Circulating concentrations of cytokines as markers for pathogenic processes in type 1 diabetes and their association with ß-cell function, ß-cell stress and metabolic status

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to

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Contents

List of abbreviations

BBDR	biobreeding diabetes-resistant
BMI	body mass index
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CTLA	cytotoxic T-lymphocyte associated
DZB	daclizumab
ELISA	enzyme-linked immunosorbent
ELISPOT	enzyme-linked immunosorbent spot
Foxp	forkhead family transcription factor
GAD	glutamicacid decarboxylase
HbA1c	haemoglobin A1c
Hsp	heat shock protein
HLA	human leucocyte antigen
ICA	islet cell antibody
IAA	insulin antibody
IA-2	insulinoma associated antigen-2
IDDM	insulin dependent diabetes mellitus type 1
IFN	interferon
IL	interleukin
MHC	major histocompatibility complex
MCP	macrophage chemoattractant protein
MFI	mean fluorescence intensity
MIP	macrophage inflammatory protein
MMF	mycophenolate mofeteil
NOD	non-obese diabetic
PBMC	peripheral mononuclear cell
PHA	mitogen phytohemagglutinin
PI	phorbol-myristate-acetate and ionomycin
PL	parameter logistic
PMA	phorbol-myristate-acetate

RANTES	regulated upon activation, normal T-cell expressed and secreted
ROR	retinoic acid-related orphan nuclear receptor
SI	stimulation index
STAT	transducer and activator of transcription
SOCS	suppressor of cytokine signaling
T-bet	T-box expressed in T-cells
TC	tissue culture medium
TGF	transforming growth factor
TLR	Toll like receptor
TNF	tumor necrosis factor
Treg	regulatory T-cells
TT	tetanus toxoid
VNTR	variable number of tandem repeats
ZnT	zinc transporter

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Introduction

Type 1 diabetes

Type 1 diabetes is an immune mediated disease characterized by the selective destruction of insulin producing ß-cells in the pancreas, leading to insulin deficiency and hyperglycemia. Patients require lifelong administration of exogenous insulin for their survival (1). Interactions between genetic and yet unknown environmental factors are thought to be the origin of type 1 diabetes. This is illustrated by the highly variable incidence of type 1 diabetes among different ethnic populations: from 0.1/100.000 per year in China to more than 40/100.000 per year in Finland. In addition, a north-south gradient with lowest incidence rates in the south has been described, but high incidence rates have been also reported in Kuwait and Puerto Rico. Of concern is the rapidly rising incidence of type 1 diabetes worldwide and the trend towards an earlier disease onset. Most striking is this development in regions with traditionally low incidence (2,3). The incidence of type 1 diabetes is estimated to be about 40% higher in 2010 than in 1997 (4).

The onset of clinically overt type 1 diabetes occurs when remaining ß-cells are not able to maintain sufficient metabolic control. However, after clinical onset and initiation of insulin therapy around 30-60% of children and adolescents develop a temporary and partial remission phase that is called "honeymoon phase". This phase develops during the first 1-6 months after starting insulin treatment and may last up to 1-2 years (5). Adults and adolescents are more likely to enter remission than very young children which probably reflects a more aggressive disease progression at younger age (6). However, the underlying mechanisms of remission are not clear and may not only be attributable to improved ß-cell function, but also to a decline of insulin resistance as result of better glycemic control (7,8).

Pathogenesis of type 1 diabetes

Autoantibodies that are directed against islet antigens such as islet cells (ICA), insulin (IAA), glutamic acid decarboxylase (GADA) and protein tyrosine phosphatase (IA-2/IA-2ß) can be detected years before the onset of clinical type 1 diabetes and identify individuals at risk to develop type 1 diabetes (9-11). Recently, a new autoantigen has been found, the zinc transporter T8 (ZnT8), and the combined measurement of ZnT8A, GADA, IA2A, and IAA raised autoimmunity detection rates to 98% at disease onset, a level that approaches the need to detect prediabetes in a general pediatric population (12). However, not all of these so

called high risk individuals will progress to type 1 diabetes, and until now the role of autoantibodies in the onset of disease is not clear (9,11). Although autoantibodies are of predictive value, the development of type 1 diabetes in a patient with congenital B-cell deficiency rather provides evidence that neither B-cells nor antibodies are indispensable in the pathogenesis of type 1 diabetes (13).

In contrast, cellular immune responses including T-cells are thought to play an important role in the pathogenesis of type 1 diabetes. Studies in the non obese diabetic (NOD) mouse model, an animal model for human type 1 diabetes, have shown that ß-cell destruction is largely mediated by T-cells (14,15). Diabetes onset could be prevented in the NOD mouse when trafficking of antigen specific T-cells to ß-cells was discontinued by blocking the CC-chemokine receptor (CCR)5 (16). Furthermore, Bradley et al induced immune mediated diabetes by islet specific $CD4^+$ T cell clones of the Th1 phenotype in an animal model, whereas the induction of a Th2 phenotype of the same islet specific T cell clone resulted in insulitits but not manifest diabetes (17).

Interestingly, Arif et al showed T-cells that are autoreactive to islet derived antigens are present in both type 1 diabetes patients and healthy controls (18). However, the characteristics of the induced immune response by T-cells were different between these two groups; the immune response upon stimulation with autoantigens showed a proinflammatory phenotype in type 1 diabetes patients, whereas in healthy controls anti-inflammatory IL-10 was induced upon stimulation that was believed to characterize T regulatory cells (Tregs). These results suggest that immune regulation plays an important role in type 1 diabetes, and Tregs that are involved in this regulation may have the potential to attenuate immune mediated pathology (19,20). Beside the adaptive immunity, innate immunity plays an important role in the pathogenesis of type 1 diabetes. Macrophages and dendritic cells are the first cell types to infiltrate the pancreatic islets and play an essential role in the development and activation of ß-cell specific T cells (21-23). Recent work done by Zipris et al showed virus-induced innate immune activation and autoimmune diabetes in the biobreeding diabetes-resistant (BBDR) rat via the Toll like receptor (TLR) 9-signaling pathway which may explain why viral infections have been found to be associated with type 1 diabetes in epidemiological studies in humans (24).

Genetics

Type 1 diabetes is strongly clustered in families with an overall genetic risk ratio (λ_s) of ~15 (25) which supports the relevance of genetic determinants. However, most type 1 diabetes patients do not have relatives with type 1 diabetes.

Recently, genome wide scans have confirmed former and found new susceptibility alleles. These discoveries were made possible by using new technologies and worldwide consortiums (Table 1) (26-28). Interestingly, most diabetes susceptibility alleles may affect functions in the immune system, especially in T-cells.

The main locus that contributes to the familial clustering resides within the major histocompatibility complex (MHC) on chromosome 6p21 which emphasizes the relevance of antigen presentation and T-cells in this disease. The human leucocyte antigen (HLA) class II genes HLA-DRB1 and HLA-DQB1 are likely to represent the primary determinants of insulin dependent diabetes mellitus (IDDM) type 1 and correlate well with the population incidence of type 1 diabetes (29). However, the MHC accounts for only 40% of the observed familial clustering. Individuals who carry the high-risk haplotypic combination DRB1*04-DQB1*0302/DRB1*03-DQB1*0201 have a risk of 5% to develop type 1 diabetes. Within affected sib pair families, this genotype-related risk increases to ~20% (30,31).

Other, non-HLA loci have been identified based on genetic association studies. One example is *IDDM2* [*INS*, 11p15 (32-35)] that encodes VNTR (variable number of tandem repeats) in the insulin gene that affect insulin expression and translation. Lower expression of insulin in the thymus is thought to impair the induction of tolerance to insulin in maturing/developing T-cells. In addition, gene loci that encode proteins which regulate T-cell activity have been linked with type 1 diabetes; *IDDM12* [*CTLA4*, 2q33 (36)] is involved in the up- and down-regulation of T-cell activity via CD28 and CTLA4 (cytotoxic T-lymphocyte associated-4), respectively; *IDDM18* [IL-12B, 5q31-33 (37-39)] and the newly found linkage signal on chromosome 4q27 [IL-2 (40,41)] may be involved in T-cell differentiation and proliferation.

Table 1: Susceptibility loci for the insulin dependent diabetes mellitus type 1 (IDDM).

Name	Chromosome	Candidate gene(s)
confirmed		
IDDM1	6p21.3	Haupthistokompatibilitätsantigen (HLA) DR/ DQ
IDDM2	11p15.5	Insulin gene region (INS-VNTR)
PTPN22	1p13	PTPN22 (LYP)
IDDM3	15q26	?
IDDM4	11q13.3	MDU1, ZFM1, RT6, ICE, LRP5, FADD, CD3
IDDM5	6q25	MnSOD
IDDM6	18q12-q21	JK (Kidd) , ZNF236
IDDM7	2q31-33	NEUROD, CTLA4
IDDM8	6q25-27	?
IDDM9	3q21-25	?
IDDM10	10p11-q11	Stromal-cell derived factor-1 (SDF-1)
IDDM11	14q24.3-q31	ENSA, SEL-1L
IDDM12	2q33	CTLA-4, CD28
IDDM13	2q34	IGF BP2, IGFBP5, NEUROD, HOXD8
IDDM15	6q21	?
IDDM16	14q32	Immunoglobulin heavy chain (IGH)
IDDM17	10q25	?
IDDM18	5q31-33	Interleukin-12p40 (IL12B)
	10p15	IL2RA/CD25
	2q24	IFIH/MDA5
newly discovered		
WTCCC Study, 2007 (40)		
	12q13	10 genes, e.g. ERBB3
	12q24	SH2B3/LNK, TRAFD1, PTPN11
	16p13	KIAA0350, dexamethasone induced transcript
	4q27	IL2, IL21
	12p13	CD69, multiple CLEC
	18p11	PTPN2 (T cell protein thyrosinase)
	5q14	?
	18q22	CD226
	5p13	CAPSL
	20q13	C20orf168
	5p13	IL7R

IDDM 14 has not been identified until now.

Role of cytokines, chemokines and adipokines

Cytokines are signaling proteins and glycoproteins with a molecular mass between 8 and 30 kDa. Cytokines act by binding to specific soluble or membrane-bound receptors and have synergistic, antagonistic, pleiotropic or redundant effects depending on cell type, cytokine concentration and environment. They are involved in virtually all cellular and metabolic processes and are critical to the function of both innate and adaptive immune responses. Thus, cytokines are given great attention in the pathogenesis of type 1 diabetes (42,43).

T-cells can be roughly divided into phenotypes based on their cytokine secretion profile (Figure 1). T-cells of the Th1 phenotype, which are able to transfer diabetes, can be distinguished by their cytokine secretion pattern from the Th2 phenotype that is believed to protect from diabetes onset (17,44). The Th1 phenotype is characterized by interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-ß secretion in contrast to the Th2 phenotype that secretes IL-4, IL-5 and IL-13 (45,46). IL-10 is often classified as a Th2 cytokine in mice, but in humans IL-10 is secreted by both Th1 and Th2 cells (47,48). It is important to note that the development of naïve T-cell to a Th1 or Th2 phenotype is cytokine driven and therefore potentially modifiable (44). IL-12 which induces changes by the transcription factor STAT4 (signal transducer and activator of transcription-4), and the transcription factor T-bet (T-box expressed in T-cells) is the key determinant for the differentiation into the Th1 phenotype (49).

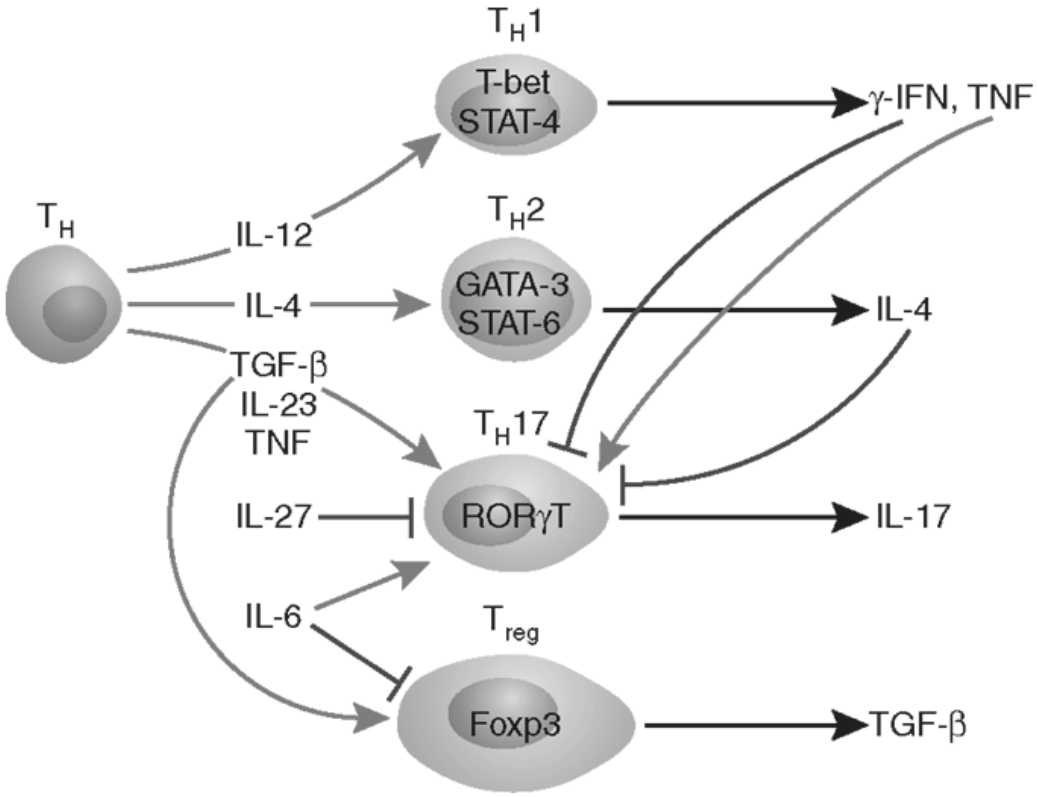
Interestingly, T-bet can convert a Th2 phenotype into a Th1 phenotype when expressed ectopically. The differentiation into a Th2 phenotype is IL-4 driven and involves activation of STAT6 and enhancer-binding protein GATA-3 (50).

Recently, a new class of T-cells has been characterized - so called regulatory T-cells (Treg). They represent a heterogeneous class of T-cells with the ability to avoid, to suppress or to stop antigen specific or unspecific immune responses. In most cases the transciption factor Foxp3 (forkhead family transcription factor3) is involved in these processes (51-57). Thus, controlling their immunoregulatory potential holds promise for the treatment of immune mediated diseases such as type 1 diabetes. Down regulation of immune reactivity by Tregs is achieved by cytokine secretion in addition to cell-cell interaction. In these regulatory processes, IL-2, IL-10 and transforming growth factor (TGF)-ß play a predominant role (58-60). Interestingly, similarly to the conversion of Th2 cells into Th1 cells, Tregs can change their suppressive phenotype which is driven by IL-6 and TGF-ß. They develop into so called Th17 T-cells which show a pro-inflammatory phenotype (61-63). Recently, it has been shown

that the retinoic acid-related orphan nuclear receptor (ROR)γT directs the differentiation program of proinflammatory IL-17+ T helper cells (64).

