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Original Article

Proinsulin, GLP-1, and glucagon are associated with partial remission in children and adolescents with newly diagnosed type 1 diabetes

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Objective: Proinsulin is a marker of beta-cell distress and dysfunction in type 2 diabetes and transplanted islets. Proinsulin levels are elevated in patients newly diagnosed with type 1 diabetes. Our aim was to assess the relationship between proinsulin, insulin dose-adjusted haemoglobin A1c (IDAA1C), glucagon-like peptide-1 (GLP-1), glucagon, and remission status the first year after diagnosis of type 1 diabetes.
Methods: Juvenile patients (n = 275) were followed 1, 6, and 12 months after diagnosis. At each visit, partial remission was defined as IDAA1C ≤9%. The patients had a liquid meal test at the 1-, 6-, and 12-month visits, which included measurement of C-peptide, proinsulin, GLP-1, glucagon, and insulin antibodies (IA).
Results: Patients in remission at 6 and 12 months had significantly higher levels of proinsulin compared to non-remitting patients ($p < 0.0001$, $p = 0.0002$). An inverse association between proinsulin and IDAA1C was found at 1 and 6 months ($p = 0.0008$, $p = 0.0022$). Proinsulin was positively associated with C-peptide ($p < 0.0001$) and IA ($p = 0.0024$, $p = 0.0068$, $p < 0.0001$) at 1, 6, and 12 months. Glucagon ($p < 0.0001$ and $p < 0.02$) as well as GLP-1 ($p = 0.0001$ and $p = 0.002$) were significantly lower in remitters than in non-remitters at 6 and 12 months. Proinsulin associated positively with GLP-1 at 1 month ($p = 0.004$) and negatively at 6 ($p = 0.002$) and 12 months ($p = 0.0002$).
Conclusions: In type 1 diabetes, patients in partial remission have higher levels of proinsulin together with lower levels of GLP-1 and glucagon compared to patients not in remission. In new onset type 1 diabetes proinsulin level may be a sign of better residual beta-cell function.

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Type 1 diabetes is a T-cell-mediated autoimmune disease characterised by destruction of the insulin-producing beta cells (1). After disease diagnosis, a variable amount of beta-cell mass is still functioning and some insulin is released together with C-peptide and uncleaved proinsulin. Elevated levels of proinsulin are seen in islet transplanted and type 2 diabetic patients. This is thought to be a result of impaired processing of proinsulin and therefore considered as a marker of stressed beta cells (2–5). The role of proinsulin in type 1 diabetes is more controversial; is proinsulin a marker of impaired/stressed beta cells or is it a marker of residual beta-cell function with good metabolic control and partial remission? Proinsulin has been shown to be elevated before diagnosis of type 1 diabetes and in first-degree relatives of type 1 diabetic patients (6, 7). This increase might be caused by cytokine-induced infiltration of the immune cells (8). Proinsulin is elevated throughout the first year of type 1 diabetes, both absolute and relative to C-peptide (9, 10). The circulating concentrations of proinsulin seem to evolve differently from C-peptide. A peak in proinsulin has been reported around 6 months after diagnosis, whereas C-peptide achieves the highest level at 3 months (9, 11). The difference in proinsulin/C-peptide ratio has been explained by impaired proinsulin processing or by circulating insulin antibodies (IA), which are thought to bind proinsulin and thereby reduce the metabolic clearance rate (9, 10, 12, 13). A low proinsulin/C-peptide ratio has been associated with a long remission phase in children, while high levels of proinsulin was associated to remission [insulin dose $\leq$0.3 U/kg/24 h and haemoglobin A1c (HbA1c) $\leq$5.5% (37 mmol/mol)] in adults (9, 12, 14). A possible association between proinsulin concentrations and remission status has not been investigated in paediatric patients.

