Diabetes Publish Ahead of Print, published online February 25, 2008

Association of IL-1ra and adiponectin with C-peptide and remission in patients with type 1 diabetes

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ON BEHALF OF THE HVIDØRE STUDY GROUP ON CHILDHOOD DIABETES

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Running Title: Association of IL-1ra with type 1 diabetes

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Received for publication 3 December 2007 and accepted in revised form 18 January 2008.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

ABSTRACT

Objective: We investigated the association of anti-inflammatory cytokines interleukin-1 receptor antagonist (IL-1ra) and adiponectin as well as pro-inflammatory cytokines IL-1ß, IL-6, CCL2 and tumor necrosis factor (TNF)-α with ß-cell function, metabolic status and clinical remission in patients with recent onset type 1 diabetes.

Research Design and Methods: Serum was obtained from 256 newly diagnosed patients (122 males/ 134 females, median age 9.6 years). Stimulated C-peptide, blood glucose and HbA1c were determined in addition to circulating concentration of cytokines at one, six and twelve months after diagnosis. Analyses were adjusted for sex, age and BMI percentiles.

Results: Anti-inflammatory IL-1ra was positively associated with C-peptide after six ($p=0.0009$) and twelve months ($p=0.009$). The beneficial association of IL-1ra on ß-cell function was complemented by the negative association of IL-1ß with C-peptide after one month ($p=0.009$). In contrast, anti-inflammatory adiponectin was elevated in patients with poor metabolic control after six and twelve months ($p<0.05$) and positively correlated to HbA1c after one month ($p=0.0004$). Proinflammatory IL-6 was elevated in patients with good metabolic control after one month ($p=0.009$) and showed a positive association with blood glucose disposal after twelve months ($p=0.047$).

Conclusions: IL-1ra is associated with preserved ß-cell capacity in type 1 diabetes. This novel finding indicates that administration of IL-1ra, successfully improving ß-cell function in type 2 diabetes, may also be a new therapeutic approach in type 1 diabetes. The relation of adiponectin and IL-6 with remission and metabolic status transfers observations from *in vitro* and animal models into the human situation *in vivo*.

Type 1 diabetes is an immune-mediated disease leading to selective destruction of insulin producing ß-cells in which cytokines play an important role (1). Cytokines related to the innate immune response such as interleukin (IL)-1ß (2-6), IL-1 receptor antagonist (IL-1ra) (7), monocyte chemoattractant protein (MCP)-1/ CCL2 (8-10), tumor necrosis factor (TNF)-α (2,11), IL-6 (3,12), and adiponectin (13,14) are thought to be associated with ß-cell destruction and disease status in humans and animal models. So far, no association of these markers with endogenous C-peptide secretion and metabolic status has been demonstrated in patients with type 1 diabetes. A recent small study has described slightly decreased circulating concentrations of IL-1ra three months after type 1 diabetes onset in patients not undergoing remission, however no data on C-peptide were available in these subjects (7).

Type 1 and type 2 diabetes are characterized by progressive ß-cell failure although the time-courses and mechanisms by which cytokines and nutrients trigger ß-cell death seem to differ (2). Nevertheless, the immune response in type 2 diabetes is thought to play a pathogenic role for disease development (15) perhaps similarly but not identically as for type 1 diabetes. Larsen et al. showed that administration of exogenous anti-inflammatory IL-1ra in type 2 diabetes patients could preserve endogenous insulin production and attenuated inflammation (16). IL-1ra is the natural antagonist of IL-1ß that induces programmed cell death (apoptosis) in ß-cells (17).

Pro-inflammatory cytokines like CCL2 and TNF-α are known to impair insulin signaling (18-20) and therefore it is not surprising that there is an association of cytokines with insulin resistance (21-23). Interestingly, adipose tissue plays an important role for cytokine secretion and may actually be a major source of pro-inflammatory cytokines (12,23-25) but is also a source of IL-1ra and adiponectin, which display anti-inflammatory and insulin sensitizing effects (17,26,27). Some cytokines, like the pro-inflammatory IL-6 and insulin sensitizing plus anti-inflammatory adiponectin, affect not only insulin signaling but also reveal an insulin independent role in glucose disposal (27,28).

In the current study we investigated in the longitudinally, prospectively performed Hvidøre study patients with recent onset type 1 diabetes. We determined how pro- and anti-inflammatory cytokines IL-1ß, IL-1ra, adiponectin, IL-6, CCL2 and TNF-α that are associated with ß-cell survival or insulin action, are related to endogenous ß-cell function, metabolic control, glucose disposal and clinical status.

RESEARCH DESIGN AND METHODS

Subjects. Patients were recruited consecutively in 18 centres throughout Europe (n=252) and Japan (n=4) from the Hvidøre Study. The design and characteristics of the Hvidøre Study has been explained elsewhere (29-31). In brief, prospective clinical and biochemical data were available from diagnosis up to one year for 256 children and adolescents (134 girls and 122 boys, median age 9.6 years, range 3 months to 16.8 years) out of 275 initially investigated patients at baseline (response rate 93.1%). Only these 256 patients entered subsequent analyses. Exclusion criteria were non-type-1 diabetes (MODY, secondary diabetes and other), or initial treatment outside the centres for more than five days. Patients were diagnosed with type 1 diabetes according to the World Health Organization (WHO) criteria (32). The study was performed according to the criteria of the Helsinki II Declaration and was approved by the local ethic committee in each centre. All patients (where applicable), their parents or guardians gave informed consent.