Figure 1: T-helper cell differentiation and regulation obtained from mice models

Arrows represent stimulation by the particular cytokines whereas lines show blockage. Transcription factors for particular lineages are placed in the nucleus.



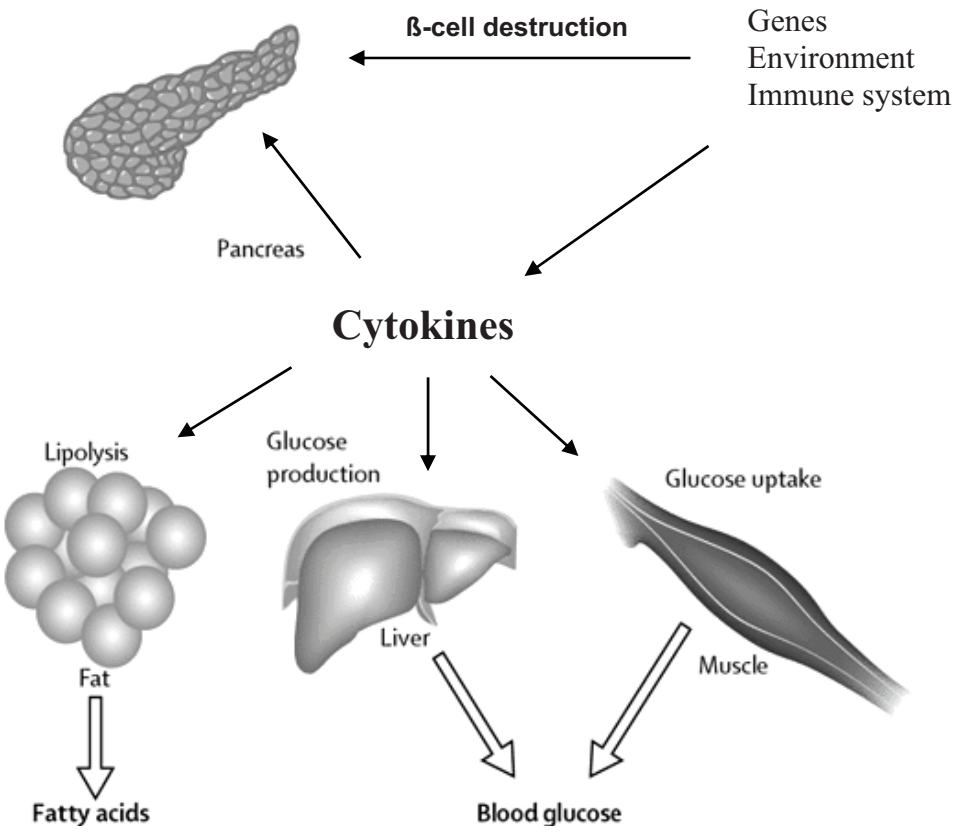
Steinman, L.; *Nat Med. 2007 Feb;13(2):139-45.*

Cytokines also reveal direct cytotoxic effects on ß-cells (Figure 2). IL-1, IFN-γ, TNF-α and TNF-ß are cytotoxic to pancreatic islets *in vitro* and the combination of at least two of these cytokines destroys ß-cells in both rodent and human islets (65).

In contrast, systemic administration of IFN-γ decreased incidence of diabetes in BB rats (66) and in combination with TNF-α decreased insulitis in NOD mice (67). IL-1 and TNF-*α* can stimulate the hypothalamic-pituitary-adrenal axis, leading to secretion of adrenocorticotropic hormone and consequently adrenal glucocorticosteroids that suppress immune and inflammatory responses (68-70).

Chemokines are cytokines, that mainly function in cell trafficking and guide cells bearing the corresponding receptor to the desired location via a concentration gradient (71,72). In the pathogenesis of type 1 diabetes, it is not only the migration of immune cells towards ß-cells, that is of particular interest, but also the migration to thymus, spleen and lymph nodes. Modulation of these migration processes may be potential therapeutic options in type 1 diabetes (73). Meagher et al showed that treatment of NOD mice at later stages of disease progression with the chemokine CC chemokine ligand (CCL)4 formerly known as macrophage inflammatory protein (MIP)-1ß protected against type 1 diabetes. This protection was associated with a Th2-like response in the spleen and pancreas, decreased recruitment of activated CD8(+) T-cells to islets and regulatory T-cell activity in the draining pancreatic lymph nodes (74). In addition, Carvalho-Pinto et al showed that the CCR5 receptor is required for migration of T-cells towards ß-cells islets (16).

Figure 2: Role of cytokines in metabolism



In addition to their role in ß-cell destruction, cytokines have an effect on insulin signaling (Figure 2). TNF-α and IL-6 have been shown to induce insulin resistance in muscle cells and hepatocytes by the protein suppressor of cytokine signaling (SOCS)-3 (75-78). This is of particular interest since obesity is associated with low grade inflammation and insulin resistance (79-82) and indeed adipose tissue has recently emerged to have a major impact on endocrine and immune mediator release (83-88). However, one has to take into account that not only adipocytes but also macrophages and T-cells are present in adipose tissue and contribute to cytokine release (89,90).

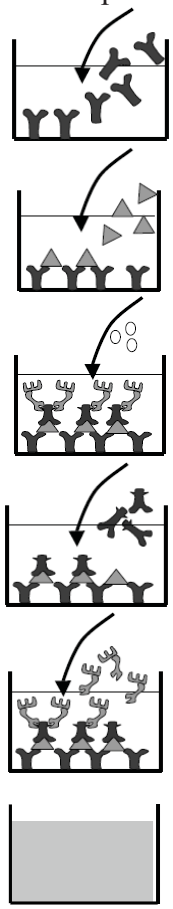
Determination of cytokines

The enzyme linked immunosorbent assay (ELISA) is the gold standard for the measurement of cytokine concentrations (Figure 3A). However, there is increasing demand for multiplexed immunoassay systems that allow for the detection of multiple cytokines so that the complex cytokine regulation and secretion pattern can be investigation while at the same time taking into account that sample volumes are often rather limited in many studies (91-94).

The commercially available Luminex-100 bead-based system, a recently developed platform that enables multiplex cytokine assays, combines the principles of sandwich immunoassay and flow cytometry (Figure 3B). A mixture of uniquely color-coded polystyrene microspheres is coated with different capture antibodies. The use of detection antibodies labeled with the fluorochrome R-phycoerythrin allows quantification of bound antigens on the microsphere surface by measurement of the mean fluorescence intensity (MFI). The system is capable of measuring up to 100 analytes simultaneously in 25 µL sample volume. Capture antibodies and the proteins of interest are not immobilized as in an ELISA, but are anchored on the surface of mobile beads, allowing for a wider dynamic range (due to an increased surface-area and enhanced freedom of antibody movement). This is of great practical value, as cytokines differ markedly in their concentrations in many biological fluids (95-97). However, the main challenge of the multiplex system is to find optimal determination conditions for *all* components in order to detect the total amount of all cytokine present in a sample (recovery) with minimal unspecific binding and minimal cross-reactivity.

Figure 3:

A: Concept of the sandwich enzym linked immunosorbent assay (ELISA)



1) capture antibody is coated on the bottom

2) capture antibody binds analyte

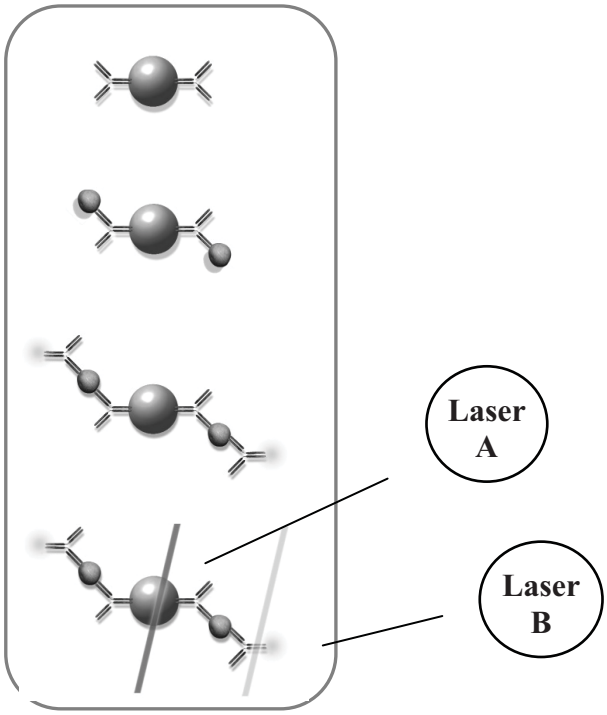
3) detection antibody binds to antibody-antigen complex

4) enzyme conjugate binds to the antibody -antigen-antibody complex

5) substrate is added

6) colour change is determined by colorimetry

B: Concept of the multiplex bead based immunoassay



1) Bead with capture antibody

2) Capture antibody binds analyte

3) Fluorophore labelled reporter antibody binds to capture analyte

4) Bead identity and reporter quantity determined by laser detector

Current and future therapies in prevention and treatment of type 1 diabetes

Treatment of type 1 diabetes is only symptomatic and involves lifelong insulin replacement. Various preparations ranging from very short to very long acting insulins are on the market to satisfy patients' needs to ensure good metabolic control (98-103). However, patients are still at risk to develop late complications (104-106). Pancreas and islet transplantation were successfully performed in adult diabetes patients and may have the potential to cure the disease. Yet, lifelong immunosuppression is needed not only to prevent rejection, but also to suppress recurrent autoimmune islet destruction (10,107,108). Since the cure of type 1 diabetes is not possible at presence without strong side effects and since substitution of insulin still cannot protect from micro- and macrovascular complications, prevention of diabetes onset or the preservation of remaining ß-cells are important goals.

Several trials have been performed that aimed at the prevention of clinically overt type 1 diabetes in high risk individuals who were identified by autoantibody status and ß-cell function (10). Nicotinamide has been used to prevent type 1 diabetes in the ENDIT study, whereas insulin has been used in the DPT-1 trial (109,110). Unfortunately, none of these trials has been successful to date.

Current intervention studies targeting newly diagnosed type 1 diabetes patients to protect remaining ß-cell involve anti-T-cell therapy via anti-CD3, ß-cell specific antigen immunization with GAD, heat shock protein (hsp)60 or DiaPep277, an hsp derived peptide, general immunsuppression by mycophenolate mofeteil (MMF) and daclizumab (DZB), and stem cell transfer (111) (http://clinicaltrials.gov). In Germany, current trials include intervention by anti-CD3, DiaPep277 and MMF/DZB in addition to vitamin D3.

So far some treatments to protect remaining ß-cells in human patients were promising:

First, treatment with an antibody directed against the CD3 receptor, which is expressed on most T-cells, led to preserved ß-cell function over 18 months probably by induction of Tregs. However, treatment was accompanied by side effects such as moderate "flu-like" syndrome and transient symptoms of Epstein-Barr viral mononucleosis (112,113).

Second, immunotherapy with DiaPep277 led to maintained insulin production with diminished need for exogenous insulin in some recent onset type 1 diabetes patients (114-116) similar to experiments in NOD mice (117-119). Treatment has induced a favorable antigen-specific immunomodulation in patients and was associated with a protective cytokine profile suggestive of the activation of regulatory T-cells confirming animal studies. Similar results have been obtained form patients treated with GAD (Diamyd) (120).

Interestingly, an anti-inflammatory approach by blocking of interleukin-1 with anakinra which has been performed in type 2 diabetes patients improved diabetes, since it is thought to protect ß-cells, improve glycemia and ß-cell secretory function and reduce markers of systemic inflammation (121). Although this study has been conducted in type 2 diabetes patients, it may open up also new ways for the treatment of type 1 diabetes.

Outline of the thesis

In the pathogenesis of type 1 diabetes T-cells and cytokines are believed to play a key role (122,123).

Although ELISAs are an established method to determine concentrations of immune mediators, recent developments such as the multiplex bead based immunoassay have improved the complex analysis of multiple cytokines.

In **Chapter 2** this thesis aimed to optimize assay conditions of the multiplex bead based system in order to obtain optimal cytokine assays in human serum. We investigated different diluents and their influence on signal intensity, assay sensitivity and background in the multiplex system in relation to the total volume-fraction of serum and protein concentration.

In **Chapter 3**, the systemic concentrations of ligands of the chemokine receptor CCR5 (i.e. CCL3, CCL4 and CCL5) and their relation to ß-cell function, ß-cell stress and metabolic control were investigated in the well phenotyped participants of the Hvidøre Study (recent type 1 diabetes patients with 12 months follow up).

In **Chapter 4,** pro- and anti-inflammatory cytokines such as adiponectin, IL-1ß, IL-1ra, IL-6, CCL2, TNF-α were investigated and their association with ß-cell function, metabolic control, glucose disposal and clinical remission in the participants of the Hvidøre Study.

In **Chapter 5**, findings regarding the relationship between circulating cytokines and ß-cell function as well as metabolic control were tested in a separate cohort. In addition, the role of antigen specific cytokine release by peripheral blood mononuclear cells (PBMC) in type 1 diabetes patients was determined in the p520/p521 DiaPep277 intervention study.

A summary and general discussion is presented in **Chapter 6**.

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2

Effect of serum content and diluent selection on assay sensitivity and signal intensity in multiplex bead-based immunoassays

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Abstract

Introduction: In the Luminex multiplexed immunoassay system, complex samples such as human serum are diluted to minimize disturbing matrix effects with a specific diluent. This diluent has to imitate the sample matrix to allow interpolation and has to provide optimal cytokine-antibody binding for all cytokines. Because diluents influence multiplex immunoassay results, this paper explores several methods to determine the quality of a chosen diluent.

Material and Methods: Two commercially available diluents, DY997 and RD6 from R&D Systems, were compared in a 19-plex immunoassay setup from Luminex.

Results: Using diluent DY997, multiplex signal intensity was reduced by 55% when spiked samples (chemokines and cytokines at 100 pg/mL) contained 50% v/v human serum, compared to samples containing 25% v/v. When using diluent RD6, signal intensity was reduced by 20% when samples contained 50% v/v human serum, compared to 25% v/v human serum. Diluent DY997 showed decreasing multiplex assay sensitivity with increasing protein concentrations, but not as low as in the presence of 50% v/v human serum.

Conclusions: In a 19-plex setup, this paper describes signal intensity, assay sensitivity and background signal levels in relation to the total volume-fraction of serum and protein concentration. For the determination of cytokines in serum samples with the multiplexed system Luminex the diluent RD6 seems more appropriate than the diluent DY997.

Introduction

There is increasing demand for multiplexed immunoassay systems that allow multiple cytokine determination by using a small amount of samples (1-4).
The Luminex-100 bead-based system combines the principle of ELISA and fluorescent bead-based flow cytometry and is theoretically capable of measuring up to 100 analytes simultaneously.
An important factor in multiplex assay optimization is the sample diluent; a protein mixture that minimizes disturbing influences such as heterophilic antibodies and unspecific binding. In addition, the diluent optimizes cytokine detection by sample dilution and, at the same time, mimics the sample matrix in the calibration curve (i.e. human serum) to allow accurate interpolation of unknown sample response in an external calibration curve. Diluents, of course, lack potentially disturbing factors such as endogenous cytokines and heterophilic antibodies (5,6).
In a 19-plex setup, this paper describes signal intensity, assay sensitivity and background signal levels in relation to the total volume-fraction of serum and protein concentration. We present several methods to determine the quality of a diluent. As an example we used two widely used diluents from R&D Systems; DY997 and RD6. We demonstrate the importance of using a diluent that adequately imitates the sample matrix.