In type 1 diabetes, the inappropriate elevation of glucagon during hyperglycaemia has been attributed to the lack of intra-islet insulin to restrict glucose's effect on the alpha cell (14, 15). It is therefore relevant to investigate the effect of insulin deficiency on glucagon secretion when studying the metabolic status of newly diagnosed type 1 diabetic patients. In addition, the gastrointestinal hormone glucagon-like peptide-1 (GLP-1) could be an important player in the remission phase of type 1 diabetes. GLP-1 has been shown to stimulate beta-cell proliferation *in vitro*, enhance beta-cell neoformation following partial pancreatectomy, protect beta cell against cytokine-induced apoptosis, enhance beta-cell replication, and stimulate insulin release in a glucose dependent way (16, 17).

The aim of our study was therefore to investigate the relationship between remission status and meal-stimulated proinsulin, C-peptide, GLP-1, and glucagon plasma concentrations in a cohort of children and adolescents during the first 12 months after diagnosis with type 1 diabetes. We tested if proinsulin, GLP-1, or glucagon measured at 1 month could predict remission status at 6 and 12 months. In addition, we examined the relationship between proinsulin, glycaemic control, and IA together with GLP-1 and glucagon.

Methods

Subjects

The Hvidoere Remission Phase Study is a prospective, long-term observational study conducted in 18 centres representing 15 countries in Europe and Japan. Between August 1999 and December 2000, 275 newly diagnosed patients (48% male, all less than 16 yr old) were recruited to the study. Clinical data (gender, duration of symptoms, height, weight, and metabolic status) were collected at the first hospital visit. The Hvidoere cohort has previously been described in details (11).

Proinsulin and C-peptide

To estimate the residual beta-cell function (C-peptide and proinsulin) a liquid-meal Boost™-test [6 mL/kg (max: 360 mL, Mead Johnson, Evansville, IN, USA; 237 mL = 8 FL OZ contains 33 g carbohydrate, 15 g protein, and 6 g fat, a total of 240 kcal)] was carried out at 1, 6, and 12 months ($\pm$ 1wk) after diagnosis. Of the patients, 133 girls and 129 boys contributed with at least one measurement (95% of total). Serum C-peptide was analysed by a fluoroimmunometric assay as described previously (18).

Mixed meal-stimulated proinsulin was analysed by a sandwich enzyme-linked immunosorbent assay using two monoclonal antibodies: Coating antibody: PEP-001 (Novo Nordisk a/s, Bagsværd, Denmark). Detecting antibody: HUI-001 (Novo Nordisk a/s, Bagsværd, Denmark), biotinylation performed as described by Berger et al. Streptavidin-peroxidase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The assay detects total proinsulin as well as the four metabolites: split(32–33), des(31–329), split(65–66), and des(64–65)-proinsulin. This assay

has no cross reactivity with insulin, C-peptide, insulin-like growth factor-I (IGF-I), and IGF-II. The detection limit was 0.3 pmol/L ('0'-response + 3 × SD), and the analytical range was 0.3–100 pmol/L. Inter-assay precision was total inter-assay coefficient of variation (CV) 4.7–8.7%.

Insulin antibody

IA was measured 1, 6, and 12 months after diagnosis. The antibodies were analysed centrally by methods described previously (11). The results were expressed as relative units (RU).

GLP-1 and glucagon

Glucagon and GLP-1 concentrations in plasma sampled at 90 min were measured after extraction of plasma with 70% ethanol (vol/vol, final concentration). The glucagon RIA was directed against the C terminus of the glucagon molecule (antibody code no. 4305) and therefore mainly measures glucagon of pancreatic origin (19). The plasma concentrations of GLP-1 were measured against standards of synthetic GLP-1(7–36) amide using antiserum code no. 89390, which is specific for the amidated C terminus of GLP-1 and therefore mainly reacts with GLP-1 of intestinal origin. The assay reacts equally with intact GLP-1 and with GLP-1(3–36) amide, the primary metabolite. Because of the rapid and intra-vascular conversion of GLP-1 to their primary metabolites, it is essential to determine both the intact hormone and the metabolite for estimation of the rate of secretion of GLP-1 (20). For both assays, sensitivity was less than 1 pmol/L, intra-assay CV less than 6% at 20 pmol/L, and recovery of standard, added to plasma before extraction, about 100% when corrected for losses inherent in the plasma extraction procedure (21). All measurements were done centrally immediately after data collection ended in year 2000.