Metabolic parameters. When diabetes was

diagnosed, blood pH was determined by routine laboratory methods and was used to assess and adjust for the severity of the metabolic disorder (33). Body mass index (BMI) percentiles were used to assess the influence of adipose tissue which is more accurate in children and adolescents than the use of BMI alone. Stimulated serum C-peptide as a marker of ß-cell function (34) was measured in a central facility at one, six, and twelve months after diagnosis. Blood samples were obtained 90 minutes after the ingestion of a standardized liquid meal (Boost drink, formerly known as Sustacal (237 ml or 8 FL OZ containing 33 g carbohydrate, 15 g protein and 6 g fat, 240 kcal): 6 ml/kg (maximum 360 ml), Novartis Medical Health, Inc., Minneapolis, MN, USA, www.boost.com). Serum samples were labeled and frozen at –20°C until shipment on dry-ice to Steno Diabetes Center for central determination of C-peptide.

Serum C-peptide was analyzed by a fluoroimmunometric assay (AutoDELFIA^{TM} C-peptide, PerkinElmer Life and Analytical Sciences, Inc, Turku, Finland). The sensitivity was below 1 pmol/l, the intra-assay coefficient of variation were below 6% at 20 pmol/l, and recovery of the standard, added to plasma before extraction, was about 100% when corrected for losses inherent in the plasma extraction procedure.

Glycemic control as assessed by HbA1c was measured at diagnosis and one, three, six, nine and twelve months after diagnosis. HbA1c was determined centrally by ion-exchange high-performance liquid chromatography (normal reference range 4.1-6.4 %) at Steno Diabetes Center, Gentofte, Denmark (31,35-37).

We used different definitions to classify patients by their clinical outcome, partial remission and improved C-peptide secretion. To define remission, values of HbA1c and insulin requirement six months after diagnosis were used. First, a more classical definition of partial remission was applied, HbA1c < 7.5% and daily insulin < 0.4 U/kg (remission 7.5) (38). However, partial remission discriminated by HbA1c < 7.5% is not always indicative for a euglycemic status. Therefore we used in addition a stricter definition of partial remission that was HbA1c < 6.5% and daily insulin < 0.4 U/kg (remission 6.5). For determination of complete remission, patients would ideally not require any insulin, however it is recommended to support patients with low doses of insulin even in case of "complete" transient remission and therefore such patients were not available. Second, patients were classified according to whether C-peptide improved or not from one to six months after diagnosis with a lower limit of 100 pmol/l. To account for interassay variation of C-peptide determination an increase of at least 20% was defined as improved C-peptide secretion.

The difference of blood glucose (Δblood glucose) was determined before and 90 minutes after ingestion of the standardized liquid meal and was taken as a measure of blood glucose disposal.

Cytokines and chemokines. Blood was drawn 90 minutes after ingestion of the standardized liquid meal. Serum samples were labeled and frozen at –20°C until shipment on dry ice to the German Diabetes Centre for determination of cytokines. Serum samples were measured at time points one, six and twelve months after diabetes diagnosis. Concentrations of IL-6 and total adiponectin were measured by ELISA. IL-6 was determined using matched antibody pairs from Sanquin (PeliKine ELISA kit, Amsterdam, The Netherlands) as described (22), total adiponectin by commercially available kits (Quantikine, R&D Systems, Wiesbaden, Germany) (39). IL-1ß, IL-1ra, CCL2 and TNF-α were determined by multiplex-bead technology using commercially available kits (Fluorokine MAP, R&D Systems). All cytokines were measured in a blinded fashion, e.g. clinical data were not known when measurements were performed. The detection limits of the assays were 6.7 pg/ml

for adiponectin, 13.6 pg/ml for IL-1ra, 0.4 pg/ml for IL-1ß, 0.15 pg/ml for IL-6, 1.8 pg/ml for CCL2 and 1.35 pg/ml for TNF-α. Determinations of cytokine concentrations lower than the detection limit were assigned a value half of the detection limit (IL-1ra n=0; adiponectin n=0; IL-6 n=0; CCL2 n=0; TNF-α n=31). Because IL-1ß was only detectable in 12% (n=86) of all samples this cytokine was treated detectable or not detectable in all analyses. The immunoassays showed inter-assay variations below 20% and intra-assay variations below 10%.

Statistical methods. Cytokine concentrations showed no Gaussian distribution and data are described by medians. Differences between cytokine concentrations during follow up were analyzed first by Friedmann test followed by Wilcoxon test in case of significance to investigate differences between two time points. Distribution and differences between follow up of IL-1ß were not investigated since too many values were below the detection limit. Log transformed cytokines were approximately normally distributed and entered Spearman correlation analysis to investigate correlations between cytokines and linear regression analysis to investigate associations between cytokines and metabolic parameters. Regression analysis included cytokines as the dependent variable and sex, age, BMI percentiles, blood pH, C-peptide and HbA1c as independent variables. Analyses investigating the influence of cytokines on Δblood glucose included cytokines as the dependent variable and sex, age, BMI percentiles and Δblood glucose as independent variables. For the analysis of IL-1ß and metabolic parameters logistic regression was performed using the same independent variables as in the linear regression analysis. Associations are descriptive and were not corrected for multiple testing. Adjustment for BMI percentiles are based on the 2000 CDC growth charts (www.cdc.gov/growthcharts) of the Centers of Disease Control and Prevention, 1600 Clifton Rd, Atlanta, GA 30333, USA. Statistical analyses were performed using SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA) and GraphPad PRISM version 4 for Windows.