Material and methods

Luminex multiplex assay

Proteins and antibodies used were purchased from R&D Systems (Wiesbaden, Germany) with the exception of recombinant proteins IL-1beta, IL-2, IL-5, IL-6, IL-8, IL-10, IL-13, IFN-gamma which were from Strathmann (Hamburg, Germany), capture antibody IL-4 was from Bioscience (Cambridge, United Kingdom), IL-6, IL-10, IL-13 were from Pelikine (Amsterdam, The Netherlands) and detection antibodies IL-4 and IL-10 were from Bioscience and Pelikine, respectively. All cytokines and proteins were reconstituted as recommended by the manufacturers.
Samples were prepared containing 25% or 50% v/v serum of a serum pool that was prepared by combining serum of 10 healthy blood donors after informed consent, in diluents DY997

and RD6 with spiking of 100 pg/mL for each cytokine. Experiments were performed two times with 4 independent runs in duplicate. Two times, calibration curves were prepared in two-fold dilution steps using the same diluent and recombinant protein standards. All assays were performed as described previously (7). Calibration microspheres were purchased from Bio-Rad (Munich, Germany), sheath fluid from Qiagen (Hilden, Germany).
Protein concentrations were quantified using a BCA protein assay kit from Pierce (Bonn, Germany) according to the specifications of the manufacturer.

Data and statistical analysis

Data were analyzed by the five parameter curve fitting in Origin 6.0 (Microcal Software Inc., Northhampton, MA, U.S.A.) and BioPlex Manager 4.1 software (Bio-Rad).

Results

Influence of different diluents on sensitivity of calibration curves

We compared calibration curves prepared in 100% v/v DY997 and in 50% v/v DY997 + 50% v/v human serum, representative of sample volume fraction used in Luminex assays (8). Calibration curves for IL-8, as an example for the effects observed in all investigated cytokines, were calculated using a 5-parameter logistic (PL) function (Fig. 1A). Interestingly, the IL-8 background mean fluorescence intensity (MFI) signal of 50% v/v DY997+ 50% v/v human serum was actually lower than DY997 alone, although serum itself contains a certain amount of IL-8. To better visualize assay sensitivity, the derivative of the 5-PL function was plotted (Fig. 1B). In 100% DY997 average Luminex assay sensitivity was approximately 30 MFI/(pg/mL) from 1 to 100 pg/mL IL-8. However, adding 50% v/v human serum Luminex assay sensitivity decreased approximately twofold to 15 MFI/(pg/mL). When comparing calibration curves prepared in RD6 and 50% v/v DY997 + 50% v/v human serum, Luminex assay sensitivities were similar (13 and 16 MFI/(pg/mL), respectively). Thus, RD6 seems to be a diluent that more adequately imitates a human serum matrix.

Protein content influences sensitivity

To test whether serum protein concentration was the main reason for lowering sensitivity, we determined the protein concentration of DY997 and human serum. Protein content was 1.5

and 85.2 mg/mL for DY997 and human serum, respectively. To further investigate the effect of protein content on Luminex assay sensitivity we measured MCP-1 calibration curves (representative of all investigated cytokines and chemokines) in the presence 50% v/v bovine serum albumin (BSA) solution, at a BSA protein concentration of 67, 100 and 133% of human serum (56 mg/mL, 85 mg/mL or 128 mg/mL, respectively) (Fig. 2). At 100 pg/mL MCP-1, 56, 85 and 128 mg/mL BSA Luminex assay sensitivity was 25, 18 and 17 MFI/(pg/mL), respectively. In the same experiment 50% v/v human serum lowered assay sensitivity to 9 MFI/(pg/mL). Using RD6 as diluent gave higher MFI/(pg/mL) values at MCP-1 concentrations below 100 pg/mL, but comparable results as DY997 + 50% v/v human serum, although RD6 had a protein concentration of 34.4 mg/mL. These results demonstrate that lowering of assay sensitivity cannot solely be attributed to protein concentration.

Comparison of signal intensity in DY997 and RD6 with respect to serum content

To investigate the effect of diluents DY997 and RD6 on Luminex assay sensitivity on samples, we spiked 19 cytokines and chemokines in diluent DY997 or diluent RD6 and human serum at a volume fraction of 25% and 50%. In DY997, samples containing 50% v/v human serum displayed 45% of MFI obtained with 25% v/v human serum (Fig. 3). In RD6, samples containing 50% v/v human serum showed 80% of MFI values obtained with 25% v/v human serum. This shows that serum matrix effects influenced the antibody-antigen equilibrium in both diluents, resulting in decreased Luminex signal intensity. However, this effect was less pronounced when using the RD6 diluent.

Conclusions

As shown in this paper for human serum, a diluent needs to mimic the sample matrix when establishing an external calibration curve to allow accurate interpolation of unknown samples. With respect to our specific assay requirements, diluent RD6 from R&D Systems appears to be a more appropriate candidate for cytokine determination using the Luminex system. Our recommendation would be that one should compare several diluents in order to optimize a multiplex setup. More and more companies are offering high quality diluents, making it easier to find an appropriate diluent.

Acknowledgements

We are grateful to Mrs. G. Gornitzka and Mr.Y. Sagik for expert help with the Luminex analyses. We thank Dr. B. Rose, Institute for Clinical Diabetes Research, German Diabetes Center, Duesseldorf, for fruitful discussions.

Figure1: Calibration curves of IL-8 prepared in RD6, DY997 and DY997 with 50% v/v serum. A) Calibration curves of IL-8 prepared in RD6 (dotted line with triangles), DY997 (thin line with squares) and DY997 with 50% v/v serum (thick line with circles). MFI = Mean Fluorescence Intensity B) Derivative of calibration curves of IL-8 prepared in RD6 (dotted line), DY997 (thin line) and DY997 with 50% v/v serum (thick line) (n = 2). Sensitivity = MFI/(pg/mL)

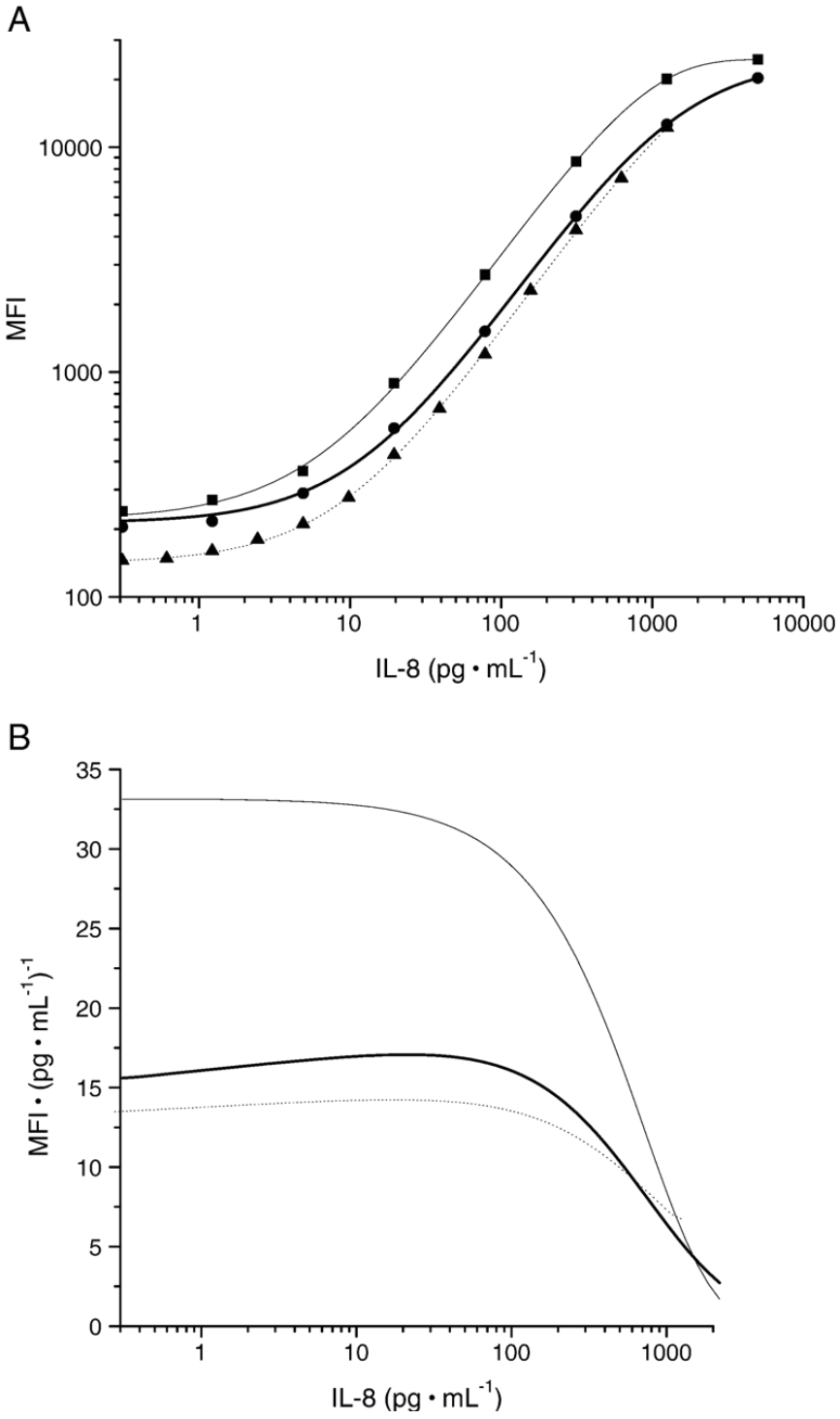


Figure 2: Derivative of calibration curves of MCP-1 prepared in diluents with different protein concentrations. Derivative of calibration curves of MCP-1 prepared in DY997 in the presence of 50% v/v serum (thick line), 50% v/v BSA solution at 56 (thin line), 85 (dashed line) or 128 mg/mL (dot-dashed line) and prepared in RD6 (dotted line) (n = 2).

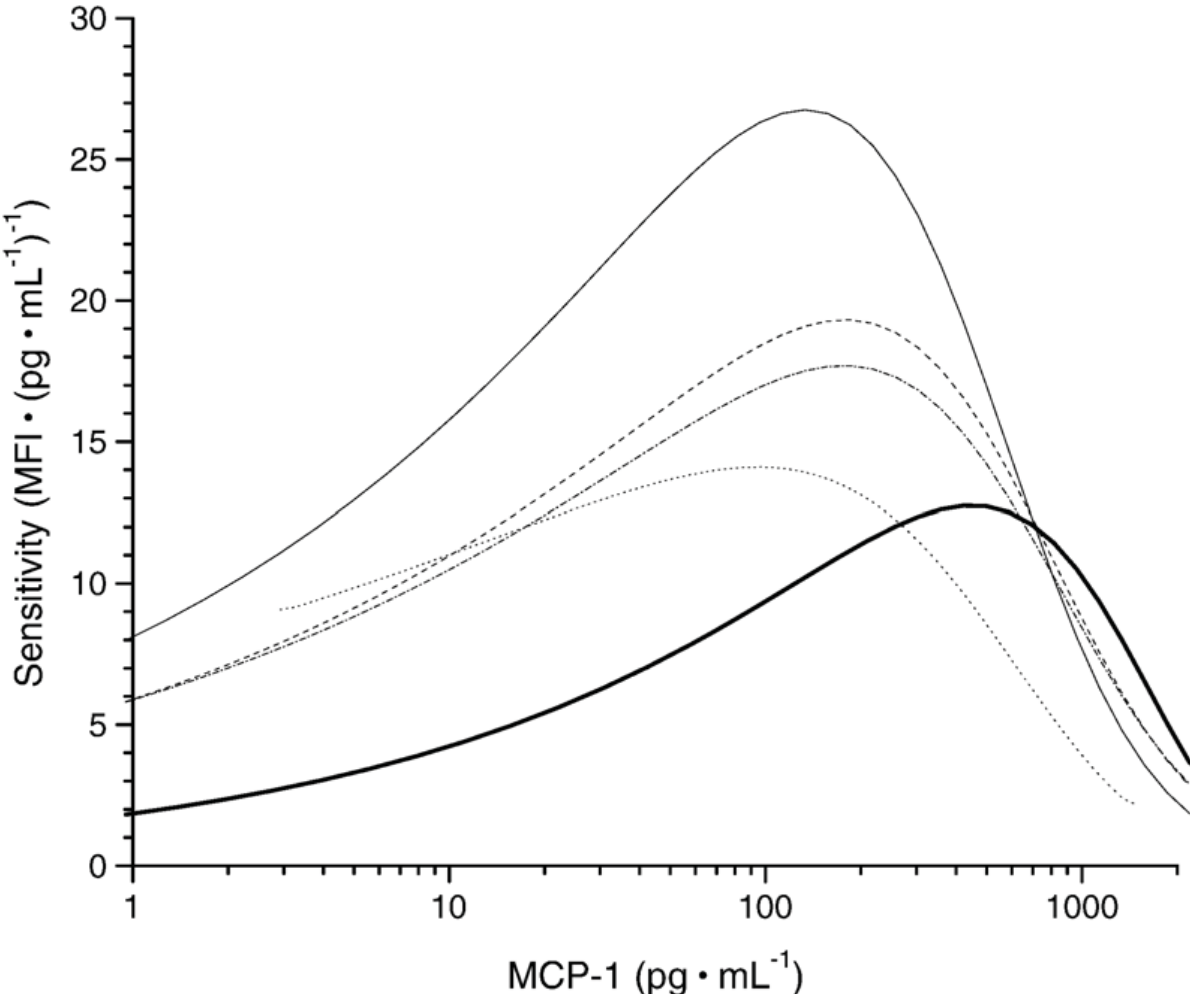
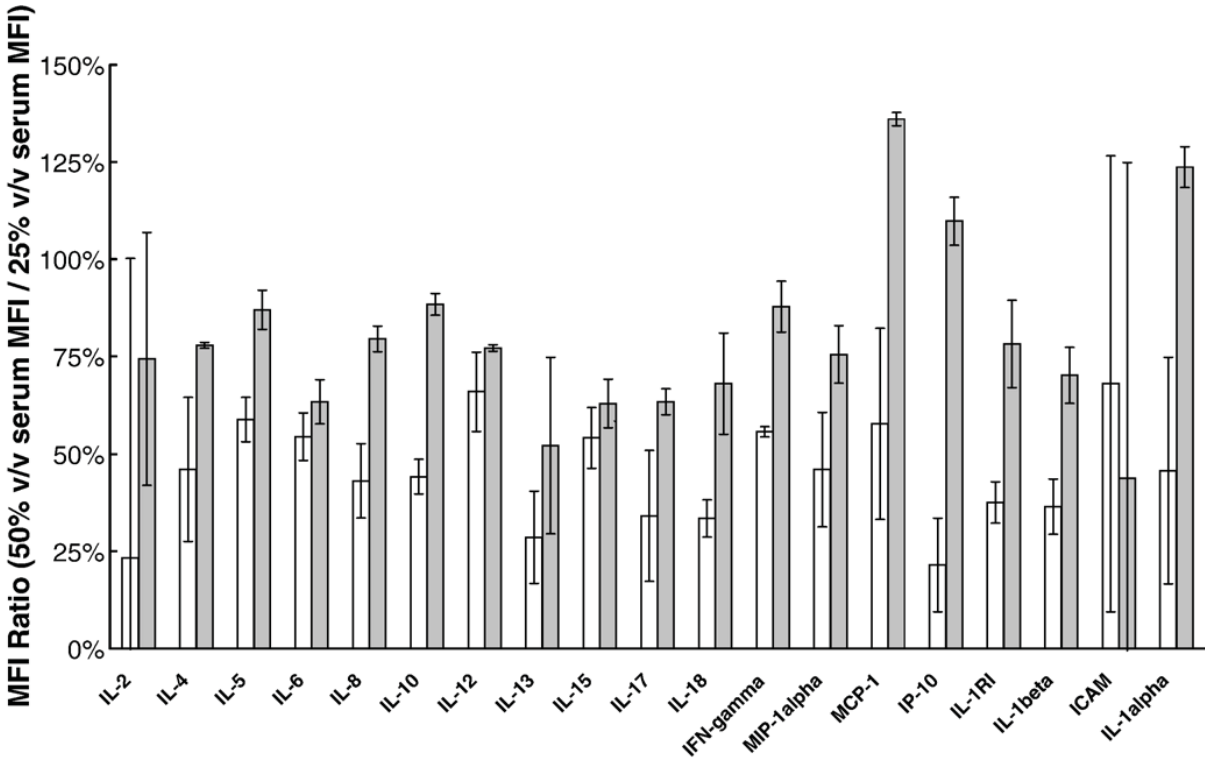


Figure 3: Comparison of MFI values ratios obtained in either DY997 (white bars) or RD6 (grey bars) for various cytokines in the presence of 50% and 25% v/v serum. DY997 and RD6 with 25% or 50% v/v serum was spiked with 100 pg/mL of cytokine (n = 2). Shown are means with standard deviation bars.



