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Increased expression of IL-18 in the serum and islets of type 1 diabetics

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Abstract

Type 1 diabetes (T1D) is a chronic disease characterized by autoimmune-mediated destruction of pancreatic insulin-producing beta cells. Interleukin (IL)-18 is a pro-inflammatory cytokine implicated in the pathogenesis of a number of inflammatory diseases. Here, we analyzed IL-18 levels in the plasma of juveniles with T1D. Compared to control subjects, IL-18 levels were significantly elevated in patients with T1D. On the other hand, levels of IL-18 binding protein (IL-18BP) and IL-37, two negative regulators of IL-18 function, remained unchanged when comparing T1D to control samples. Notably, however, although IL-18BP levels were not elevated, IL-18 and IL-18BP were found to be positively correlated in type 1 diabetics. Even so, free, unbound IL-18 remained significantly increased in diabetic patients. Additionally, correlation studies also revealed that IL-18 and IL-18BP are positively correlated with HbA1c levels in T1D patients, suggesting a potential link between IL-18 and metabolic control in these patients. Finally, we observed a significant increase in IL-18 protein expression within human pancreatic islet specimens collected from type 1 diabetics. These results further expand our knowledge of the role of IL-18 in T1D, may give insight into common pathogenic mechanisms associated with metabolic control in both T1D and T2D, and suggest that targeting this cytokine may improve therapeutic outcomes for T1D patients.

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Keywords

IL-18; type 1 diabetes; IL-18BP; IL-37; free IL-18; autoimmune diabetes

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1. Introduction

Type 1 diabetes (T1D), a disease that has risen in incidence over the past few decades, is characterized by autoimmune-mediated killing of insulin-producing β -cells within the pancreatic islet [1, 2]. Management of T1D involves administration of exogenous insulin and blood glucose monitoring. Unfortunately, despite management efforts, diabetic complications including kidney failure, heart disease and stroke may still arise in these patients [3].

Inflammatory cells invading the islet can destroy β -cells in part by releasing cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1 β , and interferon (IFN)- γ , which can induce β -cell apoptosis [4]. IFN- γ can also be induced by IL-18, a pro-inflammatory member of the IL-1 family that has been shown to activate polarized Th1 cells [5, 6]. In addition, IL-18 has also been found to enhance natural killer (NK) cell as well as macrophage activity [7-9].

The IL-18 cytokine has been implicated in the pathogenesis of inflammatory diseases, including allergy, asthma, Crohn's disease, multiple sclerosis, rheumatoid arthritis and myasthenia gravis [10-17]. Importantly, IL-18 has also been reported to be involved in the pathogenesis and progression of diabetes. IL-18-stimulated mouse islets produce nitric oxide and ultimately undergo apoptosis [18]. In non-obese diabetic (NOD) mice, pancreatic β -cells can produce IL-18, and enhanced expression of this cytokine results in destructive insulinitis in these mice [19, 20]. Additionally, our group has reported that IL-18 secretion is necessary for expansion of self-reactive T cells in NOD mice [21]. In humans, increased IL-18 levels have been observed in the serum of patients at high risk for developing T1D and type 2 diabetes (T2D) [22, 23], as well as in children and adults diagnosed with T1D and T2D [14, 24-27]. IL-18 protein expression was also observed within the pancreatic islets of patients with fulminant type 1 diabetes [28], and a significant association between elevated IL-18 serum levels and an increase in the number of autoantibodies detected was reported in new-onset type 1 diabetics (T1Ds) [29]. In addition, associations between increased IL-18 serum levels and insulin resistance in patients with T2D have been reported, as well as between higher IL-18 concentrations and obesity and the metabolic syndrome [30-34].

IL-18 activity and resultant IFN- γ production is inhibited by the IL-18 binding protein (IL-18BP) [35]. Zacccone et. al. reported that an IL-18BP fusion construct delayed diabetes development in NOD mice [36], and IL-18BP transgenic mice display extended normoglycemia while the transgenic islets are safeguarded against streptozotocin-induced apoptosis [18]. Furthermore, a second cytokine within the IL-1 family, IL-37, can bind to IL-18BP and enhance its IL-18-inhibitory function [37, 38]. IL-18BP levels have been studied in sepsis as well as systemic lupus erythematosus (SLE). Interestingly, in these patient populations, higher levels of IL-18BP, along with IL-18, are observed in the diseased

patients compared to controls [39, 40]. Importantly, these studies also report the calculated amount of free IL-18 (i.e. – IL-18 not bound to IL-18BP), which was still significantly higher in the sepsis and SLE groups despite increases in IL-18BP [39, 40].