RESULTS

Longitudinal analysis of circulating cytokine concentrations. First we investigated circulating cytokines of patients with newly diagnosed type 1 diabetes during the first year after diagnosis. Circulating concentrations of adiponectin ($p<0.0001$), IL-6 ($p=0.0008$) and CCL2 ($p<0.0001$) were significantly higher one month compared to six and twelve months after diagnosis despite a large overlap between time points (Figure 1). TNF-α and IL-1ra did not statistically differ during follow-up ($p=0.16$ and $p=0.77$, respectively), demonstrating that there is no general up-regulation of all cytokines measured one month after diagnosis. Because of too many values below detection limit differences of IL-1ß were not investigated.

Associations between serum cytokines. It is well known, that cytokines and chemokines are part of a complex network. Spearman's correlation analysis including all cytokines was performed to investigate associations between cytokines (Table 1). Interestingly, similar association patterns one and six months after diagnosis were seen, whereas twelve months after diagnosis cytokines seemed related less often. To our surprise, all statistically significant correlations between cytokines found were positive. Adiponectin was the only cytokine that exhibited no association at any time with the cytokines investigated. One month after diagnosis anti-inflammatory IL-1ra revealed associations to pro-inflammatory TNF-α, IL-6, CCL2 and IL-1ß (Table 1). Within the pro-inflammatory cytokines CCL2 was positively associated to TNF-α, IL-6 and IL-1ß, and IL-6 to IL-1ß.

Six months after diagnosis, analyses revealed a similar association pattern as in one month after diagnosis (Online appendix 1). Twelve months after diagnosis fewer

associations were found (Online appendix 2). Interestingly, the associations of anti-inflammatory IL-1ra with IL-6, CCL2 and IL-1ß were maintained.

Basic characteristics of patients classified by their clinical outcome. In the next step, we classified patients by their clinical outcome such as remission and improved C-peptide secretion (Table 2). Patients with incomplete data record with respect to classification were excluded from the respective analyses: 22 patients missed data for the classification of remissions, 45 patients had incomplete data on C-peptide values. At baseline, both definitions of remission revealed higher C-peptide and BMI percentiles and lower HbA1c for remitters in contrast to non-remitters. Non-remitter showed lower blood pH in the classification of remission 7.5; for the stricter definition of remission 6.5, age was significant higher in remitters. The classification of improved C-peptide secretion revealed no significant differences.

Association of circulating cytokines with clinical status. To assess the association of cytokines with clinical status we searched for a relation of circulating cytokines and the classification regarding clinical outcome. Regression analysis was applied to take differences of cytokine concentration due to age and sex differences into consideration. In the first approach, regression analysis was performed adjusting for sex and age; in the second approach, regression analysis was performed adjusting for sex, age and BMI percentiles since adipose tissue is known to be an origin of cytokine secretion. As before, patients with incomplete data record with respect to classification were excluded from analysis, 22 patients for the classification of remissions, 45 patients for improved C-peptide secretion.

The first regression model revealed elevated anti-inflammatory IL-1ra in remitters of both definitions of remission compared to non-remitters. Furthermore, IL-1ra was also elevated in patients with increased C-peptide secretion (Figure 2).

In contrast, the anti-inflammatory adiponectin was lower in remitters of both definitions of remission but unrelated to C-peptide classification (Figure 2).

Interestingly, the pro-inflammatory IL-6 was elevated in patients in remission 7.5 and in patients with increased C-peptide secretion (Figure 2).

In the second regression model that included BMI percentiles as co-variable in order to account for the effect of cytokine secretion by adipose tissue we observed the same association for IL-1ra with increased C-peptide one month after diagnosis (p=0.016) suggesting a BMI percentile independent association. In contrast, associations of IL-1ra with both definitions of remission were lost while adjusting for BMI percentiles.

Adiponectin showed similar associations as in the analysis without adjustment for BMI percentiles in remission 6.5 (six (p=0.037) and twelve months (p=0.019) after diagnosis). Classification of remission 7.5 showed no association anymore to adiponectin.

When adjusting for BMI percentiles, IL-6 revealed a similar association with remission 7.5 one month after diagnosis (p=0.0097) and improved C-peptide secretion six (p=0.005) and twelve (p=0.03) months after diagnosis as in analyses without additional adjustment.

Association of circulating cytokines with HbA1c and C-peptide. To address the relationship of circulating cytokines and ß-cell function in more detail, we investigated the association of cytokines with ß-cell function measured by stimulated C-peptide and glycemic control determined by HbA1c. First, regression analysis included cytokines, sex, age, blood pH, C-peptide and HbA1c. Anti-inflammatory IL-1ra concentrations revealed positive associations with C-peptide one (regression coefficient (ß)=0.00024; p=0.021), six (ß=0.00042; p=0.0001) and twelve (ß=0.00031; p=0.0013) months after diagnosis. Anti-inflammatory adiponectin concentrations showed a negative association with C-peptide twelve (ß=-

