the IFNγ genotype, in combination with serum IFNγ concentration in patients with new-onset Type 1 diabetes (mainly adolescents and adults) was associated with clinical remission defined as $HbA_{1c} < 58$ mmol/mol (7.5%) and insulin dose < 0.38 U kg^{-1} 24 h^{-1}, while there were no associations with either IL-10 levels or genotype frequencies [14]. In this study, we tested the hypothesis that IL-10 and IFNγ serum levels in combination with their respective genotypes are associated with disease progression in paediatric patients with newly diagnosed Type 1 diabetes. We investigated the relationship between cytokine serum levels, immunogenetic predisposition and β-cell function and age and different patterns of disease progression in the first year after diagnosis in paediatric patients with newly diagnosed Type 1 diabetes.

Patients and methods

The Hvidoere Study

The study was nested within the framework of the Hvidoere Study Group on Childhood Diabetes, which is a multi-centre longitudinal investigation with 275 children under the age of 16 years with newly diagnosed Type 1 diabetes (144 girls and 131 boys, 84% white Caucasians). The median age at diagnosis was 9.1 years (range 0.2–16.8) [15].

C-peptide measurements

Stimulated C-peptide was measured 1, 6 and 12 months post diagnosis. In the morning, after at least 8 h of fasting, 6 ml/kg (maximum 360 ml) of Boost/Sustacal [Mead Johnson, Evansville, IN, USA; 237 ml (8 fluid ounces) contains 33 g carbohydrate, 15 g protein and 6 g fat; 240 kcal total] was ingested in less than 10 min. In agreement with the Diabetes Control and Complications Trial protocol, capillary glucose was measured at time 0 and venous C-peptide and glucose at 90 min after the ingestion of Boost. Serum samples were frozen at –20 °C until sent to the Steno Diabetes Centre, Denmark, on dry ice for determination of C-peptide. Samples were thawed only once for radioimmunoassay determination. C-peptide was analysed in serum by an enzyme-linked immunosorbent assay (Daco, Ely, UK) [15].

IL-10 and IFNγ cytokine measurements

Serum levels of IL-10 and IFNγ were determined by multiplex-bead technology using commercially available kits (Fluorokine MAP; R&D Systems, Minneapolis, MN, USA). The samples were treated in a blinded fashion: no data of the patients were available when the serum was tested. Detection limits for the assays were 0.1 pg/ml for both IL-10 and IFNγ. Determination of cytokine concentrations lower than detection limit were assigned a value half of the detection limit (IL-10 1 month, $n = 19$; IL-10 6 months, $n = 20$; IL-10 12 months, $n = 16$; IFNγ 1 month; $n = 63$, IFNγ 6 months, $n = 59$; IFNγ 12 months, $n = 60$). The immune assays showed interassay variations $< 20\%$ and intra-assay variations $< 10\%$.

IL-10 and IFNγ genotyping

The IL-10G CA microsatellite is located in the promoter region of the IL-10 gene [13]. Different variants of the CA repeat were identified according to the length of CA repeats. All patients were genotyped for a CA repeat in the first intron of the IFNγ gene. Genotyping of IFNγ was successful for 197 (71.6%) of the patients. Different variants of the CA repeat were identified according to the length of these CA repeats. Genotyping was carried out as previously described [16]. The allele and genotype frequencies of the IL-10 and IFNγ genes were in Hardy–Weinberg proportions. There were no associations between age, gender and the variants of the IL-10 and IFNγ genes.

Definition of Type 1 diabetes progression patterns

Within the Hvidoere study population, we have shown that patients may be categorized into four progression patterns groups based on longitudinal variations in the levels of stimulated C-peptide from 1 to 6 months after diagnosis with Type 1 diabetes: (1) patients with 'stable–low' pattern, who had constant low levels (< 100 pmol/l) of stimulated C-peptide; (2) patients with a 'rapid progresser' pattern, who showed a decline of more than 20% in C-peptide 1–6 months after diagnosis; (3) patients with a 'slow progresser' pattern, who had a slow decline in the levels of C-peptide of less than 20%; and (4) patients with a 'remitter' pattern, who had an increase in the levels of C-peptide of more than 20% [4]. From the study base of 275 children (as mentioned above), 227 children (with stimulated C-peptide measurements from both 1 and 6 months) were grouped in one of the four progression pattern groups; 116 were girls and 111 boys, 85% were white Caucasians and median age at diagnosis was 9.6 years (range 0.2–16.3). The progression pattern groups have been described in detail in a previous paper. It was reported that the number of

