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Relation of circulating concentrations of chemokine receptor CCR5 ligands to C-peptide, proinsulin and HbA1c and disease progression in type 1 diabetes

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Inflammation;
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Type 1 diabetes mellitus;
Remission

Abstract Th1 related chemokines CCL3 and CCL5 and Th2 related CCL4 as ligands of the receptor CCR5 contribute to disease development in animal models of type 1 diabetes. In humans, no data are available addressing the role of these chemokines regarding disease progression and remission. We investigated longitudinally circulating concentrations of CCR5 ligands of 256 newly diagnosed patients with type 1 diabetes. CCR5 ligands were differentially associated with β-cell function and clinical remission. CCL5 was decreased in remitters and positively associated with HbA1c suggestive of a Th1 associated progression of the disease. Likewise, CCL3 was negatively related to C-peptide and positively associated with the β-cell stress marker proinsulin but increased in remitters. CCL4 associated with decreased β-cell stress shown by negative association with proinsulin. Blockage of chemokines or antagonism of CCR5 by therapeutic agents such as maraviroc may provide a new therapeutic target to ameliorate disease progression in type 1 diabetes.

Introduction

Type 1 diabetes is an immune mediated disease resulting in selective β-cell destruction. T-cells play a major pathogenic

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role in islet cell infiltration and destruction [1] and express chemokine receptors on their surface [2]. Chemokines CCL3/MIP-1alpha, CCL4/MIP-1beta and CCL5/RANTES are the natural ligands of the CC chemokine receptor 5 (CCR5) and have been shown to play an important role in immune-mediated diabetes. In the non-obese diabetic (NOD) mouse, diabetes could be transferred with T-cell clones secreting CCL3 and CCL5 that were of Th1 phenotype, whereas cells of Th2 phenotype that were unable to transfer disease secreted CCL4 [3]. However, both phenotypes were able to induce insulitis. Carvalho-Pinto [4] showed that leukocyte attraction through the CCR5 receptor controls progress from insulitis to diabetes in NOD mouse. Mice treated with neutralizing anti-CCR5 antibodies developed periinsulitis but did not progress to diabetes. These data suggest that chemotaxis via ligands of CCR5 controls the invasive as well as the destructive potential of islet infiltrating T-cells. Similarly, in a murine islet transplantation model, BALB/c islet allograft transplanted into CCR5−/− C57BL/6 recipients survived significantly longer compared to the CCR5+/+ wildtype C57BL/6 recipients [5]. Interestingly to note, β-cells do also secrete CCL3, CCL4 and CCL5 in case of stress or cell death (apoptosis) in addition to the secretion of these chemokines by infiltrating T-cells [4,6–8].

Investigations in humans revealed elevated circulating CCL3 and CCL4 concentrations in a small cohort of pre-diabetic patients [9]. In another study circulating CCL3 and CCL4 concentrations were found elevated in a subgroup of newly diagnosed patients, but patients with newly diagnosed type 1 diabetes mellitus showed reduced CCR5 expression [10].

So far, no human studies in type 1 diabetes have related the functional capacity of insulin producing β-cells and systemic concentrations of the CCR5 ligands CCL3, CCL4 and CCL5. The aim of the current study was to investigate in patients with recent diagnosed type 1 diabetes 1) the course of circulating CCL3, CCL4 and CCL5 during the first year after diagnosis 2) whether patients undergoing remission reveal differences regarding CCL3, CCL4 and CCL5 in comparison to patients not undergoing remission 3) associations of CCL3, CCL4, and CCL5 with metabolic status and β-cell function.

Materials and methods

Patients

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, [11,12]. In brief, prospective clinical and biochemical data of one year from diagnosis were available for 256 children and adolescents (134 girls and 122 boys, median age 9.6years, range 3months to 16.8years) out of 275 initially investigated patients at baseline (response rate 93.1%). 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 Organisation (WHO) criteria [13]. 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

Body mass index (BMI) percentiles were used to asses the influence of adipose tissue which is more accurate in children and adolescents than the use of BMI. Stimulated serum C-peptide and proinsulin were used as a marker of β-cell function and were measured in a central facility at one, six, and twelve months of follow up. Blood samples were obtained 90min after the ingestion of a standardized liquid meal (Boost drink, formerly known as Sustacal (237ml or 8FL OZ containing 33g carbohydrate, 15g protein and 6g fat, 240kcal): 6ml/kg (maximum 360ml.), Novartis Medical Health, Inc., Minneapolis, MN, USA, www.boost.com) [14,15]. Serum samples were labeled and frozen at −20°C until shipment on dry-ice to Steno Diabetes Center for central determination of C-peptide and proinsulin.