0.00037; p=0.0037) months after diagnosis and related positively with HbA1c one (ß=0.12; p=0.0002) months after diagnosis. Pro-inflammatory cytokine IL-1ß was negatively associated with C-peptide one month after diagnosis (ß=-0.0021; p=0.031). To account for a possible influence of adipose tissue, BMI percentiles were added to the regression analysis (Table 3). Anti-inflammatory IL-1ra concentrations were associated with BMI percentiles at all time points investigated. IL-1ra showed similar associations with C-peptide as without adjustment for BMI percentiles suggesting BMI independent associations with C-peptide six and twelve months after diagnosis (Table 3). Anti-inflammatory adiponectin was independent of BMI percentile and analysis revealed similar associations as the analysis without adjustment of BMI percentiles; adiponectin was associated with C-peptide twelve months after diagnosis and with HbA1c one month after diagnosis (Table 3). Similar to adiponectin, pro-inflammatory IL-1ß was not associated with BMI percentiles and revealed same association as without BMI percentile adjustment. IL-1ß was negatively associated with C-peptide one month after diagnosis (Table 3).

Analyses of IL-6, CCL2 and TNF-α revealed no association at any time to any metabolic parameter.

Association of circulating cytokines with Δ blood glucose in the liquid meal test. Cytokines influence not only insulin signaling, but also reveal insulin independent induction of glucose disposal in case of IL-6 and adiponectin (27,28). To assess the possible influence of cytokines on glycemic control in our study, we investigated whether Δblood glucose in the standardized liquid meal test (taken as a measure of blood glucose disposal) is associated with circulating cytokines (Figure 3). Smaller Δblood glucose is suggestive of a higher glucose disposal and likely to reflect a more healthy status. Regression analysis included cytokines, Δ blood glucose, sex, age and BMI percentiles.

Anti-inflammatory adiponectin was positively associated with Δblood glucose (ß=0.021; p=0.024) six months after diagnosis. Pro-inflammatory IL-6 and CCL2 were negatively associated with Δblood glucose twelve (ß=-0.035; p=0.047) and one and six months after diagnosis, respectively (ß=-0.021; p=0.036 and ß=-0.018; p=0.046). IL-1ra, IL-1ß and TNF-α showed no association with Δblood glucose.

DISCUSSION

Improved ß-cell function, reduced insulin resistance and improved glucose disposal are likely candidates to affect remission in type 1 diabetes. All these processes have been shown to be influenced by cytokines. Pro-inflammatory IL-1ß induces apoptosis in insulin producing ß-cells whereas the anti-inflammatory IL-1ra as the specific receptor antagonist of IL-1ß preserves beta-cells (16,17).

We here show that increased IL-1ra is associated with improved ß-cell function (stimulated C-peptide) in type 1 diabetes patients which is in line with the protective effect of IL-1ra on ß-cell in patients with type 2 diabetes (16). We observed a positive association with C-peptide in the regression models that were adjusted for sex, age and blood pH. In addition, we found that IL-1ra was elevated in patients with improved C-peptide secretion and in patients in remission 7.5. Elevated IL-1ra levels were also maintained at twelve months when more stringent criteria for remission were applied. Additional adjustment for BMI percentiles in the analyses models revealed a positive association of IL-1ra with BMI percentiles (Table 3) confirming previous studies and supporting adipose tissue as an important source of IL-1ra (40,41).

Interestingly, IL-1ra showed a BMI percentile independent protective association with C-peptide secretion when we performed regression analysis investigating metabolic parameters or patients with improved C-peptide secretion. The significant elevation of IL-1ra in

patients in remission was attenuated when adjusting for BMI percentiles.

Remitters according to both definitions of remission exhibited significantly higher BMI percentiles one month after diagnosis in contrast to non-remitters. This elevation of BMI percentiles in remitters may be due to the finding that patients with more severe symptoms at diagnosis including lower BMI and ketoacidosis have more pronounced ß-cell destruction and are less likely to undergo remission during follow-up than children with less severe symptoms. Of note, we have confirmed the protective association of IL-1ra to ß-cell function in an independent cohort of 99 recent onset type 1 diabetes patients (unpublished data C. Pfleger, N.Schloot).

Since IL-1ra is antagonistic to IL-1ß, it is interesting to note that stimulated C-peptide was negatively associated with circulating IL-1ß. However, the interpretation of these data requires caution since we could detect IL-1ß concentration in less than 15% of investigated samples.

For adiponectin, the other anti-inflammatory cytokine expressed by adipose tissue, we expected higher circulating concentrations in remitters compared to non-remitters, since it has been described that adiponectin leads to improved glucose homeostasis probably due to improved glucose disposal (42). However, we observed lower adiponectin concentrations in patients in remission of both definitions. We also observed a positive association of adiponectin with HbA1c, a negative association of adiponectin with C-peptide and with blood glucose disposal. Whether the increased adiponectin concentrations in patients with less endogenous C-peptide secretion and poorer metabolic control resulting in more oxidative stress reflect a compensatory attempt to induce glucose homeostasis cannot be investigated in this type of study. Yet, recent publications report an up-regulation of adiponectin during oxidative stress that would support our observation (43,44). Of note, adiponectin revealed no association to BMI percentiles in contrast to IL-1ra (Table 3), both secreted by adipose tissue, and was not correlated with the other immune mediators (Table 1). Both findings regarding adiponectin, missing association with BMI percentiles after multiple adjustment and lack of association with circulating concentrations of cytokines are in line with previous findings from a population-based study (45). We investigated monomeric adiponectin that has been described to be effective (27). Whether high molecular weight multimers of adiponectin would add or reveal different associations is not clear and is subject to debate (46).