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3

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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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 ß-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 ß-cell stress marker proinsulin but increased in remitters. CCL4 associated with decreased ß-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 ß-cell destruction. T-cells play a major pathogenic 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, ß-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 prediabetic 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 ß-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 ß-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 9.6 years, range 3 months to 16.8 years) 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 ß-cell function and were measured in a central facility at one, six, and twelve months of follow up. 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) (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^{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 (12).

Proinsulin was detected by Sandwich ELISA, which determines total proinsulin immuno-reactivity both proinsulin and its conversion intermediates. The detection limit is 0.3 pmol/l

and the analytical range lies between 0.3 – 100 pmol/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 90 minutes 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 90 minutes 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 2.0 pg/ml for CCL3, 3.0 pg/ml for CCL4, and 255.5 pg/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.

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 (Figure 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 ($p=0.0017$, Figure 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 ($p<0.0001$, Figure 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 10.3 years, median HbA1c at diagnosis: 10.65%) and 161 patients not in remission (74 girls and 87 boys, median age 9.4 years; median HbA1c at diagnosis: 11.1%). The stricter definition of remission 6.5 for classification showed 46 patients in remission (28 girls and 18 boys, median age 10.8 years, median HbA1c at diagnosis: 10.4%) and 204 patients not in remission (94 girls and 110 boys, median age 9.3 years; 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 (Figure 3.2). 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.

In the association model, circulating concentrations of all three CCR5 ligands revealed associations with metabolic parameters (Table 1). 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 ß-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 (Figure 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 ß-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 ß-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 ß-cell function and metabolic parameters. Both Th1 associated chemokines CCL3 and CCL5 were inversely related with ß-cell function which confirms previous work that suggested a deleterious role of these chemokines (3,27). 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 ß-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 ß-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 ß-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 ß-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) and , 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 ß-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

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

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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 pb0.0001 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.

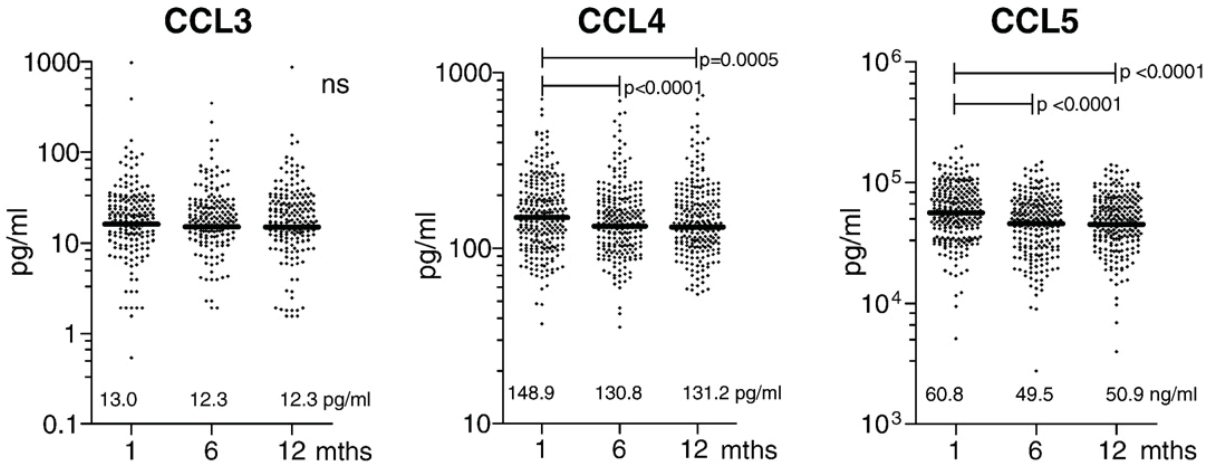

Figure 2: Circulating chemokine concentrations in patients classified remitter or non-remitter. Remission refers to the definition of remission 7.5: HbA1cb7.5 and b0.4 U/kg daily insulin; remission 6.5: HbA1cb6.5 and b0.4 U/kg daily insulin. Bars represent medians. P-values are adjusted for sex, age 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 1month, rem7.5 vs non-rem, 52.55 ng/ml vs 64.64 ng/ml; and CCL5 after 1month, rem6.5 vs nonrem, 53.45 ng/ml vs 60.33 ng/ml.

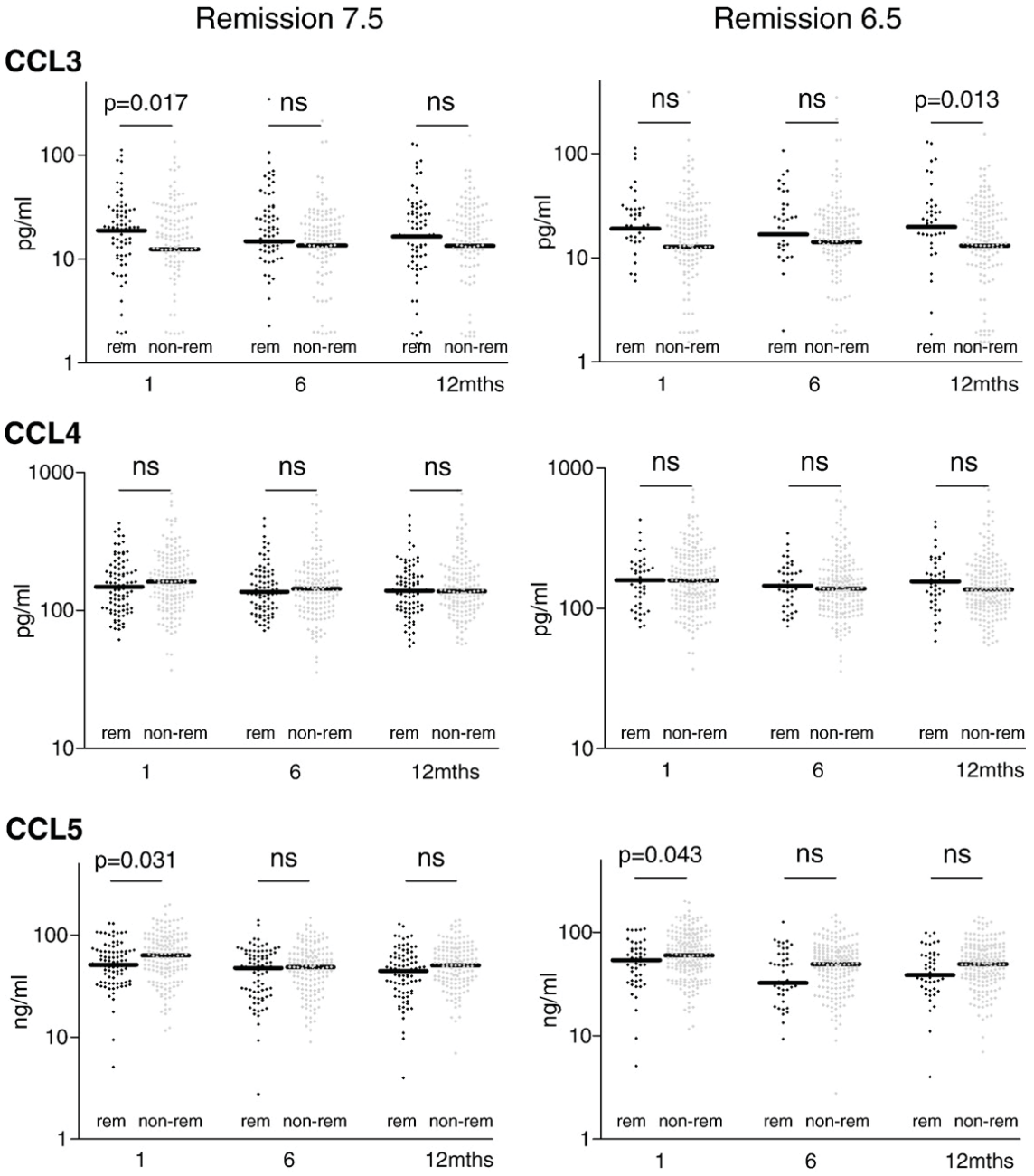

Figure 3: Distribution of chemokine concentrations and metabolic parameters of patients with respect to centers. C-peptide, HbA1c, age and chemokine concentrations of patients were classified by the enrolment of the corresponding country/center and sorted by increasing C-peptide concentrations. Medians 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.

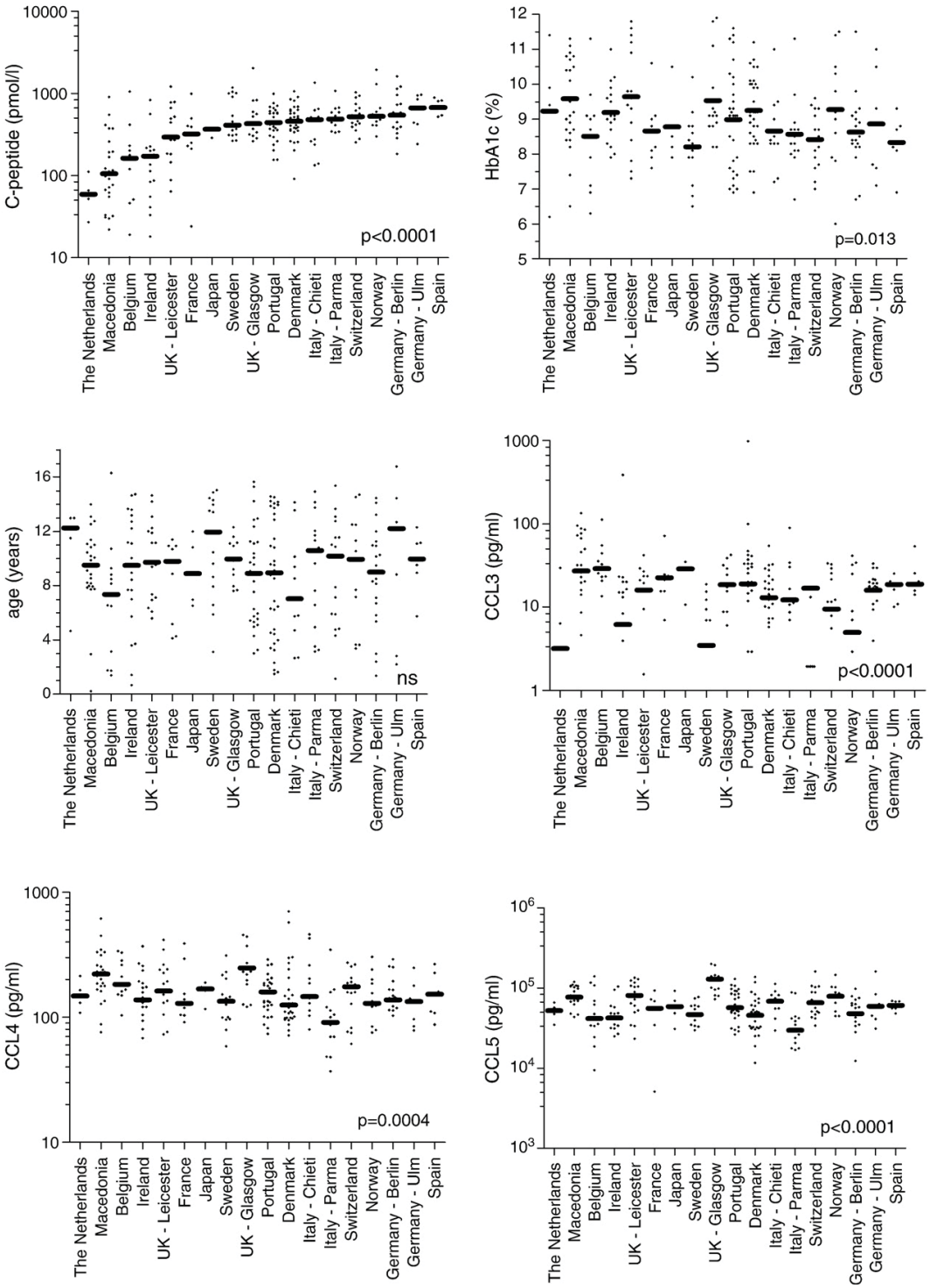

Table 1

A:"Association Model"

Chemokine	Month	Metabol. Paramt.	ß	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

Association of chemokine concentrations and metabolic parameters at each time point. Regression analysis was adjusted for sex, age and BMI percentiles. Given are the resulting coefficients (ß) with their corresponding p-values.

B: "Prospective Model"

Chemokine	Metabol.Parameter	ß	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

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

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4

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

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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*.

Introduction

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 1A). 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 (Table 1B). Twelve months after diagnosis fewer associations were found (Table 1C). 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*.

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Appendix

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

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Figure 1: Circulating cytokine concentrations of patients with type 1 diabetes 1, 6, and 12 months after diagnosis. *P* values for nonparametric testing for paired data (Friedman test) were as follows: 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.