In this report, we present our findings regarding IL-18, IL-18BP and IL-37 detected in the serum of juvenile T1Ds. Corroborating other reports [14, 24-26], we show a significant increase in the concentration of IL-18 in the serum of T1D patients compared to the control group. We also analyzed IL-18BP and IL-37 levels in these patients. Although no significant difference was seen in these cytokines between control and T1D populations, a significant positive correlation between IL-18 and IL-18BP was observed. Notably, free IL-18 was significantly increased in T1Ds. We also report a positive correlation between IL-18 and IL-18BP with HbA1c levels. Finally, we analyzed IL-18 expression in pancreatic islets of human T1D, autoantibody positive, and control and observed a significant increase in expression in the T1D group. To our knowledge, this is the first report analyzing IL-18 inhibitor expression in T1Ds as well as IL-18 expression within the islets of juvenile T1D patients. This work enhances our knowledge of the role of IL-18 in human T1D and further implicates that this cytokine is critical for T1D pathogenesis.

2. Material and methods

2.1. Patient information

Prior to collection, all donors gave informed consent in accordance with the protocols of the Internal Review Board of the University of Nebraska Medical Center. Patient data is provided in Table 1. Venous blood was then collected in BD Vacutainer® Plus K₂ EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). Next, we centrifuged whole blood for 20 minutes at 3000 x g and 20°C. We then removed the plasma layer, dividing it into multiple aliquots. Aliquots of plasma were flash frozen in dry ice and then stored at –80°C. The time from sample collection to storage at –80°C was approximately 2 hours.

2.2. ELISA

We purchased ELISA kits for IL-18 (eBioscience Inc., San Diego, CA), IL-37 (AdipoGen, San Diego, CA), and IL-18BP (Abcam, Cambridge, MA), and followed the manufacturers' protocols for each assay. Concentrations of each analyte were calculated following standard curves constructed with each assay using Gen5 software (v. 1.11.5, BioTek®, Winooski, VT). Samples below the limit of detection (LOD) were given a value of the LOD divided by the square root of 2 [41]. The alternate substitution method of LOD divided by 2 produced results of similar statistical significance. Among controls, 9 IL-18 (24%), 10 IL-37 (27%), and 2 IL-18BP (5%) samples were below the LOD. Among the T1Ds, 4 IL-18 (15%), 5 IL-37 (19%) and 1 IL-18BP (4%) samples were below the LOD.

2.3. Immunohistochemistry

We received formalin-fixed paraffin embedded slides from the Network for Pancreatic Organ Donors with Diabetes (nPOD). The samples that were analyzed in this study are as follows: Control – nPOD case numbers 6230, 6144, 6174, 6232, 6075, 6162, 6182, 6222, 6219, 6130 and 6137; autoantibody positive – case numbers 6267, 6027, 6090, 6116 and

6171; and T1D – case numbers 6209, 6228, 6051, 6052, 6224, 6212, 6113, 6025, 6211, and 6243. Histopathologic analysis obtained from nPOD shows islets are reduced in number in the T1D cases. However, all had detectable glucagon-positive islets, with 8/10 cases also showing islets with insulin positivity. Further, two cases had psuedoatrophic islets and islet atrophy was reported in one case. Patient data for nPOD samples is provided in Table 2.

To analyze IL-18 expression in these samples, slides were first de-paraffinized and rehydrated in xylene followed by soaking in graded ethanol (from 100% to 40%). Antigen retrieval was then performed using a 10 mM citrate solution (pH 6.0) and a pressure cooker. Slides were then cooled to room temperature and washed in ddH₂O. Endogenous peroxidases were then blocked in methanol/H₂O₂ and slides were subsequently washed with ddH₂O and PBS. Following a blocking step in 3% BSA/PBS, slides were incubated overnight at 4°C with IL-18 antibody (Santa Cruz Biotechnology, Inc., Dallas, TX). Slides were then washed 3X in PBS and incubated with goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). After incubation, slides were again washed 3X in PBS. Slides were then incubated with ABC solution followed by DAB, counterstained with hematoxylin, and dehydrated in graded ethanol (50%-100%) followed by xylene. Slides were then scanned using the iScan Coreo Au Slide Scanner (Ventana Medical Systems, Inc., Tucson, AZ) and analyzed using iScan Image Viewer (Ventana Medical Systems, Inc.).