Increased IL-6 has been shown to be linked with inflammation and insulin resistance especially in patients with metabolic syndrome (22). Contrary to these findings we observed in our study elevated IL-6 concentrations in patients in remission and patients with increased C-peptide secretion who are thought to be characterized by reduced insulin resistance and inflammation (47,48). The negative association of IL-6 with Δblood glucose found in our study that is suggestive of induction of blood glucose disposal by IL-6 might explain why we observed elevation of IL-6 in remission. This suggestion is supported by several studies that found induction of blood glucose disposal by IL-6 (12,28,49,50). To account for the pro-inflammatory character of IL-6 it is important to note that we observed elevated IL-6 concentrations during the first month after diagnosis suggesting pro-inflammatory processes around diabetes onset and a decrease during follow up which is in line with a previous study (14).

Similar to IL-6, CCL2 was elevated one month after diagnosis and was associated with higher glucose disposal but not associated with disease stage as had been assumed previously (9).

In an additional analysis (data not shown), we observed in a small subgroup of patients that were antibody negative higher IL-1ra concentrations six months after diagnosis ($p=0.017$). This result is in line with our

observation of preserved ß-cell function during high IL-1ra concentrations since antibody negative type 1 diabetes patients are believed to undergo a less aggressive diabetes progression (51). In addition, we observed a negative association of IL-6 with glutamic acid decarboxylase antibodies (GADA) one month after diagnosis ($p=0.03$).

Certainly, it would be interesting to see whether genotypes that have an impact on diabetes are also associated with investigated cytokines and their relation to ß-cell function and metabolic status. However, these data were not available in the current study and require future study.

The strength of the current study is that we had access to a well characterized cohort with relatively big patients' numbers of newly diagnosed patients with type 1 diabetes that were followed prospectively and longitudinally for twelve months. To our knowledge, this is the first comprehensive study relating ß-cell secretion capacity, metabolic control and remission status with circulating concentrations of cytokines in pediatric patients. Potential disadvantages come from the multicentre design of the study that combines heterogeneous patient groups throughout Europe. However, at present it will be difficult to obtain equivalent patient numbers from one region only. Also it needs to be kept in mind that the results presented here are descriptive and the outcome of associations observed from metabolic data and peripheral blood and thereby a causal relationship cannot be addressed. Furthermore, there were no clamp studies performed to investigate glucose disposal and therefore, the here reported Δblood glucose gives only an indication of the glucose disposal capacity. Another topic addresses implication of BMI percentiles. We applied BMI percentiles from the United States, although the patients investigated origin from different centers mainly in Europe. This problem of heterogeneity could be overcome by applying country specific BMI, but they were not available for all patients.

We conclude that IL-1ra is associated with preserved ß-cell capacity in type 1 diabetes. This novel finding indicates that administration of IL-1ra (anakinra), that has been successfully shown to improve ß-cell function in patients with type 2 diabetes, may also be a new therapeutical approach for type 1 diabetes patients. The relation of adiponectin and IL-6 with remission and metabolic status in patients with type 1 diabetes transfers observations from *in vitro* and animal models into the human situation *in vivo*.

ACKNOWLEDGEMENTS

We are grateful to Ms. G. Gornitzka for expert help with the Luminex analyses.

The study was supported by the German Diabetes Centre, Leibniz Centre for Diabetes Research at Heinrich Heine University Düsseldorf, which is funded by the German Federal Ministry of Health and Social Security and the Ministry of Science and Research of the State North Rhine-Westphalia and by the German Diabetes foundation "Das zuckerkranke Kind".

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TABLE 1. Correlation between cytokines during the first month after diagnosis

Variables	IL-1ß		IL-6		CCL-2		TNF-α		adiponectin	
	r	p	r	p	r	p	r	p	r	p
IL-1ra	**0.176**	**0.005**	**0.282**	**<0.0001**	**0.220**	**0.0004**	**0.196**	**0.002**	0.060	0.342
IL-1ß			**0.157**	**0.012**	**0.222**	**0.0003**	0.096	0.127	-0.01	0.874
IL-6					**0.148**	**0.019**	0.087	0.166	0.024	0.704
CCL-2							**0.167**	**0.007**	0.017	0.785
TNF-α									0.098	0.118

Given are Spearman correlation coefficients (r) and p-values. Statistical significant correlations are indicated bold.

TABLE 2: Characteristics of patients classified by remission 7.5, remission 6.5 and improved C-peptide secretion

Variables/ Classification	n	Sex (m/f)	Age (years)	BMI percentiles	pH	HbA1c (%)	C-peptide (pmol/l)
Patients in remission 7.5	89	41/48	9.9 (6.7;12.5)	**71.2*** (45.6;87.8)**	**7,390*** (7.350; 7.410)**	**8.5*** (7.6;9.3)**	**522*** (250;818)**
Patients not in remission 7.5	161	87/74	9.4 (5.4;11.3)	**52.7 (26.8;75.3)**	**7,350 (7.250; 7.400)**	**9.1 (8.3;9.99)**	**355 (230;516)**
Patients in remission 6.5	46	18/28	**10.8* (7.8;13.5)**	**74.4** (48.3;87.8)**	7,385 (7.291; 7.410)	**8.2*** (7.2;9.1)**	**529* (226;945)**
Patients not in remission 6.5	204	110/94	**9.3 (5.8;11.3)**	**55.8 (29.4;76.9)**	7,370 (7.280; 7.400)	**9.0 (8.3;9.8)**	**375 (231;559)**
Patients with improved C-peptide secretion	27	15/12	10.2 (5.9;13.1)	62.8 (48.3;87.8)	7,380 (7.250; 7.405)	9.1 (7.8;9.6)	392 (238;787)
Patients without improved C-peptide secretion	200	101/99	9.5 (6.4;11.5)	58.2 (31.8;79.3)	7,365 (7.285; 7.400)	8.9 (8.2;9.9)	410 (230;586)

