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Research Article

The Role of an iNOS Polymorphism at the Post-Diagnosis Diabetes Development in Children with Type 1 Diabetes

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Abstract:

Objective: A missense polymorphism (rs2297518) of the inducible nitric oxide synthase (iNOS) gene causing a serine to leucine substitution at amino acic position 608 has previously been associated to the development of type 1 diabetes (T1DM). Activation of iNOS is a main effector pathway in cytokine mediated beta-cell destruction. Here, we evaluate whether rs2297518 associates to interleukin-1β (IL-1β) levels, metabolic control and development of autoantibodies 12 months post-diagnosis.

Materials and Methods: The International Hvidoere cohort includes 275 children from 18 paediatric centers. The rs2297518 was tested in 256 participants and analyzed for its association to serum IL-1β levels, concentration of autoantibodies and residual beta-cell function as assessed by stimulated C-peptide, HbA1c and calculated insulin dose adjusted HbA1c (IDAA1c) at 1, 6 and 12 months post-diagnosis.

Results: At 1 month IL-1β was detected more frequently in CC-genotype individuals as compared to CT and TT individuals, 24/168 vs. 3/75 and 0/9, respectively (p=0.03). This effect was not present at 6 and 12 months post-diagnosis. The iNOS polymorphism was not associated to diabetic ketoacidosis status at diagnosis, stimulated C-peptide, measured HbA1c levels or the calculated IDAA1c at any time point during the first 12 months post-diagnosis.

Conclusion: The rs2297518 polymorphism of the iNOS gene may be involved in circulating IL-1β levels after diagnosis. However, this polymorphism does not seem to influence the metabolic outcome the first 12 months after diagnosis.

Keywords: Inducible Nitric Oxide Synthase Polymorphism; Interleukin-1β; Remission Phase; Type 1 Diabetes

Abbreviations: DKA: Diabetic Ketoacidosis; GADA: Glutamic Acid Decarboxylase; iNOS: Inducible Nitric Oxide Synthase; IAA: Insulin Auto Antibodies; IDAA1c: Insulin Dose Adjusted HbA1c; IA-2A: Insulinoma-Associated Antigen-2; ICA: IL-1β: Interleukin-1β; Islet Cell Auto antibodies; NO: Nitric Oxide; T1DM: Type 1 Diabetes; ZnT8: Zinc Transporter 8

Introduction: Type 1 diabetes (T1DM) is the result of selective beta cell destruction. Free radicals have been proposed to play an active role in this destructive process, which can be initiated by cytokine exposure of islets of Langerhans [1]. Specifically, the cytokine interleukin-1β (IL-1β) is known to regulate gene expression of the inducible form of nitric oxide synthase (iNOS) and consequently the synthesis of nitric oxide (NO). In rat islets IL-1β alone induces transcription of iNOS, while a combination of cytokines (IL-1β+TNFα+INFγ) are required to induce expression of iNOS in human islets [2-6]. Furthermore, a negative feedback mechanism has been described as NO can inhibit iNOS transcription [2].

Previously, we have demonstrated genetic association between the iNOS gene and the development of T1DM. Interaction between rs2297518 and HLA genotype was suggested as HLA DR3/4 positive individuals having the T of rs2297518 had the highest T1DM risk [7]. The amino acid substitution from C to T causes a Ser^{608} Leu shift only six amino acids from a region identified as being of importance to the Ca^{++} independency of iNOS [7,8], and is potentially damaging [8].

It is generally accepted that during the remission phase of T1DM endogenous insulin production is still present for a variable time period indicating some degree of beta-cell function and/or beta-cell recovery.

The causes of the partial remission phase have been investigated intensively, because an understanding of this phase would give an opportunity to intervene and an attempt to restore or maintain the pancreatic beta-cell function. Factors that have been associated with the remission period include age at onset, diabetic ketoacidosis (DKA) at onset, intensive metabolic control, type of diabetes specific auto antibodies [9-11] whereas association to HLA remains controversial [12]. Also mediators within the immune system have been explored. It has been shown, that the concentration of cytokines, including IL-1β, are stable from the time of diagnosis and 3-4 months post-diagnosis regardless of the patients had a partial remission phase or not [13].

Several genes have been identified to associate to development of T1DM and these genes are also shown to exert an impact on the disease progression in the remission phase [14-16].

The role of iNOS polymorphism for genetic association in the remission phase has not yet been investigated. Since, an association between iNOS polymorphism and development of T1DM has been described we hypothesized that a mutation in the iNOS gene might influence the progression of the disease the first 12 months after diagnosis.

Materials and Methods:

Subjects and consent: The Hvidoere Remission Phase Study has characterized the residual beta cell function in 275 children aged less than 16 years of age with newly diagnosed T1DM from 18 centers representing 15 countries in Europe, USA and Japan between year 1999 and 2000. Only newly diagnosed T1DM patients in the study period were included. Exclusion criteria were suspicion of non-type 1 diabetes and initial treatment outside of the centers for more than five days. Diabetes was diagnosed according to WHO criteria [17]. Older children gave their assent and all parents or guardians gave written informed consent.