2.4. Statistics

Statistical tests were performed using SPSS v21 and GraphPad Prism v. 6c (GraphPad Software, La Jolla, CA), and figures were created using GraphPad Prism v. 6c. The Mann-Whitney U Test was used to test for statistical significance unless otherwise indicated. For correlation analysis, data was first transformed to logarithmic values, and Pearson product-moment correlation coefficient (Pearson's r) analysis was then performed to test for strength of correlations. Finally, linear regression analysis was performed on our correlations to generate trend lines and to compare correlations between groups. For all statistical tests, results were considered significant when $p < 0.05$.

3. Results

3.1. IL-18 is increased and correlated to IL-18BP in T1D patients

IL-18, a pro-inflammatory cytokine shown to induce production of IFN- γ , has been linked to a number of autoimmune diseases, including T1D [16, 21, 42, 43]. Our goal was to expand the work regarding IL-18 in type 1 diabetics (T1Ds) through analysis of IL-18 in the sera of juvenile T1D patients as well as human pancreatic samples obtained from the Network for Pancreatic Organ Donors with Diabetes (nPOD).

First, we obtained venous blood samples from juvenile T1D patients (n=26) and non-diabetic controls (n=37). The patient population data is shown in Table 1. We performed ELISA assays on the plasma from these populations to analyze IL-18 levels. Confirming previous reports in adults and children [14, 26], we found that IL-18 is significantly increased in the sera of juvenile T1Ds compared to controls ($p=0.0391$; Figure 1A). Importantly, to determine if outliers were skewing the data toward a significant result, we

also performed a ROUT test ($Q=0.5\%$), to remove outliers. Two outliers were identified and removed from the control population and one outlier removed from the T1D population. Even with the removal of outliers, a significant increase in IL-18 concentration was seen in T1Ds compared to controls ($p=0.0232$; Supplemental Figure S1).

Next, we analyzed the concentrations of IL-18 binding protein (IL-18BP) and IL-37 in the T1D and control populations. IL-18BP binds to IL-18 with high affinity and inhibits its activity [40]. IL-37 is an anti-inflammatory cytokine, also from the IL-1 family of cytokines, that can bind to IL-18BP and actually enhance its ability to inhibit IL-18 activity [37, 38]. As shown in Figure 1B and 1C, the concentrations of IL-18BP and IL-37, respectively, were similar when comparing T1D to control samples.

Although IL-18 but not IL-18BP was increased in T1D, we wanted to determine if the two were correlated in T1D patients. Linear regression analysis of IL-18 vs. IL-18BP in the control population revealed no correlation between the two ($p=0.3765$; Figure 2A). Interestingly, however, a significant positive correlation between IL-18 and IL-18BP was observed in the T1D patient population ($p=0.0121$; Figure 2B). These results indicate that as IL-18 increases in T1D patients, so does IL-18BP. Importantly, however, the ratio of IL-18BP to IL-18 is significantly lower in T1D patients compared to controls ($p=0.0329$; Figure 3A). Furthermore, we also calculated the concentration of free IL-18 (i.e. – IL-18 not bound to IL-18BP) in T1Ds, using the law of mass action [39, 40, 43]. As shown in Figure 3B, the concentration of free IL-18 is significantly greater in T1Ds compared to controls ($p=0.0172$). These values were calculated by excluding samples below the limit of detection (LOD). Importantly, when all samples were included, using LOD divided by the square root of 2 as the numerical value for these samples, a statistically significant difference was still observed ($p=0.0195$; data not shown). Finally, there was no difference in the ratio of IL-18 to IL-37 when comparing T1Ds to controls (Supplemental Figure S2).