HbA1c, insulin dose-adjusted HbA1c and partial remission

Insulin dose-adjusted HbA1c (IDAA1C) is an HbA1c and insulin weighted indirect measure of residual beta-cell function. HbA1c was analysed at the Steno Diabetes Centre using the Bio-Rad HbA1c sample preparation kit (Bio-Rad Laboratories, Munich, Germany). HbA1c analysis was performed by automatic high-pressure liquid chromatography with the same calibrator lots as used in the diabetes control and complications trial (DCCT) to facilitate comparisons. Normal range for the Steno method was 4.4–6.3% (about 0.3% higher than the DCCT method).

IDAA1C is calculated on basis of the actual insulin dose and HbA1c as IDAA1C = HbA1c (percent) + [4 × insulin dose (U/kg/24 h)]. At each visit, partial remission was defined as an IDAA1C ≤9 corresponding to a predicted C-peptide response of >300 pmol/L for the corresponding stimulated C-peptide (22). At 1, 6, and 12 months 248, 231, and 230 of the patients had data available for calculating IDAA1C. Partial remission defined by IDAA1C has better stability compared with the existing definitions (22).

Statistical methods

Data are presented as median and range for parameters not normally distributed and mean ± SD for normally distributed parameters. C-peptide, proinsulin, IA, GLP-1, and glucagon were all studied on logarithmic scale. The associations between proinsulin and C-peptide and between proinsulin and IA were investigated by regression analysis with proinsulin as dependent variable accounting for gender and age, 1, 6, and 12 months after diagnosis. A prediction analysis to evaluate if proinsulin measured at 1 and 6 months could give information of C-peptide at 12 months was performed using multiple regression accounting for age and gender. The relation between proinsulin and glycaemic control was investigated by regression analysis with IDAA1C as dependent variable accounting for gender, age, and C-peptide 1, 6, and 12 months after diagnosis. The differences in mean values of proinsulin, GLP-1, and glucagon between remitters and non-remitters were analysed by the nonparametric Kruskal–Wallis test for not normally distributed parameters. To test if 1-month measurements could predict remission status at 6 and 12 months prediction analyses were performed. We used logistic regression analyses accounting for gender, age, and stimulated blood glucose. The relation between GLP-1 and proinsulin was investigated by regression analysis with proinsulin as dependent variables accounting for gender, age, IA, and C-peptide, 1, 6, and 12 months after diagnosis. The results are presented with estimates, converted into the effect of a 10% change of the independent variable ± confidence interval of the change. A p-value of <0.05 is considered significant. The analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Proinsulin, glycaemic control, C-peptide, and IA

Among all the patients the median level of stimulated proinsulin increased from 1 month (22.80 pmol/L range: 1.90–297.00 pmol/L) to 6 months (38.85 pmol/L range: 0.03–476.00 pmol/L), but subsequently decreased from 6 to 12 month (25.20 pmol/L range: 0.03–558.00 pmol/L). The median level of stimulated C-peptide decreased from baseline (1 month) to

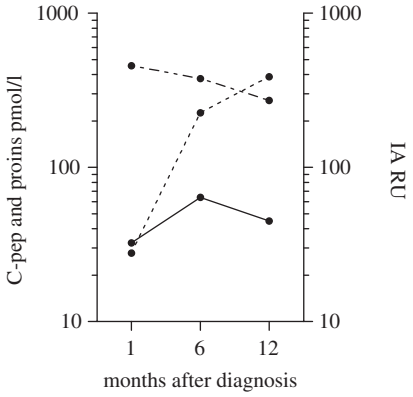

Fig. 1. The mean levels (log(10) scale) of stimulated proinsulin pmol/L (-), C-peptide pmol/l (- -), and IA relative units (RU) (···) at 1, 6, and 12 months after diagnosis. Proinsulin levels increase from 1 to 6 months but decrease from 6 to 12 months, while C-peptide level declines throughout all time points. IA levels increase throughout all time points.