Analyses: In order to estimate the residual beta-cell function as assessed by stimulated C-peptide a Boost test (formerly Sustacal; Mead Johnson, Evansville, IN, USA) was carried out at one, six and 12 months post-diagnosis. All samples were centrally analyzed as described previously [17]. DKA at onset was recorded. DKA was defined by bicarbonate value ≤ 15 mmol and/or pH<7.30.

Insulin dose-adjusted A1c (IDAA1c) was calculated: HbA1c (%) + (4 x total daily dose of insulin in IU/kg). An IDAA1c value ≤ 9 is associated to a stimulated C-peptide level 300 pmol/l during a Boost test, hence considered as remission [18].

In 256 of the 275 participants the rs2297518 in the iNOS gene was analyzed [7] (see ref 7 for further results of the mutational screening of the iNOS gene) and tested for association to DKA at onset, serum IL-1β levels above detection level (0,4 pg/ml) [19], serum values of pancreatic autoantibodies (insulin autoantibodies (IAA), glutamic acid decarboxylase (GADA), islet cell autoantibodies (ICA), insulinoma-associated antigen-2 (IA-2A), zinc transporter 8 (ZnT8) comprising two major isoforms: zink(R) and zink(W)) and stimulated c-peptide, HbA1c and calculated IDAA1c.

Statistics: The association between the iNOS genotypes and IL-1β level and pancreatic autoantibodies was determined by Kruskal-Wallis test 1, 6 and 12 months after onset. Stimulated C-peptide (logarithmic), HbA1c and IDAA1c were analyzed as dependent variables in a mixed model for repeated measurements model with unstructured covariance matrix of all time points with iNOS genotypes, age, and level of HCO_3 as co-variates. P values below 0.05 were considered statistical significant.

Results: 275 patients were included in the study and of these 256 were genotyped (93%) for the rs2297518 polymorphism. Sixty-seven percent of the patients had the CC genotype, 29% the CT genotype and 4% the TT genotype. The mean age at diagnosis was 9.1 years ± 3.7 years (mean ± SD). For further basic characterization of the cohort, please see the references Nielsen et al. 2006 [14] and Mortensen et al. 2010 [17].

Clinical parameters: DKA presentation at onset was independent of the iNOS genotype, as 20% of the CC and CT positive patients and 25% of the TT-carriers did present in DKA (p=0.94). Furthermore, no differences were demonstrated between the genotypes during the first 12 months post-diagnosis in relation to the progress in HbA1c, IDAA1c or C-peptide level (Figure 1).

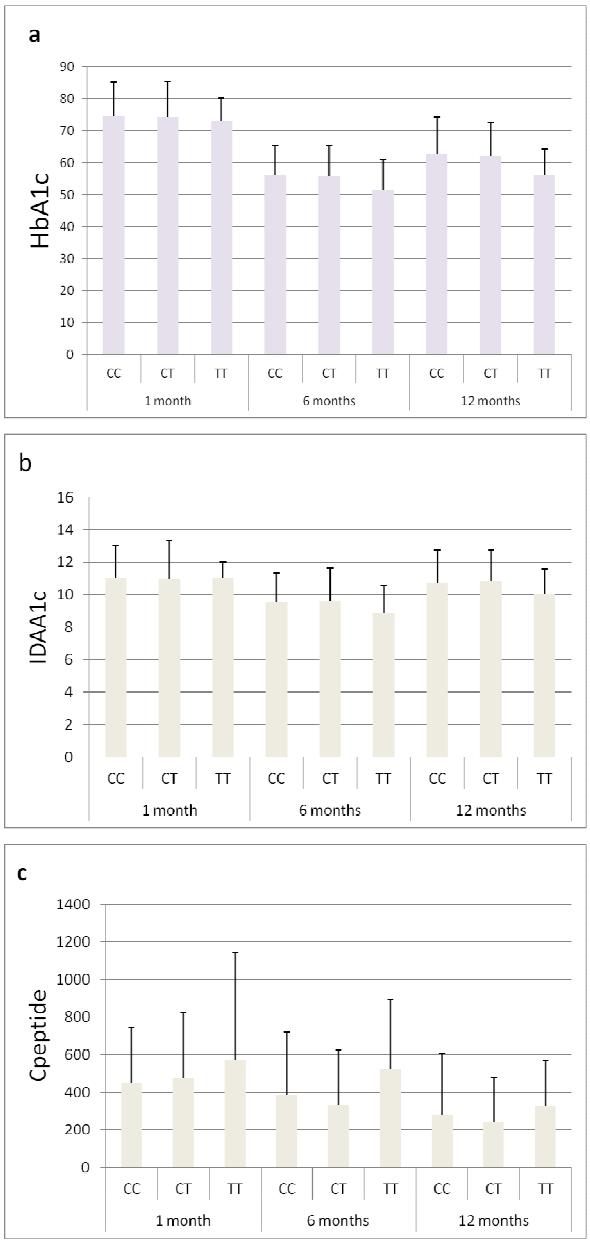

Figure 1: No difference in (a) HbA1c, (b) IDAA1c and (c) C-peptide level was demonstrated between the genotypes (CC vs. CT vs. TT) over time post-diagnosis. The lines represent standard deviation