Serum C-peptide was analyzed by a fluoroimmunometric assay (AutoDELFIA™ C-peptide, PerkinElmer Life and Analytical Sciences, Inc, Turku, Finland). The sensitivity was below 1pmol/l, the intra-assay coefficient of variation were below 6% at 20pmol/l, and recovery of the standard, added to plasma before extraction, was about 100% when corrected for losses inherent in the plasma extraction procedure [12].

Proinsulin was detected by Sandwich ELISA, which determines total proinsulin immuno-reactivity both proinsulin and its conversion intermediates. The detection limit is 0.3pmol/l and the analytical range lies between 0.3–100pmol/l. The inter-assay precision is below 8.7%.

Glycemic control as assessed by HbA1c was measured at diagnosis and one, three, six, nine and twelve months after diagnosis. HbA1c was determined in a central facility by ion-exchange high-performance liquid chromatography (normal reference range 4.1–6.4%) at Steno Diabetes Center, Gentofte, Denmark [11,16].

We used two definitions of remission to classify patients using HbA1c and insulin requirement six months after diagnosis. First, a more classical definition of partial remission was applied with HbA1c<7.5% and daily insulin <0.4U/kg (remission 7.5) [17]. However, partial remission discriminated by HbA1c<7.5% is not always indicative for a euglycemic status. Therefore we used in addition, also a stricter definition of partial remission that was HbA1c<6.5% and daily insulin <0.4U/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.

Cytokines and chemokines

Blood was drawn 90min after ingestion of the standardized liquid meal by venipuncture according to a standard protocol [12]. Thereby, an influence of catheterization on possible local production of inflammatory mediators during the 90min boost test could be excluded [18].

Serum samples were immediately labeled and frozen at −20°C until shipment on dry ice to the German Diabetes Centre for determination of chemokines. Concentrations of circulating chemokines CCL4 and CCL5 were measured by ELISA as described [9,19] using matched antibody pairs (R&D Systems, Wiesbaden, Germany). CCL3 was determined

by multiplex-bead technology using commercially available kits (Fluorokine MAP, R&D Systems, Wiesbaden, Germany). All chemokines were measured in a blind fashion, e.g. clinical data were not known when measurements were performed. The detection limits of the assays were 2.0pg/ml for CCL3, 3.0pg/ml for CCL4, and 255.5pg/ml for CCL5. Patients with chemokine concentration lower than the detection limit were assigned a value half of the detection limit (CCL3 $n=64$; CCL4 $n=0$; CCL5 $n=0$).The immunoassays showed inter-assay variations below 20% and intra-assay variations below 10%.

Statistical methods

For longitudinal follow-up, differences between chemokines concentrations were analyzed first by Friedman test followed by Wilcoxon test in case of significance to investigate differences between two time points.

Association studies were performed with log transformed chemokine concentrations, C-peptide and proinsulin that showed a normal (CCL4, CCL5, C-peptide and proinsulin) or approximately normal distribution (CCL3). Spearman correlation was applied to investigate correlations between cytokines or between metabolic parameters; multiple regression analysis was used to investigate associations between cytokines and metabolic parameters. Regression analysis included cytokines as the dependent variable and C-peptide, proinsulin and HbA1c or remission 7.5 or remission 6.5 as independent variables while adjusting for sex, age and BMI percentiles.

In the so called "association analysis" chemokines and metabolic parameters were analyzed at one time point. In the "prospective analysis" one month chemokines concentrations were associated with metabolic parameters at the later time points six and twelve months. Associations reported 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. GraphPad Software, San Diego California USA, "www.graphpad.com".

Results

Longitudinal analysis of circulating chemokine concentrations

CCL3/MIP-1alpha concentrations did not show statistically significant changes over time despite considerable variation in some patients (Fig. 1A). The two other CCR5 ligands exhibited a decrease in circulating concentrations over time. CCL4/MIP-1beta concentrations decreased from diagnosis to six or twelve months 11% or 13% respectively ($p=0.0017$, Fig. 1B). CCL5/RANTES concentrations were also significantly lower at six or twelve months after diagnosis 23% and 20% respectively compared to one month after diagnosis ($p<0.0001$, Fig. 1C).