autoantibodies (GAD, IA-2 and IC antibodies) present in patients from the four progression groups differed significantly 1 month ($P = 0.04$) after diagnosis, while no differences were found at 6 or 12 months. All three autoantibodies were present in all four groups and no difference in titres of specific autoantibodies was found between the groups. Serum concentrations of adiponectin, IL-1ra, interferon-inducible protein 10 (IP-10) and IL-6 among the patients from the four progression groups have been analysed [4]. Clinical information, including BMI, HbA$_{1c}$, diabetic ketoacidosis at diagnosis and insulin dose, is summarized in Table 1.

Definition of genotypes profiles

The IL-10G CA repeat has previously been associated with a high production of IL-10. The variant IL-10G14 has been shown to be more frequent in patients with Type 1 diabetes compared with healthy control subjects [13]. The number of CA repeats in the IL-10G microsatellite varied from 10 to 14 bp in length, corresponding to variants 123–131 (123 = 10 CA, 125 = 11 CA, 127 = 12 CA; 129 allele = 13 CA and 131 allele = 14 CA). The variants 125 and 127 were the most common in our study population (160 patients were carriers of variant 125 and 88 were carriers of variant 127). Patients were therefore categorized as carriers of two copies of the 125 allele (125/125), as carriers of two copies of 127 (127/127), as heterozygous for both variants (127/125) or as carriers of rare variants (X/X) ($n = 10$). The CA repeat in the first intron of the IFNγ gene has been associated with higher IFNγ serum levels in healthy control subjects. Further, the repeat has been associated with Type 1 diabetes in a British population but not in Danish and Finish patients [17,18]. For IFNγ genotypes, patients were categorized as non-carrier of the most common allele 190 (X/X) ($n = 58$), as carriers of one copy of the 190 allele (190/X) ($n = 107$) or as carriers of two copies of the 190 allele (190/190) ($n = 32$).

Statistical methods

We addressed the study hypotheses in four steps and tested for associations between: (1) serum levels of IL-10 and IFNγ, and levels of stimulated C-peptide as continuous variable; (2) age and cytokine levels; (3) Type 1 diabetes progression, as assessed by the progression groups and cytokine serum levels; and (4) we examined the distribution of IL-10 and IFNγ genotypes and stimulated C-peptide of the four progression pattern groups.

The possible relationship between genotypes and progression pattern groups was investigated using Fisher's exact tests. Ordinal regression analyses were used to investigate the possible relationships between IL-10 and IFNγ serum levels and C-peptide and age. Associations between cytokine and age were adjusted for C-peptide. Multiple regression analyses were used to investigate the relations of progression groups, stimulated C-peptide, age, gender, IL-10 and IFNγ serum levels and genotypes at 1, 6 and 12 months. Two models were tested: (1) a model with cytokine as the dependent variable and age, gender and C-peptide as explanatory variables; (2) a model including the cytokines levels as the dependent variable, and progression pattern groups, age and gender as explanatory variables. Cytokine and C-peptide values were log transformed to obtain normal distributions. Associations are descriptive and were not corrected for multiple testing. The statistical analysis was performed using SAS version 9.1 (SAS Institute, Cary, NC, USA).