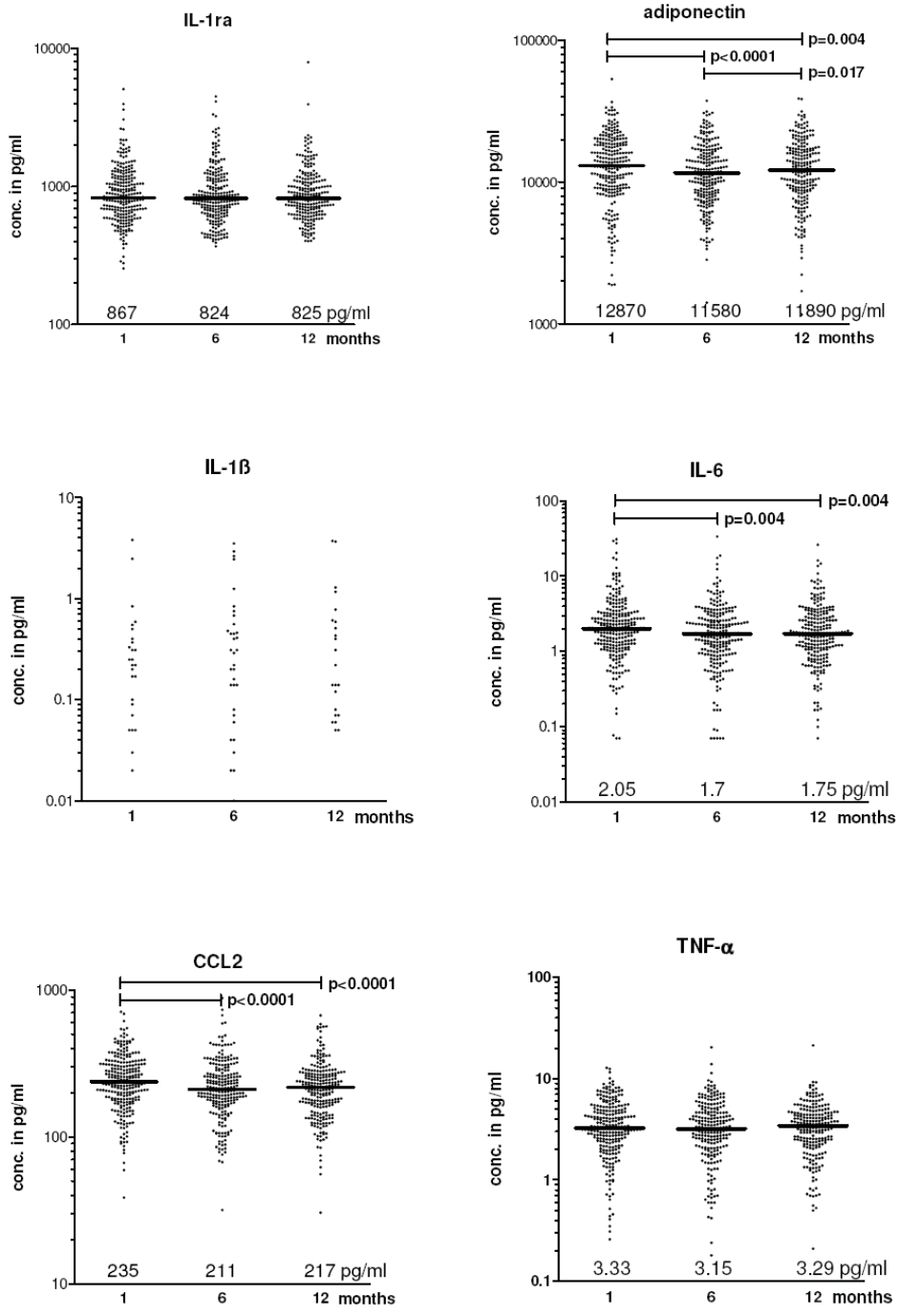

Figure 2: Follow-up of median circulating cytokine concentrations in patients classified by "remission" or "improved C-peptide secretion." Remission 7.5 (*top panel*) shows data based on the definition of remission A1C <7.5% and <0.4 units/kg daily insulin. Remission 6.5 (*middle panel*) refers to the definition of remission with A1C <6.5% and <0.4 units/kg daily insulin. Patients with improved C-peptide secretion are characterized by an increase of C-peptide from 1 to 6 months after diagnosis of at least 20% with a lower limit of 100 pmol/l. Lines represent medians of the classified groups: dashed lines for patients in "remission" or "increased C-peptide secretion" and solid lines for patients with "no remission" or "no improved C-peptide secretion." **P* < 0.05, ***P* < 0.01 adjusted for sex and age.

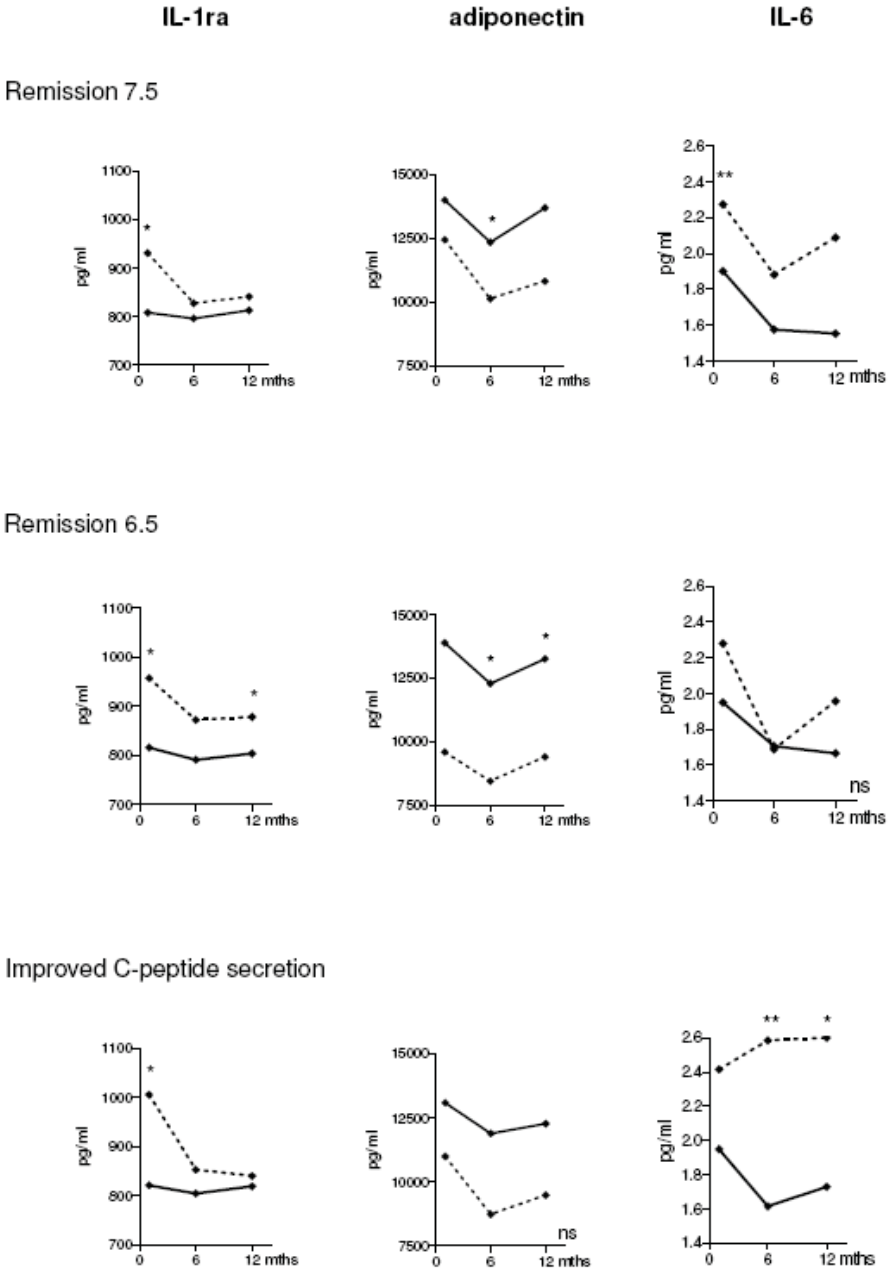

Figure 3: Correlation of circulating cytokine concentrations with Δblood glucose. Coefficient (ß) and *P* values of regression line plotted are adjusted for sex, age, and BMI percentile.

***A*:** Adiponectin vs. Δblood glucose 6 months after diagnosis.

***B*:** IL-6 vs. Δblood glucose 12 months after diagnosis.

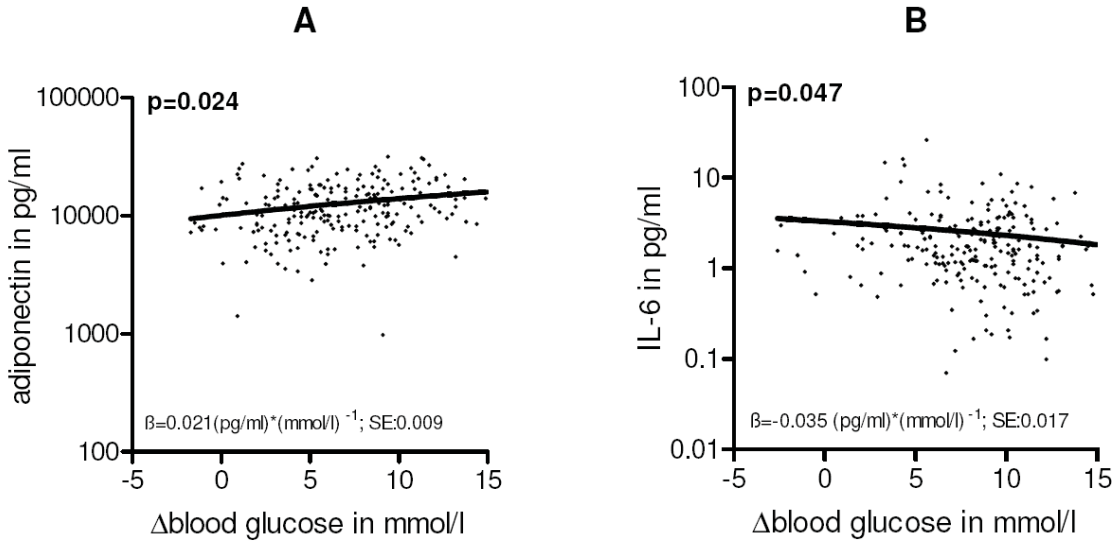

Table 1: Correlation between cytokines

A: 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

B: six months after diagnosis

Variables	IL-1ß		IL-6		CCL-2		TNF-α		adiponectin	
	r	p	r	p	r	p	r	p	r	p
IL-1ra	**0.220**	**0.0007**	**0.342**	**<0.0001**	**0.308**	**<0.0001**	**0.130**	**0.047**	-0.07	0.302
IL-1ß			**0.238**	**0.0002**	**0.134**	**0.040**	0.115	0.080	-0.02	0.762
IL-6					**0.179**	**0.006**	0.055	0.396	-0.03	0.596
CCL-2							**0.152**	**0.020**	-0.02	0.788
TNF-α									0.097	0.139

C: twelve months after diagnosis

Variables	IL-1ß		IL-6		CCL-2		TNF-α		adiponectin	
	r	p	r	p	r	p	r	p	r	p
IL-1ra	**0.130**	**0.045**	**0.329**	**<0.0001**	**0.135**	**0.038**	0.042	0.522	-0.09	0.161
IL-1ß			0.035	0.588	0.103	0.114	0.030	0.644	-0.08	0.226
IL-6					0.022	0.737	0.005	0.935	-0.04	0.511
CCL-2							**0.144**	**0.027**	-0.05	0.438
TNF-α									0.070	0.281

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	0.00104	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.

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5

Association of ß-cell function with T-cell reactivity to islet antigens in recent onset type 1 diabetes

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Abstract

Background and aims: Circulating cytokines have been suggested to associate with ß-cell function and metabolic control in recent onset type 1 diabetes patients. Aim of this study was to test this hypothesis and to investigate how antigen-reactive T cell responses relate to ß-cell function, metabolic control and treatment effects with heat shock protein peptide DiaPep277.

Materials and methods: 50 adult (mean age 27.26 ±8.1 years) and 49 children (mean age 10.9 ±2.8 years) with recent onset type 1 diabetes for less than 3 months of the p520/521 study were investigated. Secretion of interferon (IFN)-γ, interleukin (IL)-5, IL-13 and IL-10 by peripheral mononuclear cells (PBMC) upon stimulation with Islet Antigen 2 (pIA2), heat shock protein 60 (hsp60) and glutamic acid decarboxylase (GAD65) was determined applying ELISPOT in addition to lymphocyte proliferation tests. Circulating CCL3, CCL4, IL-1ß, IL-1ra, IL-2, IL-8, IL-10, INF-γ and TNF-α concentrations were determined. Endogenous ß-cell function was characterized by glucagon stimulated C-peptide. Regression analysis was adjusted for sex, age, BMI, DiaPep277 treatment, HLA and HbA1c.

Results: At baseline, PBMC of adults showed a positive association of residual ß-cell function with Th2 related IL-13 cytokine secretion upon stimulation with pIA2 ($p=0.04$) and a negative association with Th1 related IFN-γ secretion ($p=0.038$). PBMC of children at baseline exhibited a positive association of residual ß-cell function with proliferation ($p=0.04$) and with IL-13 secretion in response to stimulation with pIA2 ($p=0.03$) and hsp60 ($p=0.02$). Upon further follow up at week 8, 30, 56 and 78, similar correlations were observed and persisted longer in children (until week 30) compared to adults (until week 8. Adults showed a positive association of circulating IL-1ra with AUC C-peptide after 8 and 56 weeks ($p=0.004$ and $p=0.003$).

Conclusion: Autoantigen induced T cell responses correlated with residual ß-cell function, the production of IFN-γ was associated with lower, that of IL13 was associated with higher C-peptide secretory capacity. Among circulating cytokines analyzed, IL-1ra and CLL4 were confirmed to be associated with C-peptide secretion. Systemic cellular immune reactivity appears to be modulated by the ß-cell destructive process.

Introduction

Type 1 diabetes is an immune mediated disease, leading to selective destruction of insulin producing ß-cells. Cytokines and islet antigen reactive T-cells play an important role in this process in both humans and animals (1-3). Islet antigen reactive cytokine secretion of T-cells determined by ELISPOT technology in addition to proliferation assays have be detected in both type 1 diabetes patients and healthy controls (4,5). However, T cells in patients with type 1 diabetes exhibited polarization towards a proinflammatory Th1 phenotype, whereas healthy controls showed a T-regulatory phenotype characterized by IL-10 secretion (6). In a recent small study, it was reported that newly diagnosed children have higher mean numbers of IL-10 producing cells compared to patients in partial clinical remission patients (7). This is suggestive of an association of immunological responses with ß-cell function. However, at present, no data of T-cell reactivity and endogenous insulin production of patients with recent onset type 1 diabetes and follow up are available.

Recently, we were able to show that circulating cytokines such as IL-1ra, IL-1ß, CCL3 and CCL4 are associated with ß-cell function and metabolic parameters in children and adolescent with newly diagnosed type 1 diabetes and one year follow up (8).