Correlations between chemokines and between proinsulin and C-peptide

A close association between the three CCR5 ligands within individual patients was observed. CCL3 was positively correlated to CCL4 one ($r=0.35$, $p<0.0001$), six ($r=0.44$, $p<0.0001$), and twelve months ($r=0.42$, $p<0.0001$) after diagnosis as well as to CCL5 at one ($r=0.17$, $p=0.0188$) and six months ($r=0.28$, $p=0.0003$) of the follow up period. CCL4 was correlated to CCL5 at all time points (one month $r=0.380$, $p<0.0001$; six months $r=0.36$, $p<0.0001$; twelve months $r=0.24$, $p=0.0013$).

C-peptide and proinsulin showed a positive relation at one ($r=0.63$; $p<0.0001$), six ($r=0.61$; $p<0.0001$), and twelve ($r=0.70$; $p<0.0001$) months after diagnosis.

Associations of cytokines with remission

Patients were classified as remitters or non-remitters and associations of this classification with CCR5 ligands were investigated. Patients with incomplete data record with respect to classification were excluded from analysis. Classification by the more classical definition remission 7.5 revealed 89 patients in remission (48 girls and 41 boys, median age 10.3years, median HbA1c at diagnosis: 10.65%) and 161 patients not in remission (74 girls and 87 boys, median age 9.4years; median HbA1c at diagnosis: 11.1%). The stricter definition of remission 6.5 for

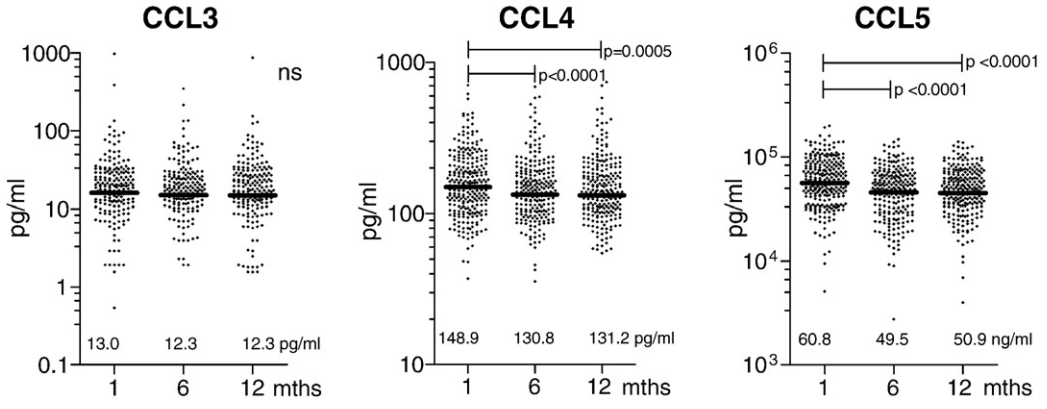

Figure 1 Circulating chemokine concentrations of patients with type 1 diabetes one, six and twelve months after diagnosis. *P*-values using non-parametric test for paired data (Friedman-test) were $p=0.0017$ for CCL3 and $p<0.0001$ for CCL5. *P*-values referring to the comparison of two time points are indicated in the graph. Bars represent medians. Exact values for medians are depicted above the *x*-axis for each time point.

classification showed 46 patients in remission (28 girls and 18 boys, median age 10.8years, median HbA1c at diagnosis: 10.4%) and 204 patients not in remission (94 girls and 110 boys, median age 9.3years; median HbA1c at diagnosis: 11.0%).

While adjusting for sex, age and BMI percentiles, associations of CCL3 and CCL5 (but not of CCL4) were observed with classification of both definitions of remission (Fig. 2). CCL3 was elevated in remitters in comparison to non-remitters, one month ($p=0.017$) and twelve months after diagnosis ($p=0.013$) in remission 7.5 and remission 6.5, respectively. CCL5 was decreased in remitters one month after diagnosis in both definitions of remission ($p=0.031$; $p=0.043$, remission 7.5 and 6.5, respectively).

Associations and prospective analysis of HbA1c, stimulated C-peptide and proinsulin with chemokines

To study associations of CCR5 ligands concentrations with metabolic parameters, we performed association and prospective analyses.