Results

Hvidoere Study Group

Of the 227 patients with stimulated C-peptide data from 1 and 6 months, 10.2% ($n = 23$) had a stimulated C-peptide below 100 pmol/l, which remained low during the first 6 months, and they were assigned to the stable–low group; 51.5% ($n = 117$) had a rapid decline in stimulated C-peptide and they

Table 1 Clinical characteristics of patients from the four progression patterns groups

Progression pattern group	n	Gender (male/female)	Age at diagnosis	BMI (kg/m^2) at 1 month	Ketoacidosis (%) at diagnosis	HbA$_{1c}$% 1 month	HbA$_{1c}$ mmol/mol 1 month	Insulin dose kg^{-1} 24 h^{-1} 1 month
All patients	227	111/116	9.6 (0.2–16.3)	17.1 (12.3–33.1)	20.3	8.9 (6.2–12.4)	74 (44–112)	0.45 (0.1–2.7)
Stable–low	23	13/10	8.8 (0.7–14.7)	16.3 (12.6–23.0)	38.1	8.9 (6.2–12.4)	76 (44–112)	0.54 (0.1–1.1)
Rapid progressers	117	58/59	9.1 (1.1–16.3)	16.8 (12.3–33.1)	21.4	8.1 (6.3–11.8)	65 (45–105)	0.44 (0.1–3.0)
Slow progressers	54	24/30	10.9 (3.1–14.6)	17.4 (13.0–27.6)	12.5	9.1 (6.9–11.8)	76 (52–105)	0.45 (0.1–1.1)
Remitters	33	16/17	9.4 (0.2–15.4)	18.1 (12.6–23.0)	24.1	8.5 (6.5–11.9)	69 (48–107)	0.57 (0.1–1.3)

Age, BMI, HbA$_{1c}$ and insulin dose are shown as median (with range). Ketoacidosis is defined as HCO^3 < 15 and/or pH < 7.3. Age and ketoacidosis are determined at diagnosis while HbA$_{1c}$, BMI and insulin dose are estimated 1 month after diagnosis. Patients from the four progression groups differed significantly in age ($P = 0.04$).

were labelled as rapid progressers; 23.8% (*n* = 54) had a slow decline and were grouped as slow progressers; while 14.5% (*n* = 33) of the patients had an increase in stimulated C-peptide and were assigned to the remitter group. As reported before, the groups differed significantly in age (*P* = 0.04), stable–low patients were younger compared with patients from the other groups [4]. There were no differences among the groups regarding ethnicity, gender, BMI, HbA$_{1c}$, insulin dose kg^{-1} 24 h^{-1} and ketoacidosis at diagnosis (Table 1).

IL-10

In all patients, the mean ± SEM level of IL-10 was higher at 1 month (0.96 ± 0.10) compared with month 6 (0.93 ± 0.11 pg/ml) and month 12 (0.88 ± 0.16 pg/ml). Among all the patients, there was an inverse association between serum levels of IL-10 and C-peptide, which was borderline significant at 1 month (*P* = 0.06, estimate = –0.14), but significant at 6 and 12 months (*P* = 0.0003, estimate = –0.27 and *P* = 0.02, estimate = –0.14), suggesting 9.7, 18.6 and 9.7% lower IL-10 level by a twofold higher C-peptide concentration at 1, 6 and 12 months, respectively (Fig. 1). The association between C-peptide and IL-10 disappeared when adjusted for age at 1, 6 and 12 months (*P* = 0.63, *P* = 0.06 and *P* = 0.73, respectively). There was an inverse significant association between IL-10 levels and age at 1, 6 and 12 months (*P* < 0.001, estimate = –0.08; *P* < 0.001 estimate = –0.11; and *P* < 0.001 estimate = –0.09 at 1, 6 and 12 months, respectively) suggesting 7.7, 10.4 and 8.6% decrease of IL-10 level per year increase in age, which was unaffected by adjustment for C-peptide. There was a positive significant association between C-peptide and age at 1, 6 and 12 months (*P* < 0.0001, estimate = 0.09; *P* < 0.0001, estimate = 0.09 and *P* < 0.0001, estimate = 0.13 at 1, 6 and 12 months, respectively) suggesting 9.6, 9.6 and 13.6% higher C-peptide concentration per year

increase in age. We found no differences of serum IL-10 across the groups, although there was a trend that patients with slow progressing diseases had lower levels of IL-10 at all time points (*P* = 0.08, Fig. 2).

Genotype frequencies of IL-10 gene variants were equal among patients from the four progression pattern groups (Fig. 3). In contrast to healthy subjects, functional genetic variants of IL-10G were not associated with IL-10 serum levels at any time point after disease onset.