In the present study we investigated the relation of circulating cytokine concentration and T-cell reactivity monitored by proliferation assay and ELISPOT with ß-cell function in children and adults with recent onset type 1 diabetes. Patients were recruited from the DiaPep277 immune intervention trials p520/p521 (6). This set up enabled us to monitor the longitudinal immune response in association with ß-cell function in addition to potential immune deviating effects of intervention with the hsp60 derived peptide DiaPep277.

Material and methods

Patients

The design and characteristics of the p520 and p521 study has been explained elsewhere (9). In brief, patients diagnosed with type 1 diabetes for less than 3 months were recruited consecutively in 12 centres throughout Hungary and Slovenia. Clinical and biochemical data were available from diagnosis up to 18 months from 50 adult patients (mean 27.26 ±8.1 years) and 49 children (mean 10.9 ±2.8 years). Different dosages of DiaPep277 were administered subcutaneously [(2,5mg; 1,0mg; 0,2mg) and (1,0mg; 0,2mg) for adults and children, respectively] in addition to placebo in a double blind setting at time intervals 0, 4, 26 and 52 weeks after inclusion into the study.

After written informed consent according to the declaration of Helsinki, venous blood was drawn at week 0, 8, 26, 56, 78 of the study and shipped immediately to the laboratory performing immunological monitoring analysis. Clinical and metabolic parameters, HLA typing and auto-antibodies ICA, GAD and IA2A were determined as described (9).

ß-cell function was measured as described previously (9). In brief, fasting C-peptide was measured at 2, 6, 10 and 20 min after stimulation with 1mg glucagon using C-peptide kit KPED1,2 (EURO/DPC Limited, United Kingdom). Primary endpoint was stimulated C-peptide as determined by the area under the curve (AUC). HbA1c was determined by the central laboratory (Quintiles Laboratories Europe, Edinburgh, UK) using a Biorad Variant II analyser. The normal reference range used was 4.3- 6.1 %.

ELISPOT

Experiments were performed not later than 24 hours after blood withdrawal according the "Peakman protocol" (5,10). In 6% of cases, blood was not available due to shipping problems. Poor blood quality and amount of cells recovered from blood samples accounted for another 15-25% data loss (depending on assay ranking in priority). ELISPOT data obtained comprise 85% of total expected (proliferation test data equal 75%).

The ELISPOT assay is described elsewhere (5,10). In brief, freshly isolated PBMC's were resuspended in Tissue Culture medium(TC) (RPMI 1640 supplemented with 2mM L-glutamine, 25mM hepes, 100IU/ml penicillin, 100µg/ml streptomycin, all Life Technologies)

with 20% human AB serum at a density of $8x10^6$/ml. Following antigen or peptide in TC were added and incubated 48 hours at 37°C, 5% CO2: DiaPep277 (10µg/ml) (courtesy of Develogen (now Andromeda), Rehovot, Israel), human hsp60 (0.5µg/ml) (courtesy of Peptor Ltd, Rehovot, Israel), human GAD65 (1µg/ml) (Diamyd, Stockholm, Sweden) and two overlapping peptides of human IA-2 (pIA-2, 10µg/ml, amino acid 531-550 and 541-560, synthesized as described (11).

Endotoxin content of hsp60 was regularly determined as <1 EU/µg protein by quantitative limulus amebocyte lysate assay (BioWhittaker, Verviers, Belgium) described by Habich et al. (12). Medium alone and a combination of phorbol-myristate-acetate (PMA) (10ng/ml) and ionomycin (1µM) (both Sigma, Steinheim, Germany) stimulation was used for negative and positive control, respectively. Cellular responses to recall antigen, tetanus toxoid (10µg/ml) (SVM, Bilthoven, the Netherlands) was included when cells were abundant.

In the next step, cells were resuspended at a concentration of $1x10^6$/300µl and 100µl dispensed in triplicate wells of 96-well ELISA plates (Nunc Maxisorp, Merck, Poole, UK). IFNγ, IL-5, IL-13 and IL-10 were determined applying the ELISPOT assay (U-CyTech, Utrecht, NL) that permits enumeration of antigen-reactive cytokine secreting cells on a single cell level using a system with good sensitivity and good balance of signal-to-noise (5). Spots were detected with an automatic reader system capable to dissolve single events on varying background conditions (BioSys, Karben, Germany) (4). Mean values of triplicates were used for comparing antigen reactive cytokine secretion versus background secretion. Ratio of mean spots of antigen stimulation divided by background was introduced as stimulation index (SI).

Lymphocyte stimulation assay (LST)

LST was performed as described previously (13). Freshly isolated PBMC were incubated for 5 days in presence of the stimulus. At the end of this incubation at 37C, 5% CO2, [methyl-^{3}H] Thymidine (Amersham, Freiburg, Germany) was added for a period of 18 hours and being taken up by the proliferating cells.

Proliferation was measured after stimulation of PBMC with two concentrations of p277 (5µg/ml and 50µg/ml), mitogen phytohemagglutinin (PHA, Murex, Dartford, England) (0.3µg/ml) and medium alone. Dependent on cell numbers available, additional diabetes relevant antigens were tested: hsp60 (5µg/ml) and pIA-2 (15µg/ml). Because of the limitation

of cells, the GAD65 and tetanus toxoid could not be included for proliferation assays. Numbers of replicates ranged from 6 (controls and optional antigens) to 12 in both p277 concentrations. The incorporated radioactivity was measured by szintillation as counts per minute (cpm) to assess cellular proliferation. Mean values of replicates were used for comparing antigen reactive proliferation versus background proliferation. In regression analysis, ratio of cpm of stimulation and background were introduced as stimulation index (SI).

Circulating cytokines

CCL3, CCL4, IL-1ß, IL-1ra, IL-2, IL-8, IL-10, INF-γ and TNF-α were determined by multiplex-bead based technology using commercially available kits (Fluorokine MAP, R&D Systems, Wiesbaden, Germany). The detection limits of the assays were 0.8 pg/ml for CCL3, 1.8 pg/ml for CCL4, 0.29 pg/ml for IL-1ß, 13.9 pg/ml for IL-1ra, 0.2 pg/ml for IL-2, 0.8 pg/ml for IL-8, 0.08 pg/ml IL-10, 0.13 pg/ml INF-γ and 0.6 pg/ml for TNF-α. Determinations of cytokine concentrations lower than the detection limit were assigned a value half of the detection limit (CCL3 n=50, CCL4 n=2, IL-1ra n=0, IL-2 n=5, IL-10 n=54, INF-γ n=34 and TNF-α n=0). Because IL-8 was only detectable in 18% (n=129) of all samples this cytokine was treated detectable or not detectable. IL-1ß was detectable in only 6% (n=28) of all samples and was not investigated any further. Immunoassays showed inter-assay variations below 20% and intra-assay variations below 10%.

Statistical analysis

For results from ELISPOT and LST, differences between stimulation and background were first analyzed by Kruskal-Wallis test. In case of significance Mann-Whitney test followed.
Log transformed stimulation indices, circulating cytokine concentrations and AUC C-peptide concentrations were applied to regression analysis. Stimulation index (SI) was calculated as the ratio of the means of triplicate wells upon stimulation with antigen divided by stimulation with background. Regression analysis of ELISPOT and LST results included sex, age, HLA, treatment, AUC C-peptide and HbA1c in addition to BMI for adults or BMI percentiles for children. Regression analysis investigating antibodies included sex, age, BMI/ BMI percentiles for adults and children respectively, HLA, treatment, GADA and IA-2A. Regression analysis investigating circulating cytokines included sex, age BMI/ BMI percentiles, HLA, treatment, AUC C-peptide and HbA1c.

Adjustment for BMI percentiles are based on the 2000 Centers for Disease Control growth charts (www.cdc.gov/growthcharts). Associations are descriptive and were not corrected for multiple testing. Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC) and GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Antigen reactivity measured in ELISPOT and LST

Studies p520 and p521 were planned and conducted in parallel but independently performed addressing two different populations, adults and children with recently diagnosed type 1 diabetes. Therefore, both studies p520 and p521 are presented separately throughout the manuscript.

Before first application of DiaPep277 at week 0, we investigated the functionality of applied immunoassays in both cohorts. ELISPOT assay displayed significantly increased numbers of spots upon stimulation with PMA and ionomycin (PI) and recall antigen tetanus toxoid (TT) in comparison to background, all $p<0.0001$ (Figure 1A, B). IL-10 induced spots in children were significantly decreased upon stimulation with TT or PI ($p=0.02$). LST assay showed for both adults and children increased proliferation upon stimulation with positive control PHA (all, $p<0.0001$) (Figure 1C, D).

Further, we investigated immune responses *in vitro* upon stimulation with ß-cell associated antigens at week 0 of the trial. Compared to background, significantly increased immune response upon stimulation with diabetes associated antigens for both adults and children were found.

In both groups immune response was increased for Th1 related INF-γ and Th2 related IL-5 and IL-13 upon stimulation with pIA-2 and GAD65 when compared to background responses (all, $p<0.05$) (Figure 2A, B). IL-10 spots upon stimulation with pIA2 were elevated in children ($p=0.046$). Upon stimulation with DiaPep277, INF-γ and IL-5 was elevated in comparison to background in children, whereas in adults only IL-5 was significant elevated (all, $p<0.05$).

LST showed increased proliferation upon stimulation with diabetes associated antigens

similar in children and adults: hsp60 and pIA2 increased proliferation compared to background (all, $p<0.02$) whereas proliferation did not change upon stimulation with DiaPep277, neither at low (5µg/ml) nor at high (50µg/ml) concentration (Figure 1C, D).

Association of ß-cell function with T cell reactivity and circulating cytokines

As a measure of ß-cell function, area under curve (AUC) of glucagon stimulated C-peptide was investigated.in relation to the immune response. As sex, age, BMI/BMI percentiles (14,15) and HLA are potential confounders that influence immune responses, we here report the relation with these confounders and then report on results obtained from regression model adjusting for these factors.

In adults, we measured statistically significant lower response in males compared to females regarding hsp60 stimulated secretion of IL-13 ($p=0.03$), GAD65 stimulated IL-13 ($p=0.03$), DiaPep277 stimulated IFN-γ ($p=0.005$) and TT stimulated IFN-γ ($p=0.03$). Secretion of IL-5 and IL-13 upon stimulation with pIA2 was negatively associated with age ($p=0.02$) and BMI ($p=0.02$).

Whereas in adults sex was the most prevalent parameter influencing T cell responses, in children most associations were observed with age. We found a negative association of age with pIA2 stimulated IL-13 secretion ($p=0.02$), PI stimulated IL13 ($p=0.04$) and hsp60 stimulated IL-10 ($p=0.04$). In LST, proliferation upon stimulation with DiaPep277 5mg was positively associated with age, and lower upon stimulation with DiaPep277 50mg in males in comparison to females.

HLA DR3/4 and DR4/4 are diabetes susceptible genotypes and play an important role in antigen-presentation (16-18). HLA DR3/4 and DR4/4 were associated with lower secretion of Th2 related cytokines upon stimulation in children more frequent than in adults (Table 1). In addition, children carrying diabetes susceptible haplotypes revealed higher secretion of Th1 related IFN-γ. Thus, HLA was also included in regression analysis.

The analysis of ß-cell function, circulating immune mediators and in vitro stimulated immune responses showed several associations after adjustments for potential confounders: Adults showed positive association of AUC C-peptide with Th2 related IL-13 and negative association with Th1 related IFN-γ until week 8 of follow up (Table 2). Interestingly, while after 30 weeks immune response showed no association with AUC C-peptide, stimulation with treatment DiaPep277 showed positive association of IL-10 secretion with AUC C-peptide after 56 weeks and negative association of IL-5 secretion after 78 weeks.

Children, in contrast, showed association with AUC C-peptide with more antigens but only until week 30 and no association were observed upon stimulation with DiaPep277. At week 0, children showed positive association of Th1 related IFN-γ and Th2 related IL-13 and proliferation upon stimulation with ß-cell specific and unspecific antigens. After 8 weeks, the picture is distinct; IFN-γ showed negative association with AUC C-peptide while Th2 related IL-5 was positively associated both upon stimulation with ß-cell specific antigens. Interestingly, IL-13 showed negative association with AUC C-peptide after 30 weeks upon ß-cell specific and unspecific stimulation.

Similar to our previous study, circulating cytokines revealed associations with AUC C-peptide. Adults showed a positive association of IL-1ra with AUC C-peptide at week 8 and 56 of the study ($p=0.0043$ and $p=0.0027$). Circulating cytokines were not determined after 30 weeks. In addition, IL-8 was negatively associated with AUC C-peptide after 8 weeks ($p=0.032$) and CCL4 showed a positive association with AUC C-peptide ($p=0.006$). In contrast, children revealed negative association of IL-10 with AUC C-peptide after 56 weeks.

Association of ELISPOT and LST with antibody

Whereas T cell responses and cytokines are considered important players during pathogenesis of type 1 diabetes, islet associated autoantibodies are the gold standards for risk assessment in the prediabetes phase. Increased numbers and titres of autoantibodies are associated with pronounced disease progression (19,20).

After detecting associations of cytokine secretion and proliferation with ß-cell function we were interested whether cytokine secretion and proliferation of PBMC is also associated with antibody concentrations. At week 0, associations could not be investigated since data were incomplete. However, cytokine secretion and proliferation was associated with antibody concentrations during follow up (Table 3). Adults showed only associations of Th2 related cytokine secretion with both GADA and IA2A whereas in children most association were observed with Th1 related IFN-γ secretion. Interestingly, both adults and children showed association of LST upon stimulation with hsp60 with antibody concentration at week 30.

Association of ELISPOT and LST with treatment

In the next step, we investigated the influence of DiaPep277 treatment versus placebo on immune response in pediatric and adult patients. At week 0, no differences regarding immune response between treatment groups were observed (data not shown). Both, adults and

children showed associations of immune response with treatment though association were more prevalent in adults than in children (11 and 5 associations, respectively). In addition to the difference in frequency, adults showed most associations of treatment with IL-5: after 8, 30 and 78 weeks upon stimulation with GAD, PI and hsp60, respectively (Figure 3A) and after 30 weeks upon stimulation with TT ($p=0.027$); while in children IL-5 was not significantly associated.

Associations of treatment with IL-13 and IFN-γ were observed in both adults and children. In adults, increased dose of treatment was associated with increased secretion of IL-13 upon stimulation with TT after 30 and 78 weeks ($p=0.035$ and $p=0.043$, respectively) while secretion of IFN-γ upon stimulation with pIA2 was negatively associated with treatment dose (Figure 3A).

In contrast, children showed positive association of treatment dose with IFN-γ secretion upon stimulation with PI after 8 weeks ($p=0.014$) but similar to adults positive association of IL-13 secretion upon TT stimulation ($p=0.01$). After 56 weeks, treatment dose was negative associated to IL-13 secretion upon stimulation with GAD ($p=0.047$).

Of course, of more relevance is the change of immune response over time due to treatment. Therefore we investigated ratio of stimulation indices of week 0 as enumerator and week 78 as denominator and its association with treatment dose. Both, adults and children revealed positive association of treatment dose with IFN-γ ratio but upon stimulation with DiaPep277 ($p=0.025$) or TT ($p=0.008$) respectively. In addition, adults showed also association of treatment dose with IL-5 ratio upon stimulation with hsp60 (Figure 3B).