Due to incomplete data record for classification 22 patients for both definition of remission and 45 patients for improved C-peptide secretion were excluded from analysis. Variables are reported as median and IQR. For sex, absolute numbers are given. Dichotomous variables and variables with non-Gaussian distribution were compared within the classification using Fisher's exact test and Mann-Whitney tests, respectively. $*P < 0.05$, $**P < 0.01$; $***P < 0.001$. Statistical significant correlations are indicated bold. Sex, age and pH have been determined at diagnosis, BMI percentiles, HbA1c and C-peptide one month after diagnosis.

TABLE 3: Association between cytokines and metabolic parameters

Months after diagnosis	Variables/ models	Sex		Age (years)		BMI percentiles		C-peptide (pmol/l)		HbA1c (%)	
		ß	p	ß	p	ß	p	ß	p	ß	p
1	IL-1ra (pg/ml)	-0.11	0.059	-0.010	0.358	**0.0036**	**0.001**	0.00011	0.279	0.006	0.788
6	IL-1ra (pg/ml)	-0.062	0.307	**-0.038**	**0.0001**	**0.0027**	**0.048**	**0.00037**	**0.0001**	0.028	0.318
12	IL-1ra (pg/ml)	**-0.107**	**0.049**	**-0.022**	**0.009**	**0.0023**	**0.019**	**0.00026**	**0.009**	**0.044**	**0.046**
1	IL-1ß (pg/ml)	0.528	0.268	0.145	0.082	-0.0117	0.210	**-0.00263**	**0.009**	-0.095	0.639
6	IL-1ß (pg/ml)	-0.189	0.655	-0.054	-0.432	0.0050	0.521	0.001004	0.146	-0.057	0.781
12	IL-1ß (pg/ml)	-0.275	0.589	0.073	0.390	0.0051	0.602	-0.00041	0.665	-0.384	0.119
1	adiponectin (pg/ml)	**-0.172**	**0.027**	**-0.058**	**<0.0001**	0.0011	0.444	0.00004	0.784	**0.121**	**0.0004**
6	adiponectin (pg/ml)	-0.098	0.161	**-0.044**	**<0.0001**	-0.0002	0.891	-0.00018	0.143	0.051	0.111
12	adiponectin (pg/ml)	**-0.197**	**0.005**	**-0.035**	**0.0014**	0.0022	0.090	**-0.00047**	**0.0004**	0.038	0.182

The table gives regression coefficients (ß) and p-values. Linear regression analyses were performed for IL-1ra and adiponectin. Cytokines entered the models as log transformed variables. Logistic regression analyses were applied for IL-1ß. Cytokine entered the model as detectable or not detectable. Statistical significant correlations are indicated bold.

FIGURE LEGENDS

Figure 1. Circulating cytokine concentrations of patients with type 1 diabetes 1, 6 and 12 months after diagnosis. P-values for non parametric testing for paired data (Friedman- test) were: adiponectin and MCP-1 ($p<0.0001$), IL-6 ($p=0.0008$), TNF-α ($p=0.16$) and IL-1ra ($p=0.77$). In case of significance, p-values were calculated from comparison of two time points that are indicated in the graph. Bars represent medians. Exact values for medians are depicted above the x-axis for each month and time point [pg/ml]. For IL-1ß neither medians nor differences were investigated because of too many values below the detection limit.

Figure 2. Follow up of median circulating cytokine concentrations in patients classified by "remission" or "improved C-peptide secretion". Remission 7.5 (top panel) shows data based on the definition of remission HbA1c < 7.5 and < 0.4U/kg daily insulin. Remission 6.5 (medium panel) refers to the definition of remission with HbA1c < 6.5 and < 0.4U/kg daily insulin. Patients with improved C-peptide secretion are characterized by an increase of C-peptide from one to six months after diagnosis of at least 20% with a lower limit of 100pmol/l. Lines represent medians of the classified groups; dashed lines for patients in "remission" or "increased C-peptide secretion", black lines for patients with "no remission"or "no improved C-peptide secretion". *, $P < 0.05$; **, $P < 0.01$ adjusted for sex and age.

Figure 3. Correlation of circulating cytokine concentrations with Δblood glucose. Coefficient (ß) and p-values of regression line plotted are adjusted for sex, age and BMI percentile. A) Adiponectin vs Δblood glucose six months after diagnosis; B) IL-6 vs Δblood glucose twelve months after diagnosis.