12 months after disease onset (1 month: 409.50 pmol/L range: 10.00–2040.00 pmol/L, 6 months: 287.00 pmol/L range 10.00–1663.00 pmol/L, 12 months: 206.00 pmol/L range: 10.00–2721.00 pmol/L). IA increased because of the introduction of exogenous insulin treatment throughout the study period [1 month: median with range: 12.91 RU (0-588.91), 6 months: 117.51 RU (0-3005.98), 12 months: 181.98 RU (0-3143.28)] (Fig. 1).

As expected, analyses showed a positive significant association between proinsulin and C-peptide at all time points; 1 month (estimate: 0.54, $p < 0.0001$) suggesting 5.3% (3.9–6.7%) higher C-peptide by a 10% increase in proinsulin, 6 months (estimate: 0.39, $p < 0.0001$) suggesting 3.8% (2.9–4.8%) higher C-peptide by a 10% increase in proinsulin, and 12 months (estimate: 0.46, $p < 0.0001$) suggesting 4.4% (3.6–5.3%) higher C-peptide by a 10% increase in proinsulin, when adjusted for gender and age. There was a positive significant association between proinsulin and IA at all time points (estimate: 0.10, $p = 0.002$, estimate: 0.15, $p = 0.007$, and estimate: 0.21 $p < 0.0001$) suggesting 0.9% (0.3–1.5); 1.4% (0.4–2.5); and 2.0% (1.0–3.0) higher proinsulin level by a 10% increase in IA at 1, 6, and 12 months after diagnosis, respectively.

We found a negative significant association between IDAA1C and proinsulin at 1 and 6 months (estimate: −0.66, $p = 0.0008$ and estimate: −0.39, $p = 0.0022$) suggesting 6.1% (2.6–9.5) and 3.7% (1.4–5.9) lower IDAA1C by a 10% increase in proinsulin, respectively. Prediction analyses showed that the best predictor of C-peptide at 12 months was C-peptide at 6 months (estimate: 0.70, $p < 0.0001$) suggesting 6.8% (5.1–8.7) higher C-peptide at 12 months by a 10% increase in C-peptide after 6 months. However, in addition to C-peptide total proinsulin at 6 months had a significant predictive value on C-peptide at 12 months (estimate: 0.14 ($p = 0.03$) suggesting 1.4% higher C-peptide at 12 months by a 10% increase in proinsulin after 6 months.

Remission status related to proinsulin, GLP-1, and glucagon levels

Remission status was defined at 1, 6, and 12 months after diagnosis in all patients with complete data available for calculating the IDAA1C (HbA1c and insulin dose). At 1 month, 35 patients (14.1%) were in partial remission (IDAA1C ≤9), at 6 months 104 (45.0%), and at 12 months, 41 (17.8%) of the patients were in partial remission (Table 1). Analysis revealed that patients in remission had higher levels of proinsulin compared to patients not in remission at all time points. This difference was not significant at 1 month, whilst it was highly significant at 6 ($p < 0.0001$), and 12 months ($p = 0.0002$), after diagnosis. Also, GLP-1 and glucagon levels differed significantly between patients in remission and patients not in remission. Patients in remission had significantly lower levels of GLP-1 and Glucagon compared to patients not in remission at both 6 and 12 months (Table1). No statistically significant difference was found at 1 month after diagnosis for both variables. Prediction analyses using the 1 month measurements revealed that proinsulin and GLP-1 could predict remission status at 6 month, suggesting 6.7% (1.8–11.7%) better possibility for remission at 6 months by a 10% increase in proinsulin at 1 month (estimate: 0.68, $p = 0.005$), 8.5% (3.2–13.5%) lower possibility for being in remission at 6 months by a 10% increase in GLP-1 at 1 month (estimate: −0.93, $p = 0.001$) and 16.9% (8.6–24.5%) lower possibility for remission at 6 months by a 10% increase in stimulated glucose at 1 month (estimate: −1.95, $p < 0.0001$). There was no effect of age, gender, and glucagon (Table 2). At 12 months after diagnosis only glucagon and stimulated blood glucose measured at 1 month could predict remission status, suggesting 6.2% (1.0–11.2%) lower possibility for remission by a 10% increase in glucagon at 1 month (estimate: −0.67, $p = 0.02$) and 12.0% (3.0–20.1%) lower possibility for remission by a 10% increase in stimulated blood glucose (estimate: −1.33, $p = 0.009$). At this time point age, gender, proinsulin, and GLP-1 no longer had any predictive power (Table 2).