DiaPep277 treatment revealed also association with circulating cytokines. DiaPep277 was associated with CCL4 in adults after 56 weeks (Figure 3C). In addition, we observed decreased IL-10 concentrations with increased dose of DiaPep277 treatment after 78 weeks ($p=0.041$). Interestingly, no associations of treatment with circulating cytokines were observed in children.

Discussion

In the previously described cohorts of studies p520 and p521, we investigated associations of immune response and immune markers with ß-cell function, antibodies and DiaPep277 treatment in patients with recent onset type 1 diabetes. In both immunoassays, ELISPOT and LST, stimulation with ß-cell specific and unspecific antigens of mononuclear cells obtained from peripheral blood was detected. The mild but statistically significant stimulation with ß-cell associated autoantigens is in line with recent publications (10,21).

Important to note is the association of sex, age, body weight and HLA with antigen-reactivity *in vitro* described here. Most studies on ELISPOT or LST to our knowledge have not performed regression analysis to account for these confounders. One of the reasons could be that these studies have been performed with rather small patients groups and compared healthy persons with matched patients with type 1 diabetes that made such regression analysis not necessarily compulsory. In our study, however, we compared patients with different disease stage and ß-cell function and found that indeed age, sex, HLA and BMI influence immune responses to autoantigens. These observations are not surprising, since it is clinically well known, that patients with adult onset of type 1 diabetes show a less dramatic clinical progress and faster C-peptide decline after diagnosis compared to children (9). Also, body weight has been proposed to affect disease progression as is currently discussed in the accelerator hypothesis and may link observations in type 1 diabetes to results obtained with patients who have type 2 diabetes (22-24)

The diabetes susceptible HLA DR3/4 and 4/4 were associated with higher Th1 and lower Th2 response and were more prevalent in children which is in line with recent work (17,25,26). One could argue that not all different HLA risk haplotypes and combinations have been analyzed however, we wanted to relate to previous reports (9). In addition, separation of patients groups due to their haplotypes would result in little statistical power. Observed associations of circulating cytokines IL-1ra and CCL4 with AUC C-peptide confirmed previous results of Delovitch's and our group suggestive of a protective impact of these cytokines on ß-cell function (8,27,28).

Cytokine secretion and proliferation of PBMC upon stimulation with ß-cell specific antigens was associated with ß-cell function during the first months after diagnosis of type 1 diabetes and lasted longer in children compared to adults. Increased Th1 related IFN-γ response was

associated with lower ß-cell function whereas increased Th2 related IL-5 or IL-13 response was associated with better ß-cell function except for the latest time point. The loss of ß-cell function related PBMC responses over time may be due to regression of insulitis activity or caused by exogenous insulin treatment that may dampen reactivity of immune cells to ß-cell antigens. This might explain why children showed longer association with AUC C-peptide since it has been suggested by others that children display a more aggressive disease progression than adults (9). This suggestion is supported by two findings; first, proliferation was increased upon stimulation with pIA2 only in children which suggests a higher reactivity to ß-cell antigens, second, ß-cell unspecific stimulation of Th1 related IFN-γ with PI was positively associated with AUC C-peptide at the beginning of the trial that might reflect a fully activated immune response.

In this study, PBMC reactivity showed association with DiaPep 277 treatment in both adults and children. As expected for a randomized trial, treatment arms did not differ in their immune response at week 0. However, during follow up cellular effects *in vitro* were seen after 8 weeks, and statistically significant serum cytokine changes were found after 56 weeks.

Proliferation upon stimulation with DiaPep277 was negatively associated with treatment dose in both adults and children probably reflecting downregulation of immune reactivity at week 8. Associations with AUC C-peptide upon stimulation with DiaPep277 were seen initially after 56 week and only in adults (Table 2). However, treatment with DiaPep277 caused immunologically neither a shift from Th1 to Th2 nor was this effect ß-cell specific as proposed earlier (29). Even more, analysis of the change of immune response over time due treatment rather suggested an increased Th1 and decreased Th2 response. These immunological results are in line with clinical observation of DiaPep277 intervention that showed clinical benefit for treated patients in only some but not all (9). Intervention with DiaPep277 might require an adjuvant that enhances protective inflammation for initially priming of immune response (30,31) since DiaPep277 revealed immunological effects first on a cellular basis then in circulation and metabolism, but this remains speculative.

When considering the strength of this study the well described study cohort, the study duration and the parallel performance of ELISPOT, LST and cytokine determination from serum s has to be underlined. The low stimulation indices upon stimulation with ß-cell specific antigens and the low patient number for each treatment arm have been the shortage when investigating associations. 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.

In summary, we have shown for the first time that PBMC activity is related to ß-cell function and that age, sex, body weight and HLA are important to adjust for. In addition, we could confirm the previously reported positive association of circulating cytokines with preserved ß-cell function. DiaPep277 showed a rather mild treatment effect immunologically which is in line with the clinical results (9). We conclude, immune responses determined by ELISPOT and LST are associated with endogenous ß-cell function and may be used to monitor intervention trials in type 1 diabetes. However, since workload and monetary requirements are enormous to perform assays with freshly isolated cells, circulating cytokines from serum might be considered alternatively to investigate disease progression and success of immune intervention.

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Figure 1: Cytokine secretion and proliferation upon stimulation in relation to background is shown in A+B and C+D, respectively. Bars represent median. *$P < 0.05$, **$P < 0.01$, ***P <0.001.

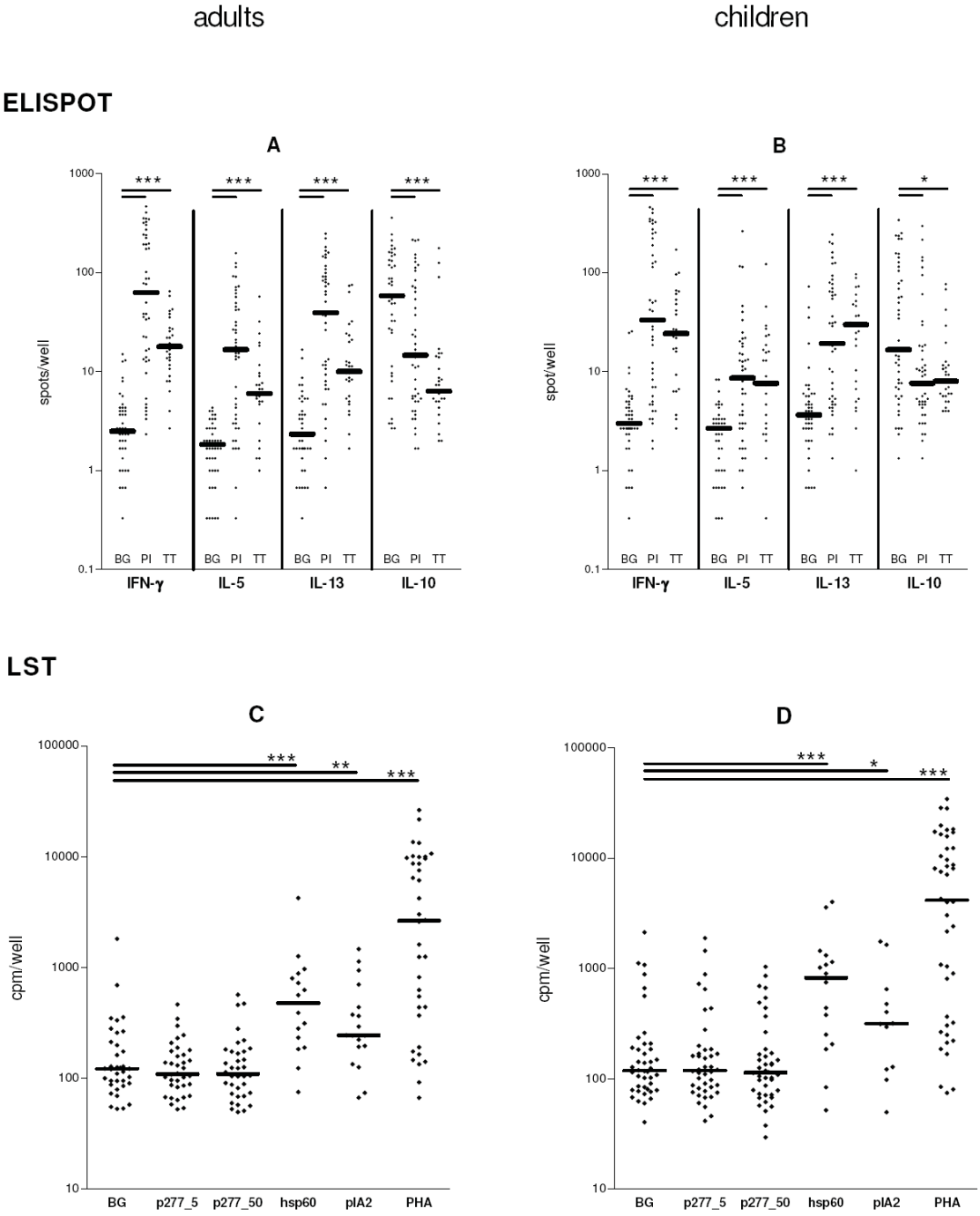

Figure 2: ELISPOT results of adult and pediatric patients of study p520/p521 at baseline. Shown are individual mean spot numbers per cytokine and antigen stimulations. BG background. Bars represent median. *$P < 0.05$, **$P < 0.01$.

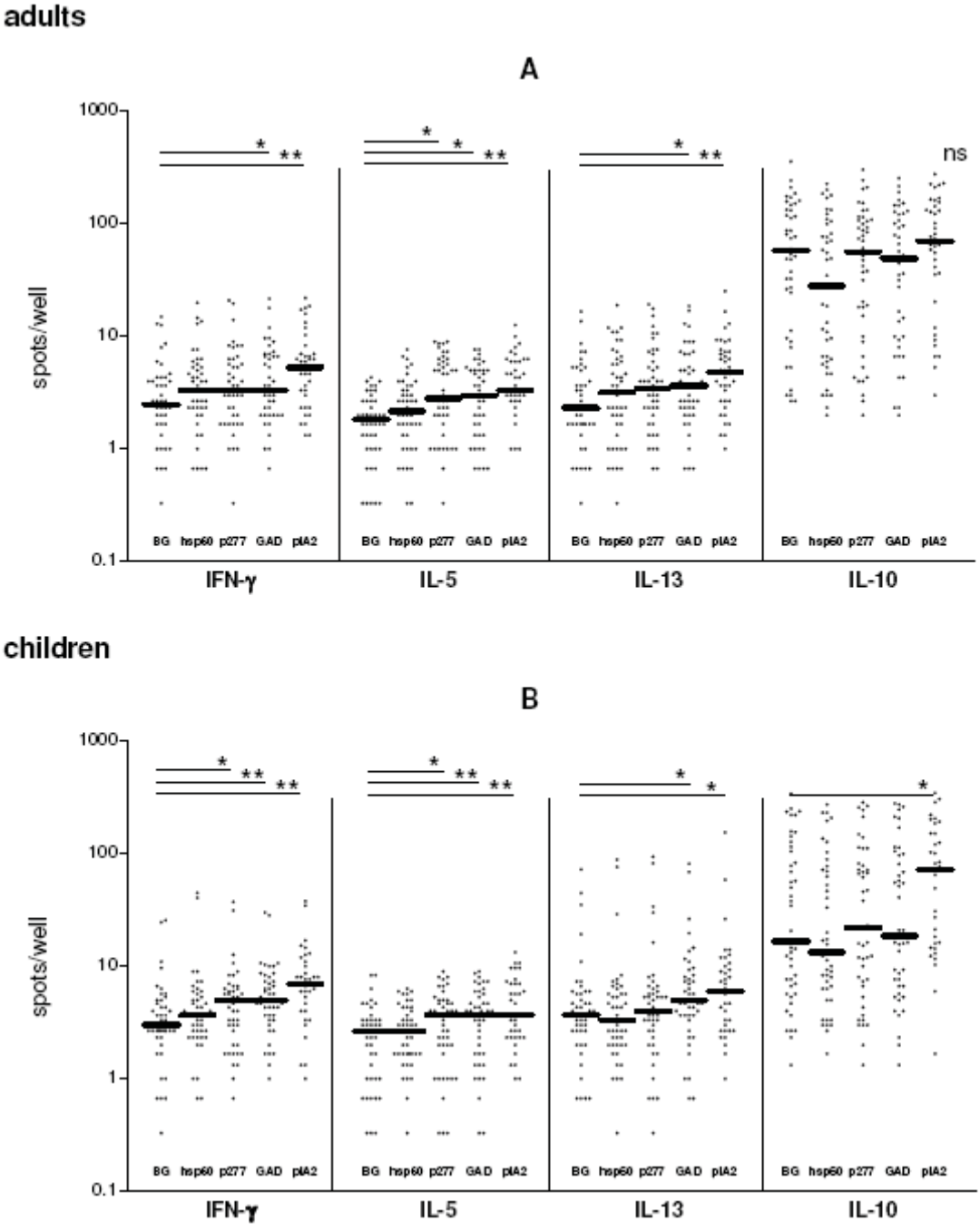

Figure 3: Immune responses in different treatment groups (0mg= placebo, 0.5mg, 1.0mg, 2.5mg DiaPep277. (A) ELISPOT results upon different antigen stimulation and different time points (B) longitudinally change of cytokine response to hsp60 (C) and circulating CCL4 concentrations. Graphs show individual data, P-values are adjusted for sex, age, BMI and, HLA.