Figure 1

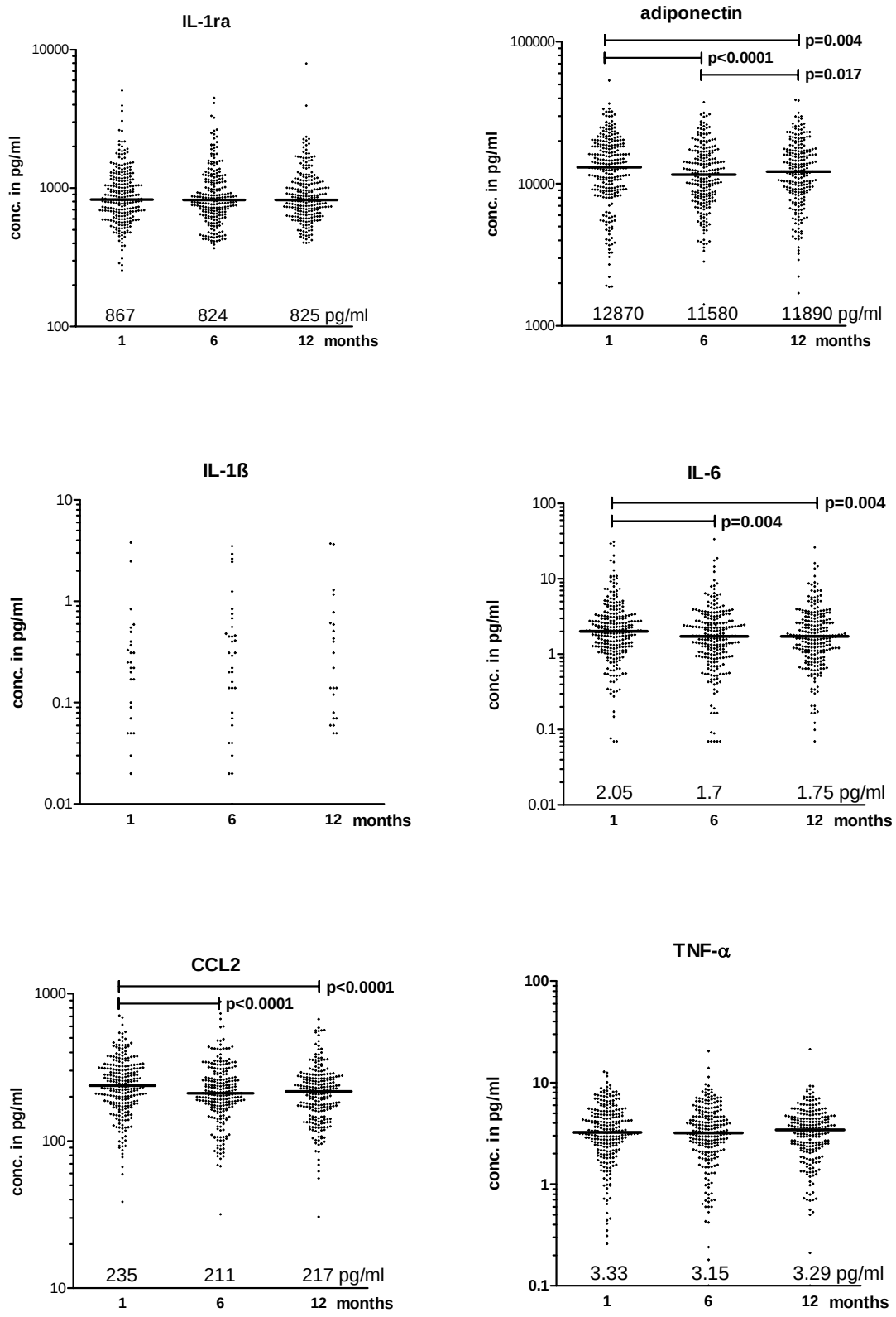

IL-1ra
conc. in pg/ml
10000
1000
100
867
824
825 pg/ml
1
6
12 months
adiponectin
100000
10000
1000
p=0.004
p<0.0001
p=0.017
12870
11580
11890 pg/ml
1
6
12 months
IL-1ß
10
1
0.1
0.01
1
6
12 months
IL-6
100
10
1
0.1
0.01
p=0.004
p=0.004
2.05
1.7
1.75 pg/ml
1
6
12 months
CCL2
1000
100
10
p<0.0001
p<0.0001
235
211
217 pg/ml
1
6
12 months
TNF-α
100
10
1
0.1
3.33
3.15
3.29 pg/ml
1
6
12 months