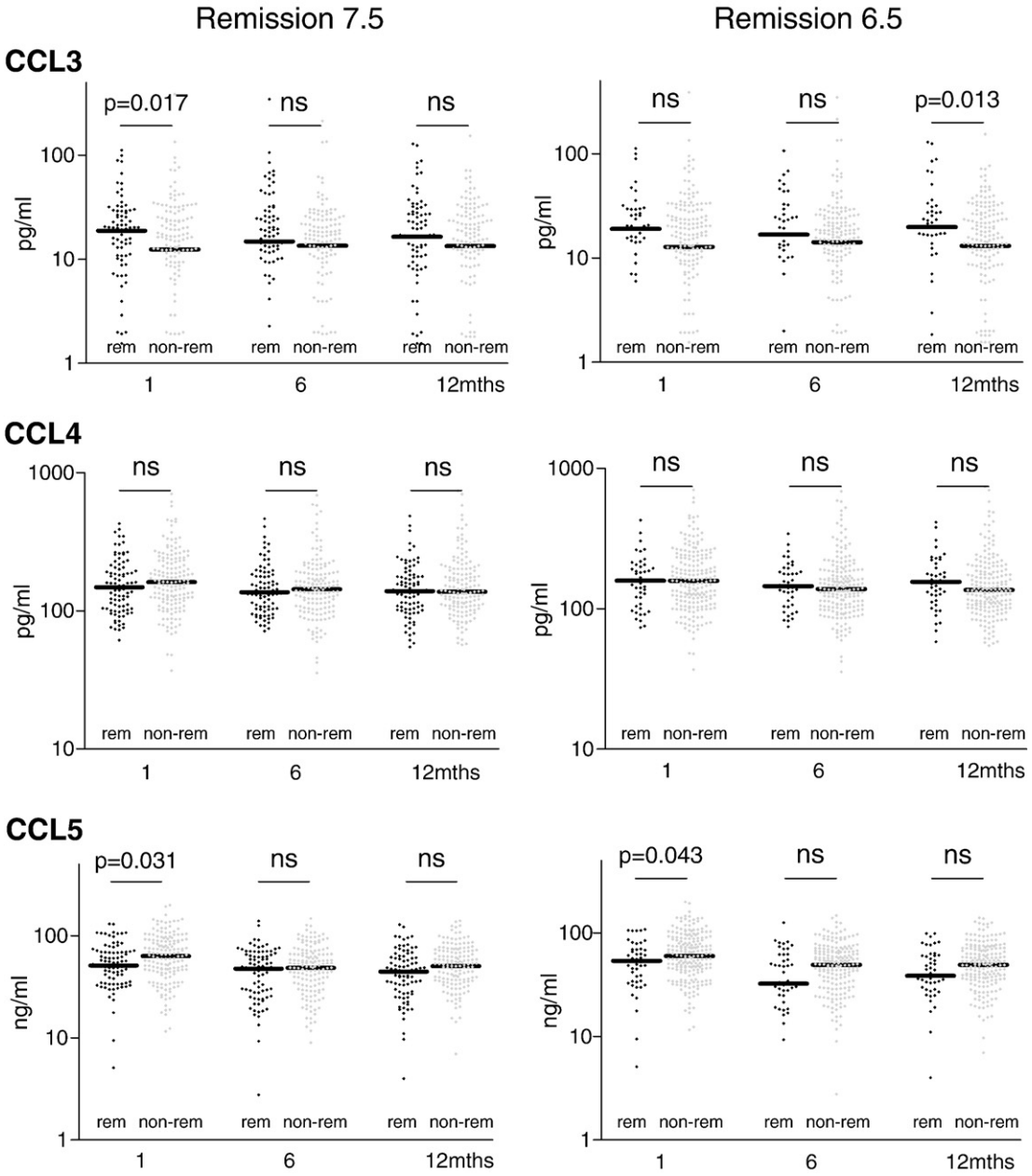

Figure 2 Circulating chemokine concentrations in patients classified remitter or non-remitter. Remission refers to the definition of remission 7.5: HbA1c<7.5 and <0.4 U/kg daily insulin; remission 6.5: HbA1c<6.5 and <0.4 U/kg daily insulin. Bars represent medians. *P*-values are adjusted for sex, age and BMI percentiles and indicate cross sectional statistical significant differences at the corresponding time. At the four time points where the values were significantly different, the median values for remitters vs non-remitters were as follows, CCL3 one month after diagnosis, rem 7.5 vs non-rem, 18.77 pg/ml vs 12.5 spg/ml; CCL3 after 12 months, rem 6.5 vs non-rem, 19.83 pg/ml vs 13.11 pg/ml; CCL5 after 1 month, rem 7.5 vs non-rem, 52.55 ng/ml vs 64.64 ng/ml; and CCL5 after 1 month, rem 6.5 vs non-rem, 53.45 ng/ml vs 60.33 ng/ml.