IFNγ

Among all patients, the mean values of IFNγ serum concentrations (mean ± SEM) at 6 months (0.78 ± 0.09 pg/ml) and 12 months (0.77 ± 0.12 pg/ml) were higher than at 1 month (0.66 ± 0.06 pg/ml). In addition, there was no association between IFNγ serum concentrations and age or stimulated C-peptide at any time point. However, serum levels of IFNγ differed among patients from the four progression pattern groups at 6 months (*P* = 0.02). Rapid progressing patients showed the highest serum levels (mean ± SEM: 0.96 ± 0.16 pg/ml), followed by patients from the remitter group (0.68 ± 0.13 pg/ml) and the slow progresser group (0.62 ± 0.16 pg/ml), while stable–low patients had the lowest concentration (mean ± SEM: 0.44 ± 0.13 pg/ml). At 12 months, the differences between the four progression groups remained significant (*P* = 0.01), with rapid progressing and remitting patients having the highest levels, compared with patients from the slow progresser and stable–low groups (Fig. 2). Genotype frequencies of the IFNγ gene were equal among patients from the four progression pattern groups (Fig. 3). IFNγ genotypes and serum levels of IFNγ were moderately associated: patients homozygous for the 190 variant had higher levels at 1 and 6 months (*P* = 0.07 and *P* = 0.02, respectively), compared with heterozygous and non-carriers of 190.
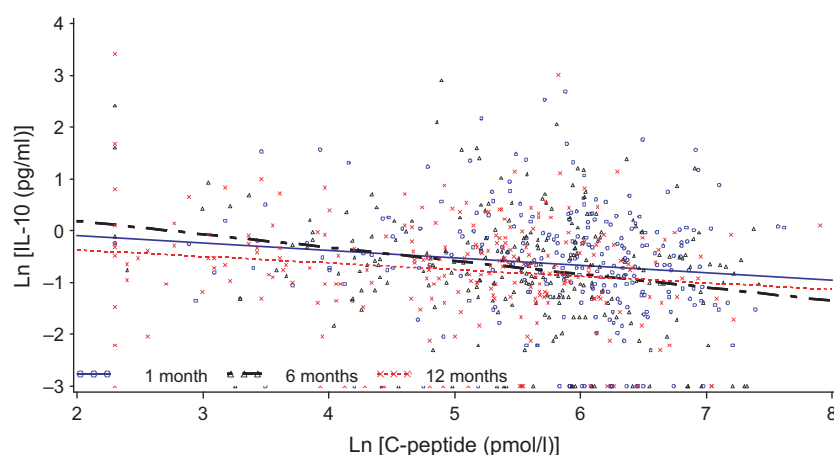