Figure 2

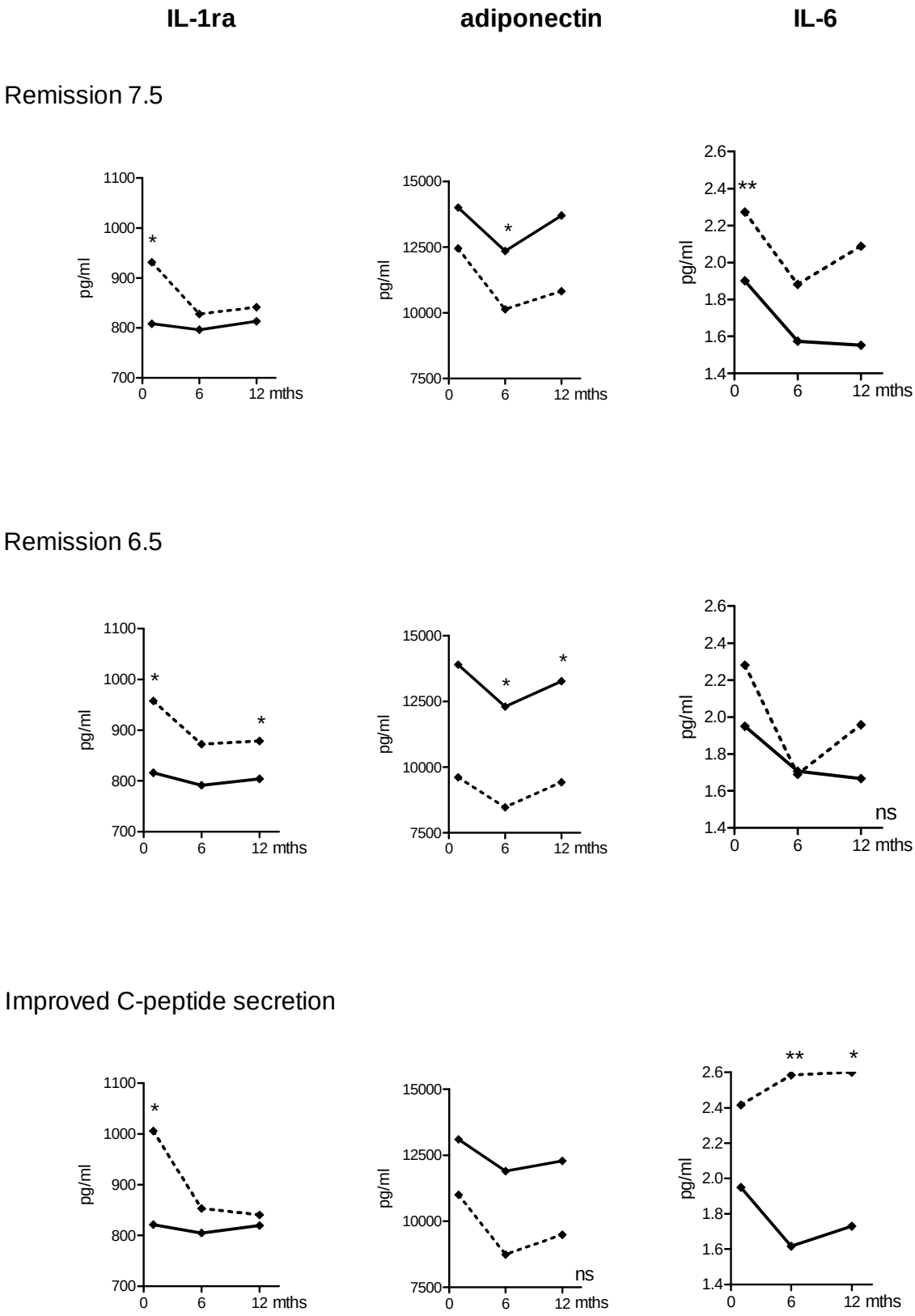
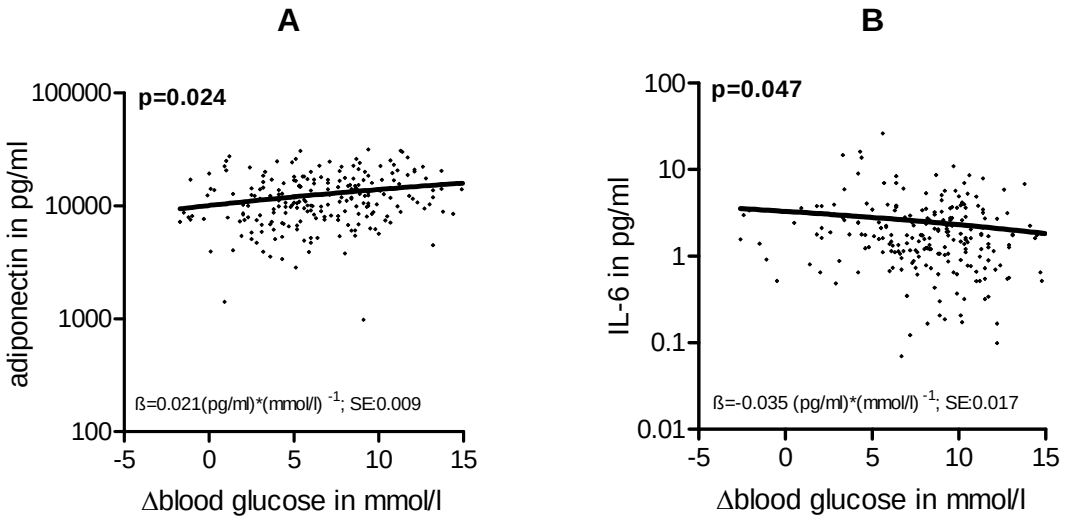

IL-1ra
adiponectin
IL-6
Remission 7.5
Remission 6.5
Improved C-peptide secretion
pg/ml
0
6
12 mths
*
**
ns

Figure 3


A
p=0.024
adiponectin in pg/ml
100000
10000
1000
100
ß=0.021(pg/ml)*(mmol/l) -1; SE:0.009
-5
0
5
10
15
Δblood glucose in mmol/l
B
p=0.047
IL-6 in pg/ml
100
10
1
0.1
0.01
ß=-0.035 (pg/ml)*(mmol/l) -1; SE:0.017
-5
0
5
10
15
Δblood glucose in mmol/l