Table 1A "Association Model", association of chemokine concentrations and metabolic parameters at each time point

Chemokine	Month	Metabolic parameters	β	*p*-value
CCL3	1	C-peptide	−0.448	0.0006
CCL3	1	Proinsulin	0.368	0.014
CCL3	6	C-peptide	−0.446	0.002
CCL3	12	C-peptide	−0.280.	0.022
CCL4	1	C-peptide	−0.100	0.042
CCL4	12	Proinsulin	−0.070	0.037
CCL5	1	HbA1c	0.093	0.005

Regression analysis was adjusted for sex, age and BMI percentiles. Given are the resulting coefficients (β) with their corresponding *p*-values.

In the association model, circulating concentrations of all three CCR5 ligands revealed associations with metabolic parameters (Tables 1A and 1B). Circulating concentrations of Th1 associated CCL3 were negatively associated with C-peptide one, six and twelve months after diagnosis ($p=0.0006$, $p=0.002$ and $p=0.022$ respectively) and positively related to proinsulin one month after diagnosis ($p=0.014$). CCL5 concentrations were positively related to HbA1c one month after diagnosis ($p=0.005$) (Table 1A). Th2 associated CCL4 revealed a negative association with proinsulin twelve months after diagnosis ($p=0.037$) and with C-peptide one month after diagnosis ($p=0.042$).

In the prospective model, only the Th1 associated chemokines CCL3 and CCL5 revealed associations of their baseline concentrations with later β-cell function and metabolic control. CCL3 concentrations one month after diagnosis were negatively associated with C-peptide ($p=0.007$) and HbA1c ($p=0.026$) six months after diagnosis and with C-peptide ($p=0.009$) and proinsulin ($p=0.018$) twelve months after diagnosis (Table 1B). One month after diagnosis CCL5 concentration showed positive association with proinsulin twelve months after diagnosis ($p=0.007$).

Chemokine and C-peptide concentrations separated by centers

It is known from epidemiological studies that the incidence and metabolic characteristics of type 1 diabetes varies geographically [20,21] and therefore we investigated patients for center differences. Centers showed differences for C-peptide, HbA1c, and the CCR5 ligands CCL3, CCL4, CCL5 ($p<0.0001$, $p=0.013$, $p<0.0001$, $p=0.0004$, $p<0.0001$ respectively) but not for age (Fig. 3).

Discussion

CCR5 and its ligands CCL3, CCL4, and CCL5 are thought to play a role in immune mediated diabetes in several animal models. We investigated the association of circulating CCL3, CCL4, and CCL5 with different definitions of remission and β-cell function in the well characterized prospective Hvidøre cohort of newly diagnosed juvenile type 1 diabetes patients during the first year [11,22].

Circulating CCR5 ligands were positively correlated with each other during the investigated time but further analysis revealed a distinct role of CCL3, CCL4 and CCL5 in type 1 diabetes.

Patients showed decreasing CCL4 and CCL5 concentrations with highest concentrations one month after diagnosis. In contrast, CCL3 remained stable during follow up suggestive of differential regulation of CCR5 ligands early after diagnosis of type 1 diabetes without general up regulation of systemic immune mediators confirming a previous study [17]. Although these observations suggest a change of cytokines overtime, it is unclear whether this is of clinical or biological meaning. Due to the considerable overlap of measurements at the different time points these cytokine measurements do not qualify as diagnostic markers on an individual basis. However, they may offer insights on the role of chemokines in type 1 diabetes and our findings such as associations with remission or metabolic parameters are in line with observations made in animal experiments *in vivo* [4,23,24].