Relationship between proinsulin and GLP-1

Multiple regression analyses showed that proinsulin was significantly positively associated with GLP-1 at 1 month (estimate: 0.22, $p = 0.004$) suggesting 2.1% (0.7–3.5) higher proinsulin level by a 10% increase in GLP-1 and significantly negatively associated with GLP-1 at 6 and 12 months after diagnosis

Table 1. Concentrations of proinsulin, C-peptide, GLP-1, and glucagon (median with range) at 1, 6, and 12 months among patients in remission and patients not in remission at the three different time points

	Patients in partial remission	Patients not in partial remission	p Values
1 month			
Number	35 (14.11%)	213 (85.89%)	
Proinsulin (pmol/L)	31.50 (6.90–297)	22.00 (1.90–136.00)	NS
C-peptide (pmol/L)	457.00 (22.00–1620.00)	392.00 (10.00–2040.00)	NS
GLP-1 (pmol/L)	18.00 (3.00–65.00)	17.00 (4.00–134.00)	NS
Glucagon (pmol/L)	10.00 (2.50–22.00)	9.00 (2.50–34.00)	NS
6 months*			
Number	104.00 (45.04%)	127 (54.96%)	
Proinsulin (pmol/L)	58.8 (3.60–476.00)	23.90 (0.03–474.00)	<0.0001
C-peptide (pmol/L)	414.00 (26.00–1663.00)	233.00 (10.00–1600.00)	<0.0001
GLP-1 (pmol/L)	16.00 (4.00–62.00)	22.00 (1.00–68.00)	0.0001
Glucagon (pmol/L)	10.00 (2.50–31.00)	12.00 (2.50–28.00)	<0.0001
12 months*			
Number	41 (17.83%)	189 (82.17%)	
Proinsulin (pmol/L)	38.5 (0.20–204.00)	21.2 (0.03–558.00)	0.0002
C-peptide (pmol/L)	417.00 (10.00–1364.00)	178.00 (1.00–2721.00)	<0.0001
GLP-1 (pmol/L)	19.00 (6.00–66.00)	24.00 (5.00–64.00)	0.002
Glucagon (pmol/L)	11.00 (2.50–23.00)	13.00 (2.50–58.00)	0.02

GLP-1, glucagon-like peptide-1; NS, not significant.
*The parameters differed significantly between patients in remission and patients not in remission by use of the nonparametric Kruskal–Wallis test.

Table 2. Results of the prediction analysis on remission status (IDDA1c ≤9)

Contribution of 1 month measurements*	6 Months		12 Months	
	Est.	p	Est.	p
Age	−0.0388	NS	0.0570	NS
Sex	−0.3179	NS	−0.7936	NS
Ln (proinsulin)*	0.6780	0.005	0.4248	NS
Ln (GLP-1)*	−0.9283	0.001	−0.0638	NS
Ln (Glucagon)*	−0.2318	NS	−0.6724	0.02
Ln (stim. BG)*	−1.9457	<0.0001	−1.3347	0.009

Est, estimate; GLP-1, glucagon-like peptide-1; IDAA1c, insulin dose-adjusted haemoglobin A1c; NS, not significant; stim. BG, stimulated blood glucose.
Results of the prediction analyses on remission status (IDAA1c ≤9) at 6 and 12 months after diagnosis with type 1 diabetes are shown in the table.
Age, gender, and the 1-month measurements of proinsulin, GLP-1, glucagon, and stimulated blood glucose are used as covariates.