3.2. IL-18 and IL-18BP are positively correlated to HbA1c in T1D patients

HbA1c is an indicator of metabolic control as it provides an index of average blood glucose over the 120-day life-span of red blood cells [44]. HbA1c levels were measured in the T1D patients we studied (Table 1), and so we therefore wanted to determine if HbA1c was correlated to IL-18 in these patients. As shown in Figure 4A, HbA1c is positively correlated to the concentration of IL-18 in the sera of T1Ds, as determined by linear regression analysis ($p=0.0114$). Notably, a significant positive correlation is still observed when values below the LOD are included ($p=0.019$; Supplemental Figure S3A). Similarly, HbA1c is also positively correlated to the concentration of IL-18BP detected in the serum of T1D patients (Figure 4B; Supplemental Figure S3B). These results suggest that IL-18 levels may increase with decreased metabolic control.

3.3. IL-18-positive cells are significantly increased in T1D islets

Finally, after determining that the concentration of free IL-18 is significantly increased in T1D patients, we wanted to analyze IL-18 expression in the pancreatic islets of T1Ds, since this cytokine may play a role in pancreatic β -cell destruction by promoting inflammation. We obtained human control ($n=11$), autoantibody positive ($n=5$), and T1D ($n=10$) pancreatic

samples from the Network for Pancreatic Organ Donors. Immunohistochemistry was performed on these samples to analyze IL-18 protein expression within and around the islets. Representative staining is shown for each of these populations in Figure 5A-C. As shown, we observed an increase in IL-18 positive cells in the T1D samples compared to controls. We identified islets based on morphology and quantitated the IL-18-positive (IL-18⁺) cells in 25 islets per sample. As described in the Material and Method section, islets were detectable in all samples, including the T1D samples. The mean percentage of islets with 2 or greater and 5 or greater IL-18⁺ cells is shown in Figure 5D. As shown in this figure, a significant increase in islets with 2 or 5 IL-18⁺ cells is observed in T1D pancreatic tissue compared to control tissue ($p=0.0147$ and $p=0.0239$, respectively).

Overall, our results show that IL-18 levels are increased in the sera as well as the pancreatic tissue of human T1D patients. IL-18 levels in the serum correlate to blood glucose levels as indicated by a correlation with HbA1c. Furthermore, although IL-18BP concentrations increase along with the concentration of IL-18, there is still a significantly greater amount of free circulating IL-18 in T1Ds compared to controls. Increased levels of the pro-inflammatory IL-18 cytokine may be a contributing factor to the autoimmune destruction of pancreatic β -cells in patients with T1D.

4. Discussion

In this study, we analyzed IL-18, IL-18BP and IL-37 expression in the serum of juvenile patients with T1D compared to age-matched healthy controls. IL-18 has been shown to be involved in the pathogenesis and progression of T1D. We found, as has been previously reported [14, 24-26], that IL-18 is significantly increased in T1Ds. Since IL-18 is a pro-inflammatory cytokine, shown in mice to recruit inflammatory cells to islets and to be involved in β -cell destruction [18-21], it is likely that the elevated levels of IL-18 observed in our T1D patient cohort are contributing to the β -cell destruction occurring in this population. In addition, we also report a significant positive correlation between IL-18 and IL-18BP in T1Ds, as well as between HbA1c and both IL-18 and IL-18BP. Finally, we observed a significant increase in IL-18 expression within the islets of T1Ds compared to controls.

As mentioned above, even though IL-18BP and IL-37 levels were no different between control and T1D populations (Figure 1B and 1C), a positive correlation between IL-18 and IL-18BP was observed within the T1D patient cohort (Figure 2B). On the other hand, no significant correlation between IL-18 and IL-18BP was observed in the control population (Figure 2A). Therefore, it appears that as IL-18 levels begin to rise in T1D patients, IL-18BP is subsequently activated as a compensatory mechanism to reduce active levels of this cytokine. Importantly, however, the levels of free IL-18, as calculated by the law of mass action [43], are still significantly greater in T1Ds compared to controls (Figure 3B). Thus, negative regulation by IL-18BP may not be able to fully eliminate IL-18-induced inflammation. Notably, increased free IL-18 in conjunction with elevated levels of IL-18BP was also observed in another autoimmune disorder, systemic lupus erythematosus [39, 43], as well as in patients with sepsis syndrome and with sporadic amyotrophic lateral sclerosis [40, 45].