FIGURE 1 There was a significant inverse association between serum levels of IL-10 and stimulated C-peptide at 1 month (blue), 6 months (black) and 12 months (red), *P* = 0.06 (estimate –0.14), *P* = 0.0003 (estimate –0.27) and *P* = 0.02 (estimate –0.14), respectively, suggesting 9.7, 18.6 and 9.7% lower IL-10 level at a twofold higher C-peptide concentration.
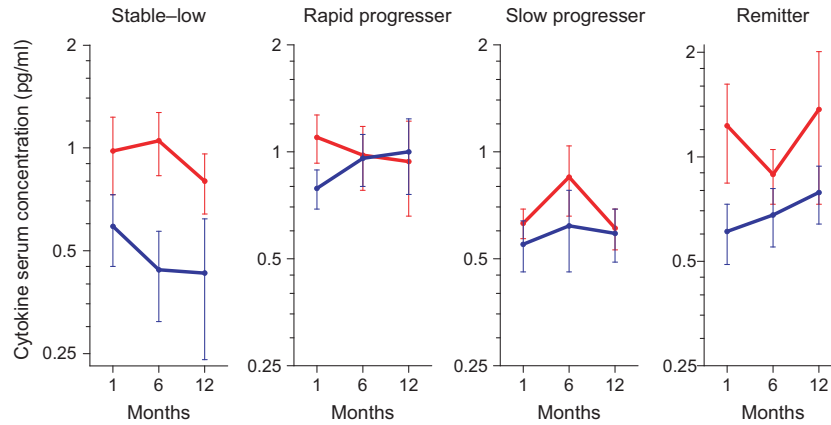
FIGURE 2 Serum concentrations of IL-10 (red) and IFNγ (blue) for the four progression pattern groups at 1, 6 and 12 months. The values are presented as mean serum concentrations with SEM. Serum IFNγ differed significantly between the groups at 6 months ($P = 0.02$) and at 12 months ($P = 0.01$), rapid progressing patients having the highest level, followed by patients from the remitter and slow progresser group, while stable–low patients had the lowest level. There was also a significant difference at 12 months ($P = 0.01$), where rapid progressing and remitting patients had the highest levels followed by patients from slow progressing and stable–low groups.
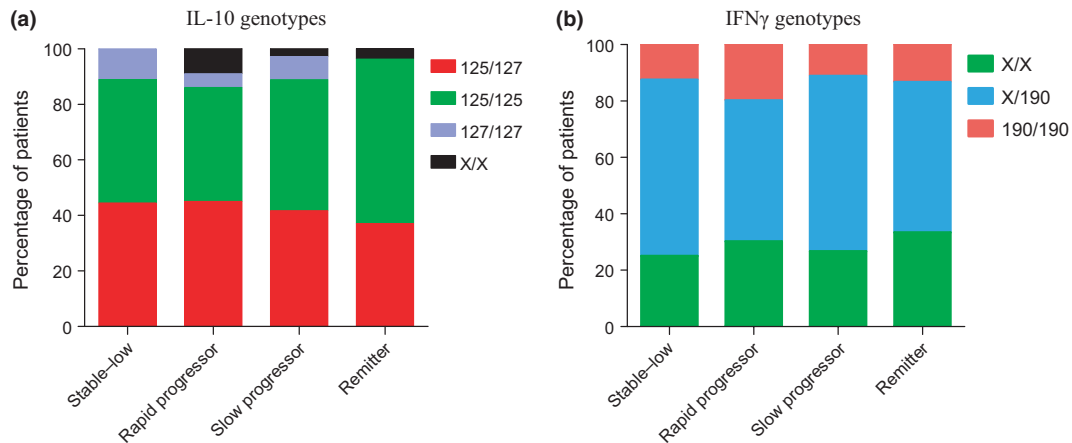

FIGURE 3 (a) The most comment variants of the IL-10 gene among patients from the four progression pattern groups were 125, present in 168 of the patients, and 127, present in 93 patients. Even although none of the patients from the stable–low group had X/X and none of the patients from the remitter group had 127/127, there were no significant differences in the distribution. (b) The most common variant of the IFNγ gene among patients from the four groups was 190, which was present in 139 of the patients. The genotypes were equally distributed among the four progression pattern groups.

There were no associations between stimulated C-peptide and variants of the IL-10 and IFNγ genes or between the serum levels of the two cytokines.

Discussion

The results presented in this study could point to a trinity between C-peptide, IL-10 serum concentration and age. While IL-10 is thought to have a protective effect on development of Type 1 diabetes through its down-regulating effect on Th1 cytokines and inhibitory effect on MHC class I expression [8,13,19], our findings suggest negative associations between IL-10 and C-peptide and age. We also report an association between IFNγ and different progression patterns.

The negative association between IL-10 and C-peptide is counter-intuitive. Indeed, the frequency of natural regulatory T-cells has been shown to increase with age [20]. IL-10 inhibits inflammation and could therefore reflect attempts for regulation of the autoimmune process. Deficiency in peripheral tolerance has been proposed to be important in the autoimmune process leading to Type 1 diabetes [21]. In the healthy immune system, auto-reactive T-cells are suppressed by regulatory T-cells [8]. Studies have shown that the number of regulatory T-cells is similar in patients with newly diagnosed or long-standing Type 1 diabetes and healthy age-matched control subjects [22,23]. Yet, the regulatory capacity of regulatory T-cells is markedly reduced in patients with Type 1 diabetes, while more recent studies suggest that effector T cells are

relatively resistant for regulation by regulatory T-cells [22,23]. As regulatory T-cells may inhibit effector T-cells via IL-10 production [24], it could be speculated that high levels of IL-10 seen in patients with low C-peptide production reflects resistance of auto-reactive T-cells to immune regulation requiring more IL-10. The rate of islet infiltration has been shown to be similar in regulatory T-cell-deficient and normal non-obese diabetic (NOD) mice [25]. This accords with the lack of association between the progression pattern groups and IL-10. Our findings are in line with previous reports that show low levels of IL-10 at diagnosis correlate with later clinical remission [26]. Malfunction of regulatory T-cells as an explanation for the negative association between IL-10 and C-peptide warrants further investigation.