(estimate: −0.33, p = 0.004 and estimate: −0.50, p = 0.0009) suggesting 3.1% (1.0–5.1) and 4.5% (1.9–7.1) lower proinsulin level by a 10% increase in GLP-1, respectively (Fig. 2).

Discussion

We investigated the association between partial clinical remission in children with newly diagnosed type 1 diabetes and levels of proinsulin, GLP-1, and glucagon.

Patients in remission at 6 and 12 months after diagnosis have significantly higher proinsulin compared to patients not in remission. A possible explanation could be that higher circulating levels of proinsulin, which is a primary autoantigen in type 1 diabetes, might generate more regulatory T-cells (Tregs) followed by a relative hyposensibilisation towards this autoantigen and a slower autoimmune destruction of the beta cells (23) in the same way as immunisation with L7–24 peptide from preproinsulin has been shown to induce Tregs and significantly reduce diabetes incidence in non-obese diabetic mice (24). Previously, another study has shown a positive association between remission and proinsulin levels in an adult population, where partial remission was defined as an insulin dose ≤0.3 U/kg/24 h and HbA1c ≤5.5% (37 mmol/mol) at a given time point. We confirm this finding in a paediatric population using IDAA1C as a measure of remission. IDAA1C has proven to be a very stable definition of remission. It is expected that the number of patients in remission increases between 1 and 6 months, independent of remission definition, however, only one patient entered remission between 6 and 12 months when IDAA1C was used to define remission in this cohort (22). In addition, we used a standardised liquid-meal Boost™-test to obtain stimulated values of proinsulin. In line with that, we report a significant association between proinsulin and good metabolic control, as assessed by IDAA1C. The relation between proinsulin and C-peptide in our cohort is in consensus with previous reports (9, 11). Serum concentrations of proinsulin peaked at 6 months, while C-peptide declined from 1 to 6 months. As stimulated C-peptide has been described to be the best measure of preserved beta-cell function (25), it is interesting to

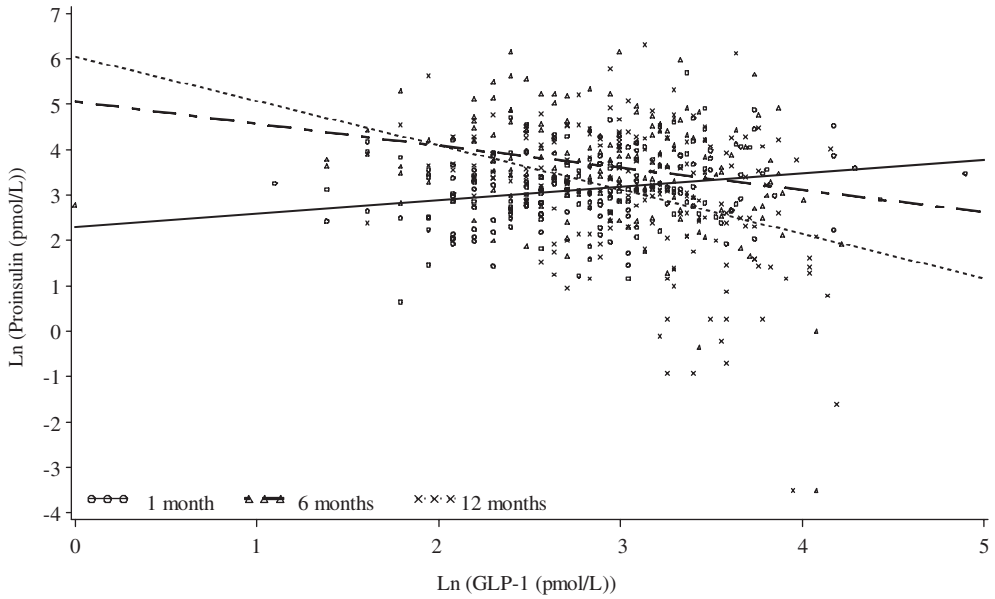

Fig. 2. The association between proinsulin and glucagon-like peptide-1 (GLP-1) levels at 1 (-), 6 (- – -), and 12 (---) months after diagnosis.