Autoantibodies: For the autoantibodies IAA, IA2A, ICA and Zinc(R) no difference were demonstrated between the genotypes over time. At one month post-diagnosis GAD concentration was higher among the CC positive subjects compared to the TT genotype (p=0.015). However, no allele-dose effect was seen as CT genotype was higher than the CC genotype. No differences were seen at six and 12 months. At 12 months Zinc(W) level within the CT genotype was lower compared to the homozygous genotypes (p=0.01). No differences were seen at one and six months.

Cytokine IL-1β: Serum IL-1β was more frequently detected one month post-diagnosis among the CC genotype compared to CT and TT genotypes: 14%, 4% and 0%, respectively (p=0.03) (Figure 2). No difference in the number of patients with detectable levels of IL-1β in serum was demonstrated at six and 12 months post-diagnosis, p=0.27 and p=0.16 respectively.

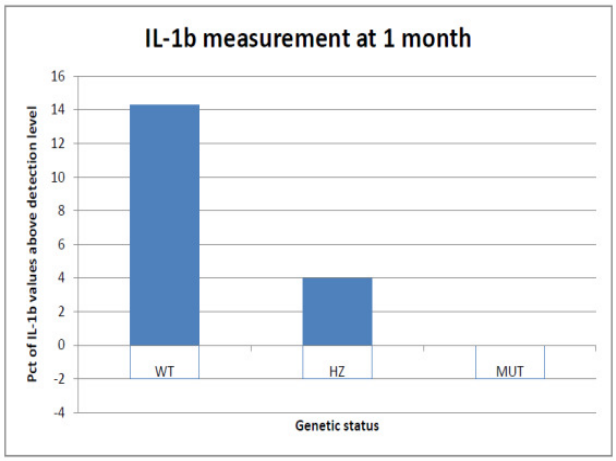

Figure 2: Number with detectable IL-1β level across the various genotypes at one month post-diagnosis. WT: Wild Type (CC). HZ: Heterozygous (CT). MUT: Mutated Genotype (TT)

Discussion: We here have demonstrated that the iNOS polymorphism, rs2297518, is associated to detectable levels of IL-1β at one month post-diagnosis. This association disappeared over time and was not detectable at six and 12 months post-diagnosis. At one month post-diagnosis serum IL-1β was more frequently detected among the CC-carriers compared to the CT and TT carriers, p=0.03. We do not believe that the detected serum IL-1β have a significant role for the outcome of the remission phase, as serum IL-1β levels is only detectable in a fraction of the population. However, the identified association may be of general functional interest as IL-1β induces the transcription of the iNOS gene and thereby NO production. In spite of ongoing presence of IL-1β a progressive decrease in iNOS expression have been shown by Eizirik et al, probably due to a negative feedback by NO on iNOS transcription [2].

An explanation of the low IL-1β expression level at one month post diagnosis among the TT genotype could be that the iNOS activity was high due to the polymorphism leading to increase NO level that negatively feeds-back at the cytokine level. However, this needs to be addressed in functional studies.

We hypothesized that the iNOS polymorphism was associated to the outcome of the remission phase as (i) NO being an effector molecule in cytokine mediated beta-cell destruction and (ii) the iNOS gene polymorphism has previously been demonstrated to be associated to the development of T1DM [7]. No association was found for iNOS genotype and DKA at onset and the residual beta cell function assessed by stimulated C-peptide, HbA1c and calculated IDAA1c during the first 12 months post-diagnosis (Figure 1).

Finally, the concentrations of the autoantibodies GAD and Zinc(W) were higher at one month (CC genotype) and lower at 12 months (CT genotype), respectively. No differences were seen for the other tested autoantibodies compared to the genotypes over time. As two different genotypes are associated to high expression of two different autoantibodies, we believe this finding to be spurious. As autoantibodies are believed to represent a marker of ongoing beta-cell destruction we would have expected the iNOS genotype associated to high NO (TT) and low IL-1 expression (due to negative feedback)to be associated to high autoantibody expression, indicating this being a type 1 error.

Overall, iNOS gene polymorphism has previously been associated to development of T1DM and our results might indicate that the iNOS gene may play a role in the pathogenesis in the very early phase of overt T1DM. We demonstrated a higher IL-1β level in CC genotype in the very early phase after diabetes onset. However, no association to clinical presentation with DKA or to the metabolic outcome the first 12 months after diagnosis was demonstrated. In conclusion, iNOS is to our knowledge the first gene to be selectively associated to T1DM development but not to metabolic outcome during the first 12 months after diagnosis.

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