A positive association between stimulated C-peptide and age is well established [27], but to our knowledge this study is the first to report an inverse association between age and IL-10 serum concentrations in a paediatric population with newly diagnosed Type 1 diabetes. We propose a triangular interaction between age, IL-10 and C-peptide in children with newly diagnosed Type 1 diabetes.

Positive correlations between age and IFNγ, IL-2 and tumour necrosis factor alpha (TNFα) have been described in healthy individuals [27]. We found no association between IFNγ and age in paediatric patients shortly after clinical manifestation of Type 1 diabetes. The negative association between IL-10 and C-peptide could be mediated to some extent via the effect of age on IL-10 and possibly more resistant auto-reactive T-cells at young age, as suggested by our data.

The functional variant was not associated with serum IL-10 among patients, as opposed to what has been found in healthy individuals [14]. Our study is not conclusive to exclude an association, but we speculated that genetic influence on IL-10 production is masked by distinct disease mechanisms during islet inflammation. It could be of interest to investigate IL-10 variants in another paediatric population with Type 1 diabetes, in particular longer after disease manifestation.

IFNγ is produced by natural killer cells and Th1 cells in response to antigen recognition. IFNγ is involved in the development of Type 1 diabetes. However, conflicting reports on whether IFNγ genotypes influence the development of Type 1 diabetes exist. In this study, we found no association between IFNγ serum levels and stimulated C-peptide and age. While genotypes of IFNγ influenced the serum concentration of IFNγ among the patients, which is in line with previous findings in healthy control subjects [14], IFNγ genotypes were not associated with distinct patterns of disease progression. Yet, even although the distribution of the genetic variants was equal among the progression groups, rapidly progressing patients had significantly higher levels of IFNγ compared with other progression groups. Conversely, we have previously shown that patients in clinical remission, defined by $HbA_{1c} \leq 58$ mmol/mol (7.5%) and insulin requirement ≤ 0.38 U kg^{-1} day^{-1}, had significantly lower IFNγ levels than patients not in remission, in spite of the presence of IFNγ genotypes associated with high IFNγ production [14]. IFNγ has been described as a key molecule in β-cell destruction [7]. However, a possible dichotomous action of IFNγ has been suggested [28]. Thus, IFNγ deficiency in NOD mice did not prevent Type 1 diabetes, but only delayed disease onset [29]. Also, long-lasting protection against Type 1 diabetes after exogenous treatment with IFNγ was shown in BioBreeding (BB) rats [30]. From a functional perspective, our data support the proposed association of IFNγ with β-cell destruction in humans. Rapidly progressing patients are believed to have a more active inflammatory process and could therefore be expected to display higher levels of IFNγ compared with patients with other disease progression profiles. At 12 months, remitting patients also had higher levels of IFNγ, possibly indicating an end to the partial remission phase. Together with our aforementioned findings on IL-10, our results support earlier reports that disease mechanisms overrule functionality of IFNγ gene polymorphisms, as only rapidly progressing patients showed high serum IFNγ concentrations in two independent studies.

In conclusion, we propose that changes in stimulated C-peptide can be used to stage different kinetics of Type 1 diabetes progression, which may prove valuable for understanding factors affecting residual β-cell function and preservation after diagnosis. High levels of IL-10 correlated with low levels of residual β-cell function, which may reflect malfunction of regulatory T-cells. Further, we contend that age is important when studying IL-10 and disease progression. The difference in IFNγ serum concentrations among patients with different progression pattern suggests distinct mechanisms of disease progression.

Appendix

Members of the Hvidoere Study Group on Childhood Diabetes who have contributed to the Remission Phase Study:

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Competing interests

Nothing to declare.

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