note that while the residual beta-cell function decline the level of proinsulin increases. This could indicate that proinsulin processing is not functioning well and that the remaining cells could be under elevated physiological stress. It has, however, been suggested that binding of proinsulin by IA is the explanation for the change of C-peptide/proinsulin ratio after diagnosis of the disease (9, 12). As opposed to that, our data show an increase in IA from 6 to 12 months, which most likely is a result of insulin treatment, while proinsulin declines in the same time period. It is not possible to make a firm conclusion from our data on whether the proinsulin C-peptide relationship reflects stressed beta cells, and further investigations are needed to elucidate this. However, proinsulin (6 months) has a predictive effect on later C-peptide (12 months) and we find that proinsulin measured at 1 month could predict remission status at 6 months. This indicates that proinsulin could be regarded as an important future endpoint.

Glucagon levels were lower among patients in remission compared to patients not in remission at 6 and 12 months, and glucagon measured at 1 month could predict remission status after 12 months. This fits very well with our previous publication on the intra-islet hypothesis, where we propose that glucagon secretion is stimulated by postprandial glucose in an islet environment where the beta cells no longer produce insulin and thereby have no inhibitory effect on the alpha cells (21). In this study, the hypothesis proves its value in regards to remission status. Patients in partial remission still produce insulin after meal stimulation and glucagon secretion is inhibited.

GLP-1 differed significantly between patients in remission and not in remission, with lower levels found in remitting patients at 6 and 12 months after disease onset. Proinsulin and GLP-1 associated positively at 1 month but inversely at 6 and 12 months. As we have previously shown that GLP-1 associate positively with postprandial glucose, this could be expected, but the results are also in consensus with a previous study showing that GLP-1 can reverse the anti-proliferative effect mediated by inflammatory cytokines such as IFNg in rat islets (26). Thus, it could be speculated that the cytokine inflammation is less aggressive in patients in remission and that GLP-1 level as a response to that is lower. Further, it is very interesting that GLP-1 measured at 1 month could predict remission status at 6 months, even when there was adjusted for postprandial glucose in the analyses. This could indicate that the actions of GLP-1 do influence partial remission. The shift in proinsulin and GLP-1 association between 1 and 6 months might relate to a gradual improvement of the residual beta-cell function in this period.

High levels of proinsulin and low levels of GLP-1 resemble the pattern seen in patients with type 2 diabetes before treatment or with poor metabolic disease status (3). Several reports have shown that incretin-based therapies to type 2 diabetic patients lowers proinsulin level and HbA1c, which leads to a better metabolic control (17, 27–29). Keeping in mind that type 2 diabetic patients display a very low grate immunological inflammation in the islets, it could be speculated that islets of type 1 diabetic patients in clinical partial remission resemble the islet-condition of type 2 diabetes – a disease condition where the islets are somehow protected against immunologic-induced killing of the beta cells. We propose that high proinsulin and low GLP-1 could be a marker of this.

Several reports suggest treating prediabetic or newly diagnosed type 1 diabetic patients with GLP-1 (27, 30, 31). Based on our data it could be speculated that the potential effect of GLP-1 on proinsulin/C-peptide ratio would be important to take in to consideration. To our knowledge this is the first report that describes both a significant relation between proinsulin and GLP-1, and between remission status and GLP-1.

In conclusion, our data suggest that proinsulin, GLP-1, and glucagon are significantly associated with partial clinical remission, patients in remission having higher proinsulin and lower GLP-1 and glucagon levels compared to patients not in remission. In addition, proinsulin associate positively with C-peptide and inversely with IDAA1C, which could point to, that in our cohort of juvenile patients with newly diagnosed type 1 diabetes, relatively high levels of proinsulin could be regarded as a marker of a slower disease progression.

Appendix

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

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