The analysis of patients' subgroups showed that both Th1 related chemokines CCL3 and CCL5 revealed opposite associations with remission whereas the Th2 related CCL4 was not associated with remission, regardless whether the more classical or stricter version of remission was applied. CCL5 showed decreased concentrations in remitters in comparison to non-remitters which is in line with a recent publications that describes a key role in the process of leukocyte invasion in islets [4]. Interestingly, CCL3 which attracts leukocytes to the site of inflammation [25] was elevated in remitters. This observation was unexpected since remission is characterized by a rather less aggressive progression whereas elevated CCL3 has been shown to be associated with increased insulitis [23]. In addition, protection from immune-mediated diabetes in animal models was associated with decreased CCL3 concentrations [26]. Whether elevated CCL3 concentrations in remitters reflect an enforced leukocyte attraction due to the presence of more insulin producing β-cells remains speculative. Interestingly, we could not observe a statistical significant elevation of the Th2 related CCL4 in remitters that has been described to prevent diabetes in NOD mice [24].

We investigated the relation of CCR5 ligands in type 1 diabetes in more detail by analyzing association of CCR5 ligands with β-cell function and metabolic parameters. Both Th1 associated chemokines CCL3 and CCL5 were inversely related with β-cell function which confirms previous work that suggested a deleterious role of these chemokines [3,27].

Table 1B "Prospective Model", prospective analysis with association of baseline chemokine concentrations (one month after diagnosis) and subsequent course of the disease (i.e., metabolic parameters six and twelve months after diagnosis)

Chemokine	Metabolic parameters	β	*p*-value
1 month CCL3	6 months HbA1c	−0.225	0.026
1 month CCL3	6 months C-peptide	−0.410	0.007
1 month CCL3	12 months C-peptide	−0.345	0.009
1 month CCL3	12 months proinsulin	0.244	0.018
1 month CCL5	12 months proinsulin	0.120	0.007

Regression analysis was adjusted for sex, age and BMI percentiles. Given are the resulting coefficients (β) with their corresponding *p*-values.