In addition to detecting increased circulating levels of IL-18 in T1Ds compared to healthy controls, we also observed a significant increase in IL-18 protein expression within the islets of pancreatic tissue collected by nPOD from deceased T1D patients (Figure 5). Due to low sample availability, we have been unable thus far to determine which cells within the islet are actually producing IL-18. Aida et. al. analyzed enterovirus-infected pancreatic specimens from subjects with fulminant diabetes and found that β -cells as well as a population of non- β -cells express IL-18 [28, 46]. Moreover, IL-18 mRNA is constitutively expressed in islet β -cells harvested from mice and rats [47]. Additionally, we observed IL-18⁺ cells within the inflammatory population of cells around the islet in an autoantibody positive patient specimen (Figure 5B, case # 6267). Current studies are underway to determine if the surrounding infiltrating immune cells are IL-18 receptor (IL-18R) positive. If so, these data would then suggest that the increased IL-18 expression within and around the islets of T1D patients may act to recruit IL-18R⁺ inflammatory cells, which may contribute to T1D pathogenesis and progression through autoimmune destruction of resident β -cells.

IL-18 is cleaved to its active form by capase-1, which is activated by a multiprotein complex known as the inflammasome [48]. Interestingly, palmitic acid (PA), a free fatty acid, can induce inflammasome activation, which results in impaired insulin sensitivity [49]. We have found that inducing glucolipotoxicity using PA and glucose in human islets results in inhibition of autophagy and, ultimately, β -cell death (Mir et al, data submitted for publication). Active inflammasomes can be eliminated through the process of autophagy [50], and thus IL-18 secretion is elevated when autophagy is inhibited [51, 52]. IL-18, in turn, elicits an inflammatory response that can impair autophagy [53]. Hence, the increased expression of IL-18 found in the islets of T1D samples compared to controls may be due to impaired autophagy within the islets of these patients, and may also cause further inhibition of autophagy, further propagating β -cell death. Preserving β -cells in diabetics may therefore be possible by concomitantly stimulating autophagy and inhibiting IL-18 expression.

IL-18 and IL-18BP expression were also found to be significantly, positively correlated to HbA1c levels in the T1D population (Figure 4). Other groups have reported no correlation between IL-18 and HbA1c [14, 25], which may be due to differences in the populations studied. For example, Ryba-Stanislawowska's group sampled T1Ds with a mean disease duration of 7.39 years [14], whereas the mean disease duration in our T1D population was significantly lower, at 9.58 months. Unfortunately, childhood obesity is a predictor of T1D diagnosis, and approximately 40% of children with T1D are obese [54, 55]. Notably, IL-18 levels are associated with obesity and are increased in patients with metabolic syndrome and are further elevated with increasing severity of the syndrome [33, 34]. Relevantly, BMI levels in our patient cohort were significantly higher in the T1D compared to the control group (Table 1; $p=0.0087$), and analysis of nPOD patient information also revealed a significantly increased mean BMI when comparing the T1D to controls that we studied (Table 2; $p=0.0279$). Furthermore, perturbations of IL-18 are also associated with dyslipidemia, hypertension and insulin resistance [33], and Esposito's group found that acutely inducing hyperglycemia in human subjects significantly increases circulating IL-18 levels [56]. The correlation between IL-18 and HbA1c that we observed suggests that reduced metabolic control may contribute to increased IL-18 expression in T1Ds. On the

other hand, chronic low grade inflammation is detected in those with metabolic syndrome, and eventual systemic inflammation in these patients may consequently lead to obesity-related comorbidities [57]. Further, depletion of IL-18, a pro-inflammatory cytokine, results in obesity and insulin resistance in mice [58]. Thus, it is possible that not only is the metabolic syndrome affecting IL-18 levels, but amplified IL-18 concentrations are also exacerbating the improper metabolic control seen in those with metabolic syndrome and diabetes.

5. Conclusion

In summary, this study shows that free IL-18 levels are significantly increased in juvenile T1D patients compared to controls, and that IL-18 expression in T1Ds is also elevated within the pancreatic islets. Furthermore, IL-18 and IL-18BP levels are positively correlated to HbA1c in the T1D population. These results further expand our knowledge of the role of IL-18 in T1D and suggest that targeting this cytokine, such as with exogenous IL-18BP, may improve therapeutic outcomes for T1D patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We analyzed IL-18 levels in the plasma of juveniles with type 1 diabetes (T1D).
- Increased IL-18 expression, but not IL-18BP or IL-37, was found in T1D vs. control.
- Free IL-18 also determined to be significantly increased in T1D samples.
- IL-18 and IL-18BP positively correlated to HbA1c levels in type 1 diabetics.
- Significant increase in IL-18 protein expression in T1D human pancreatic islets.