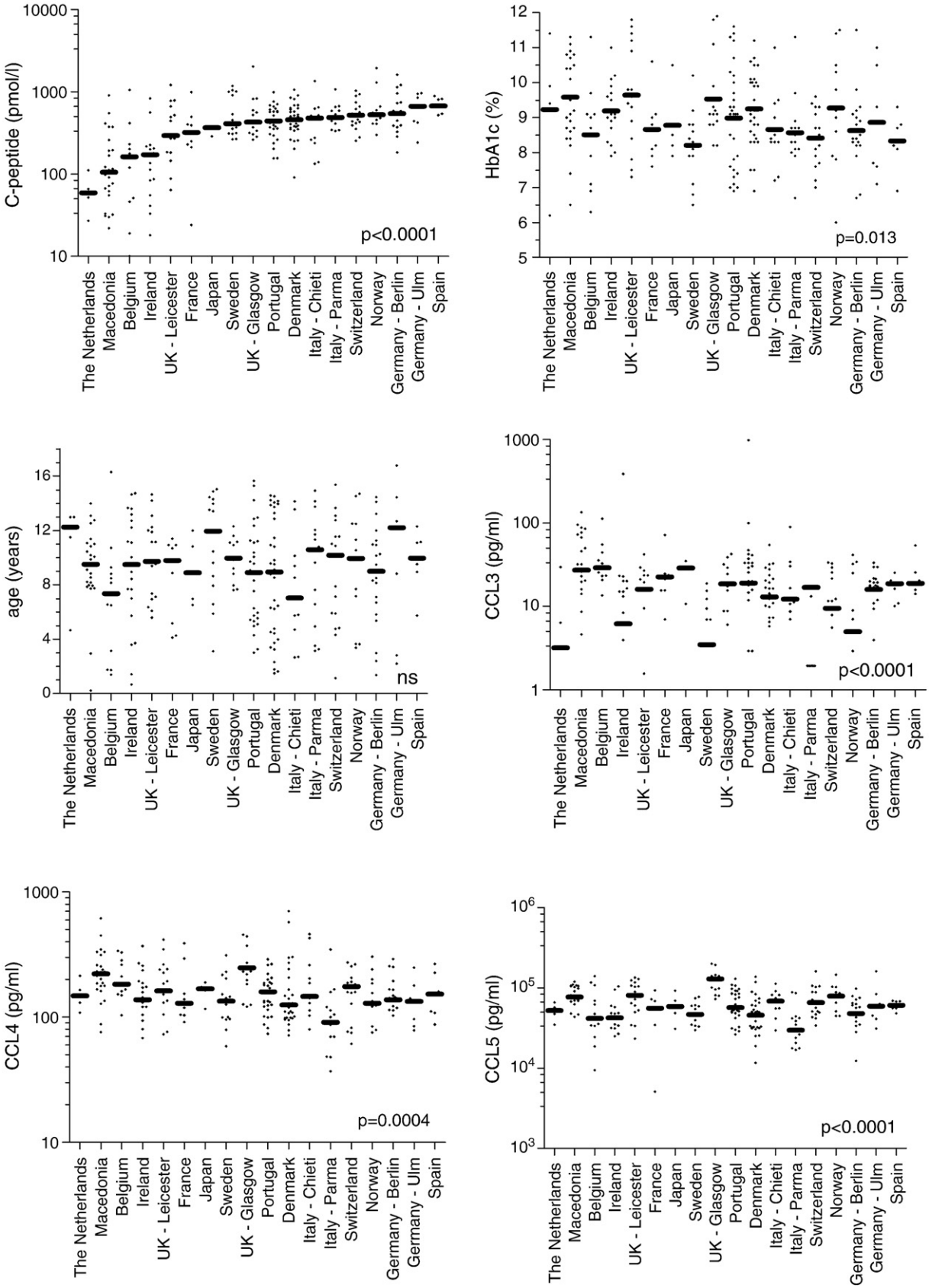

Figure 3 Distribution of chemokine concentrations and metabolic parameters of patients with respect to centers. C-peptide, HbA1c, age and chemokine concentrations of patients were classified by the enrolment of the corresponding country/center and sorted by increasing C-peptide concentrations. Medians are presented and calculated p-values indicate that medians differ significantly (Kruskal Wallis test). Of note, all *y*-axes show log values beside age which gives linear values.

We observed different associations of CCL3 and CCL5 with metabolic parameters suggesting a differential role of these Th1 associated chemokines in type 1 diabetes. CCL3 showed the most prominent contribution in the association and prospective model despite the rather stable course over time in the entire cohort. This conception underscores the importance of analysis of clinically different subgroups. Indeed, when classifying for remission, CCL3 revealed an opposed longitudinal course for remitters versus non-remitters.

CCL3 showed a negative association with C-peptide and a positive association with proinsulin that is not only a precursor of C-peptide but has also been described as a marker for β-cell stress [28–30]. Interestingly, in the prospective model CCL3 was negatively associated with HbA1c and might support the assumption of enforced leukocyte attraction due to the presence of more insulin producing β-cells and confirms observations between CCL3 and remission. In contrast, CCL5 showed positive association with HbA1c and proinsulin but not with C-peptide. CCL4 that is related to Th2 was negatively associated with proinsulin. This finding supports the suggestion of a protective role of CCL4 to β-cell during diabetes progression [23,24]. The missing association of CCL4 with remission that was defined by metabolic control and insulin requirement might be explained by the reason that remission is caused by different factors than just β-cell function [31,32].

Of note, the standardized protocol of the Diabetes Control and Complications Trial was applied to determine the peak C-peptide concentrations after mixed meal stimulation [14,15], appreciating that some patients may have a peak response at a slightly different time point.

Interestingly, patients separated by country/enrolling centers revealed statistical significant differences with respect to metabolic parameters and chemokine concentrations. Whether differences occurred due to more aggressive disease, early diagnosis due to good health care system or environmental factors beside many others cannot be answered in this study design. As samples were obtained after a standardized protocol and were sent frozen to a central facility for determination, differences of these parameters due to centres can be excluded. Our findings are in line with observations from others reporting country or even site specific differences due to several reasons [21,33]. However, our data need to be interpreted with caution since patient numbers recruited varied considerably between centres. As discussed and suggested by others [34], multiple regression analyses were not adjusted for centres because of standardized protocol, central determination of outcome variables, low center enrollment and determination of local caused immune markers in circulation.

Taken together in this well-characterized, prospective and unique Hvidøre cohort we present different associations of the CCR5 ligands CCL3, CCL4 and CCL5 in type 1 diabetes. Both Th1 related chemokines CCL3 and CCL5 were associated with decreased β-cell function. Associations with remission and metabolic parameters suggest a differential role in disease progression of these two chemokines. CCL4 that revealed negative association with proinsulin as a stress marker might play a rather benign role.

However, 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. 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 direct blockage of CCL3 and CCL5 or the antagonism of CCR5 by maraviroc currently applied for HIV patients may provide a new therapeutic target to ameliorate disease progression in type 1 diabetes as has been shown in animal models [4,5].

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Appendix

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