Fig. 1.

IL-18 levels, but not IL-18BP and IL-37, are increased in the sera of T1D patients. The concentration of (A) IL-18, (B) IL-18BP, and (C) IL-37 in the sera of control (n=37) and T1D patients (n=26) was determined by ELISA. Bars represent the median. Significance was determined using the Mann-Whitney U test. *p=0.0397; NS=not significant.

A

B

Fig. 2.

IL-18 and IL-18BP concentrations are positively correlated in T1D patients. (A) Regression analysis showing no correlation between [IL-18] (log(ng/ml of IL-18)) versus [IL-18BP] (log(ng/ml of IL-18BP)) in control subjects ($p=0.3765$). (B) Regression analysis showing a significant positive correlation between [IL-18] (log(ng/ml of IL-18)) versus [IL-18BP] (log(ng/ml of IL-18BP)) in T1D patients ($p=0.0121$). Significance was determined by Pearson's r analysis.

Fig. 3.

The concentration of free IL-18 is elevated in T1D patients. (A) The ratio of IL-18BP:IL-18 is significantly decreased in T1D patients compared to controls. Mann Whitney U test was used to determine significance. * $p=0.0329$. Mean ratios are shown, and bars represent SEM. (B) The concentration of free IL-18 (i.e. – IL-18 not bound to IL-18BP) is significantly greater in T1D patients compared to controls. Mann Whitney U test was used to determine significance. * $p=0.0172$. Mean concentrations are shown, and bars represent SEM.

Fig. 4.

The concentrations of IL-18 and IL-18BP are positively correlated to HbA1c in T1D patients. (A) Regression analysis showing a significant positive correlation between [IL-18] (log(ng/ml of IL-18)) and HbA1c in T1Ds ($p=0.0114$). (B) Regression analysis showing a significant positive correlation between [IL-18BP] (log(ng/ml of IL-18BP)) and HbA1c in T1D patients ($p=0.0275$). Significance was determined by Pearson's r analysis. Values below the limit of detection were excluded; analysis including all samples is shown in Supplemental Figure S3.

D

Fig. 5.

The percentage of IL-18 positive cells within islets is significantly increased in T1D patients. Human pancreatic samples were obtained from the Network for Pancreatic Organ Donors with Diabetes (nPOD). Immunohistochemistry was performed to analyze IL-18 expression within islets of pancreas. Representative IL-18 staining of islets is shown for samples collected from (A) control, (B) autoantibody positive, and (C) T1D donors. nPOD case numbers are provided in the upper left corner for reference. Arrows point to IL-18⁺ cells. All images are shown at 400X magnification. (D) IL-18⁺ cells were quantitated in 25 islets per specimen. Islets were identified morphologically. Mean percentage of islets with 2 or more IL-18⁺ cells (light gray columns) and 5 or more IL-18⁺ cells (black columns) is shown for control (n=11), autoantibody positive (n=5), and T1D groups (n=10). Bars represent SEM. Significance was determined using the Mann Whitney U test. *p=0.0239 compared to control; **p=0.0147 compared to control.

Table 1

Patient population data.

	Control	T1D
n (n female)	37 (22)	26 (8)
Mean age, years (range)	9.9 (2-15)	10.8 (5-18)
Mean disease duration, months (range)	n/a	9.58 (0-57)
Mean BMI (range)	17.72 (13.9-30.1)	21.19* (13.2-33.5)
Mean HbA1c at draw (range)	n/a	8.78 (5.5-15)

* Significantly different compared to control (p=0.0087)

Table 2

nPOD population data.

	Control	Aab+	T1D
n (n female)	11 (3)	5 (3)	10 (4)
Mean age, years (range)	10.3 (0.17-22.7)	9.7 (0.17-23)	16.5 (5-24)
Mean T1D duration, years (range)	n/a	n/a	5.0 (0-19)
Mean BMI (range)	19.54 (14.9-28.9)	20.12 (14.8-23.6)	23.23* (17.4-29.1)

* Significantly different compared to control (p=0.0279)