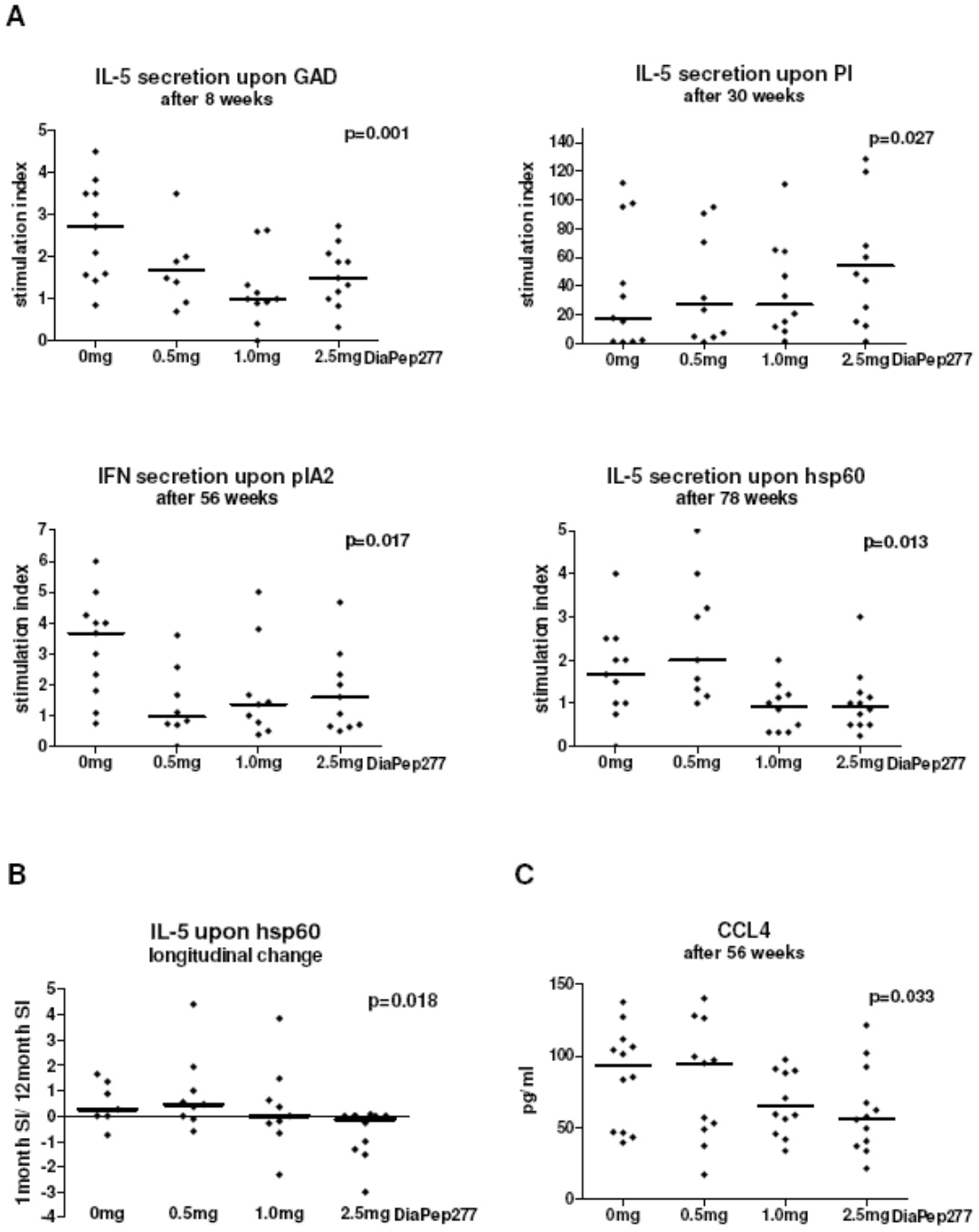

Table 1: Associations of ELISPOT outcome and proliferation (LST) with HLA.

A: adults

	week 0 HLA 3/4; 4/4		week 8 HLA 3/4; 4/4		week 30 HLA 3/4; 4/4		week 56 HLA 3/4; 4/4		week 78 HLA 3/4; 4/4	
	ß	p	ß	p	ß	p	ß	p	ß	p
IL-5										
IL-10										
IL-13										
DiaPep277					-0.42	0.041				
GAD					-0.6	0.025				
PI	1.53	0.024								
IFN-γ										

B: children

	week 0 HLA 3/4; 4/4		week 8 HLA 3/4; 4/4		week 30 HLA 3/4; 4/4		week 56 HLA 3/4; 4/4		week 78 HLA 3/4; 4/4	
	ß	p	ß	p	ß	p	ß	p	ß	p
IL-5										
pIA2					-0.63	0.031				
PI									1.14	0.025
TT										
IL-10										
pIA2									-0.57	0.042
Hsp60	0.55	0.022								
IL-13										
pIA2									-0.82	0.018
DiaPep277									-0.96	0.029
GAD									-0.59	0.056
Hsp60									-0.77	0.0066
IFN-γ										
PI			1.5	0.022						
TT			1.22	0.03						
LST										
DiaPep277 5µg	-0.4	0.004	-0.43	0.046						
DiaPep277 50µg			-0.57	0.009						

The table gives regression coefficients (ß) and p-values. Linear regression analyses were performed including stimulation index (SI), sex, age, BMI/BMI percentiles, HLA, HbA1c, AUC C-peptide and treatment. Positive associations are grey shadowed

Table 2: Associations of cytokine secretion and proliferation with AUC C-peptide.

A: adults

	week 0 AUC C-peptide		week 8 AUC C-peptide		week 30 AUC C-peptide		week 56 AUC C-peptide		week 78 AUC C-peptide	
	ß	p	ß	p	ß	p	ß	p	ß	p
IL-5										
DiaPep277									-0.67	0.0145
IL-10										
DiaPep277							0.40	0.018		
IL-13										
pIA2	0.88	0.04								
Hsp60			0.54	0.038						
IFN-γ										
pIA2	-0.8	0.038								
TT			-1.9	0.015						
LST										
pIA2										

B: children

	week 0 AUC C-peptide		week 8 AUC C-peptide		week 30 AUC C-peptide		week 56 AUC C-peptide		week 78 AUC C-peptide	
	ß	p	ß	p	ß	p	ß	p	ß	p
IL-5										
Hsp60			0.91	0.044						
IL-10										
IL-13										
pIA2	0.88	0.034								
GAD					-0.54	0.019				
Hsp60	0.822	0.024								
PI	2.42	0.022								
TT					-1.6	0.019				
IFN-γ										
pIA2			-1.05	0.047						
GAD			-0.56	0.045						
PI	3.0	0.023								
LST										
pIA2	5.79	0.038			2.19	0.017				

The table gives regression coefficients (ß) and p-values. Linear regression analyses were performed including stimulation index (SI), sex, age, BMI/BMI percentiles, HLA, HbA1c, AUC C-peptide and treatment. Positive associations are grey shadowed

Table 3: Associations of cytokine secretion and proliferation with antibodies

A: adults

	week 8 IA2A		week 30 GAD		week 30 IA2A		week 56 GAD		week 56 IA2A		week 78 GAD		week 78 IA2A	
	ß	p	ß	p	ß	p	ß	p	ß	p	ß	p	ß	p
IL-5														
PI													-0.33	0.023
IL-10														
GAD			-0.12	0.047										
Hsp60											-0.13	0.053		
TT											-0.21	0.036		
IL-13														
pIA2							-0.12	0.045			0.19	0.036		
PI									0.32	0.0476				
TT	0.36	0.0039												
IFN-γ														
LST														
pIA2											-0.08	0.055		
Hsp60			-0.43	0.0009	-0.23	0.049								

B: children

	week 30 GAD		week 30 IA2A		week 56 GAD		week 56 IA 2A		week 78 GAD		week 78 IA2A	
	ß	p	ß	p	ß	p	ß	p	ß	p	ß	p
IL-5												
GAD											-0.16	0.38
Hsp60											-0.2	0.005
IL-10												
IL-13												
IFN-γ												
P277									-0.19	0.043		
GAD									-0.2	0.025		
Hsp60	0.2	0.017										
TT					0.31	0.0235						
LST												
DiaPep277 5µg									-0.2	0.016		
DiaPep277 50µg									-0.18	0.03		
Hsp60			0.79	0.029								

The table gives regression coefficients (ß) and p-values. Linear regression analyses were performed including stimulation index (SI), sex, age, BMI/ BMI percentiles, HLA, treatment, GADA and IA-2A.

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6

Summary and General discussion

Summary

Selective destruction of insulin producing ß-cells in type 1 diabetes is mediated by islet reactive T-cells and cytokines. The aim of thesis was to investigate systemic cytokine concentrations and cytokine secretion of T-cells and their relation to ß-cell function, ß-cell stress, and metabolic control.

In **Chapter 2** we adapted the method of the multiplex bead based immunoassay for the requirements of cytokine measurements in serum.

In **Chapter 3**, we presented different associations of the CCR5 ligands CCL3, CCL4 and CCL5 that had been shown previously to be important in mouse models of type 1 diabetes in recent onset type 1 diabetes patients of the Hvidøre Study. Increased concentrations of both Th1 related chemokines CCL3 and CCL5 were associated with decreased ß-cell function. Associations with remission and metabolic parameters suggest a differential role in disease progression of these two chemokines. CCL4 showed a negative association with proinsulin as a stress marker and might play a rather benign role. Blockage of chemokines or antagonism of CCR5 by therapeutic agents such as Maraviroc may provide a new therapeutic target to delay disease progression in type 1 diabetes. In **Chapter 4**, we demonstrated in the Hvidøre Study that 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. This is of particular interest since the counter player of IL-1ra, the pro-inflammatory IL-1ß that is involved in ß-cell destruction, was negatively associated with ß-cell function. The positive association of adiponectin with metabolic control and the relation with remission may reflect a compensatory effort to improve the metabolic situation. In addition, association of proinflammatory IL-6 with remission and glucose disposal indicated also a benign role of IL-6 in type 1 diabetes. In **Chapter 5**, we showed for the first time that T-cell activity measured *in vitro* in addition to circulating cytokines is related to ß-cell function in the prospective, double blind, placebo controlled immune intervention study p520/521 using heat shock protein 60 peptide derived DiaPep277 as the therapeutic agent. Although the clinical benefit of this treatment was rather mild, DiaPep277 treatment showed effects on T-cell activity and circulating cytokine concentrations that were in line with the clinical observations.

General discussion

Method of multiple cytokine determination

The recently developed multiplex bead based immunoassay provides a great tool to investigate the complex network of cytokines and secretion patterns in small samples volumes (1).

Still, further optimization of this method is required to overcome problems due to high protein content, presence of heterophile antibodies and the wide range of cytokine concentrations varying from very high (ng/µl or µg/ml) to very low (pg/ml).

This thesis has shown that in the multiplex bead based system diluent RD6 is more appropriate when investigating cytokines in human serum compared to diluent DY997 that has been used before. RD6 mimics human serum allowing correct interpolation of external calibration curves and minimizes unspecific binding by heterophile antibodies and proteins. This is of particular interest because i) heterophile antibodies are capable of binding primary and secondary antibody multivalently and can thereby cause false positive signals (2) and are more prevalent in type 1 diabetes patients than in healthy controls (3); ii) the high protein content in serum causing unspecific binding usually requires dilution that may lead to undetectable concentrations of cytokines that are present at very low concentrations in the sample.

The wide range of cytokine concentrations is of no major concern in the multiplex bead based system because capture antibodies and the proteins of interest are not immobilized, as in an ELISA, but are anchored to the surface of mobile beads, allowing for a wider dynamic range (due to an increased surface and enhanced antibody freedom of movement).

However, the potential for optimization is limited when many cytokines are to be included. Grouping of cytokines with similar requirements regarding cytokine-antibody binding could be one option to achieve lower unspecific binding and higher sensitivity. One commercial company has chosen this option and offers different cytokine kits which has the advantage of better assay characteristics for the different analytes, but limits the possibilities to screen for all cytokines of interest in a single assay.

Circulating cytokines and their role in type 1 diabetes

It has been shown in many animal models and *in vitro* experiments that cytokines play a key role in immune mediated ß-cell destruction.

The work of this thesis shows associations of circulating cytokines with ß-cell function and ß-cell stress in humans (Figure 1). Moreover, circulating cytokines revealed also associations with metabolic parameters such as HbA1c or glucose disposal and clinical remission. This is of particular interest since the investigated cytokines CCL3, CCL4, CCL5, IL-1ß and IL-1ra have been shown to contribute to ß-cell destruction in several *in vitro* and *in vivo* models, whereas adiponectin and IL-6 influence both immune system and metabolism. Therefore, our observed associations were in line with results obtained from *in vitro* (4,5) and *in vivo* (6-8) models.

Many publications have described associations of cytokines with proinflammatory diseases such as coronary heart disease, metabolic syndrome, Parkinson's disease, rheumatoid arthritis or type 2 diabetes (9-21). Thus, it was not unexpected to find associations of cytokines with type 1 diabetes that also has important proinflammatory components.

For type 1 diabetes, former studies described differences of systemic cytokines predominantly between patients and healthy controls. This thesis presented relationships of cytokines with endogenous processes and different disease stages in patients with recent onset of type 1 diabetes. More interestingly, associations of cytokines with endogenous processes have included ß-cells and their functional capacity, which are the major target of the immune driven process in type 1 diabetes.

It needs to be kept in mind that results in this thesis are descriptive and the outcome of observational studies that estimate associations between metabolic data and biomarkers in peripheral blood. In humans, the question of causality or whether findings reflect a direct or indirect association cannot be accurately addressed mainly because this would require (sometimes ethically questionable or impossible) intervention studies and because effects on pancreatic ß-cells can only be assessed by surrogate markers, whereas the cells are not directly accessible.

However, several observations speak for a potential relevance of circulating cytokines in the pathogenic process of disease development.

Associations of chemokines with ß-cell function and ß-cell stress were shown in **Chapter 3** and associations of pro- and anti-inflammatory cytokines with ß-cell function, metabolic control, glucose disposal and remission were presented in **Chapter 4.**

The reported associations in this thesis may be meaningful because circulating cytokines may have the potential to serve as markers for disease risk, disease progression in type 1 diabetes, or better assessment of the metabolic situation. In addition, circulating cytokines might serve as markers to provide new insights in the pathogenesis of autoimmune diabetes in humans and may provide the rationale for cytokine-directed immune intervention studies.
This would be of particular interest since (i) *in vitro* models cannot imitate the complex environment in which ß-cell destruction takes place in vivo (22), (ii) most observations from animal models cannot be extrapolated to humans and (iii) not all human tissues are accessible for scientific research.

Confounders of circulating cytokine concentrations

Data presented in this thesis showed significant associations between circulating cytokines and parameters such as sex, age and BMI that have been adjusted for in the regression analysis. Earlier studies that investigated the role of cytokines in type 1 diabetes did not adjust for anthropometric variables but used sex and age matched cohorts of type 1 diabetes patients and healthy controls (23,24). This definitely underestimated the influence of anthropometric variables on circulating cytokine concentrations. Moreover, adipose tissue which has recently emerged as an endocrine organ with high cytokine secretory capacity was not included in previous considerations. However, the study design requires sufficient patient numbers to enable powerful regression analysis.
In addition, circadian rhythm, food intake, physical exercise and psychosocial stress are all well known factors influencing concentrations of circulating cytokines (25-31) which again underlines the central role of cytokines in the regulation not only of immune responses, but of many other physiological processes in the body. Thus, it is very important to consider all critical parameters that may influence circulating cytokine concentrations by study design or adjustment.

Determination of circulating cytokines -clinically useful for the individual patient?

In the analysis of large cohorts, concentrations of circulating cytokines showed significantly different time courses during the study period and differences depending on classification of remission or C-peptide secretion.
These observations suggest a change of cytokine concentration over time due to disease progression, but it is unclear whether this is of “clinical” meaning, i.e. whether it has

implications for treatment, monitoring or secondary prevention. The considerably overlap of measurements at different time points or between patient groups precludes the use of circulating cytokines as diagnostic markers on an individual basis at present. Further research is needed to investigate whether the analysis of more complex cytokine patterns can extend these findings and be of relevance for the individual patients and the treating physician.

Currently available cytokine based intervention strategies

Intervention by cytokine directed therapy was successfully introduced in rheumatic diseases by blockage of cytokines such as IL-1, TNF-α, IL-6 and IL-15 (32). In particular anti-TNF-α therapy (Enbrel®, Infliximab®) is successful with response rates of over 80% in early-stage disease and prevention of joint pathology when given in combination with methotrexate (33,34). Drawbacks are (i) clearly increased risk of infection (ranging from increased frequency of respiratory infections to tuberculosis or opportunistic infections that occasionally are lethal), (ii) cure of symptomes, but not of the disease (relapse after withdrawal of anti-TNF-α therapy), therefore requiring life-long treatment, (iii) development of anti-nuclear antibodies (in rare cases leading to drug-induced systemic lupus erythematosus), (iv) high costs (32).

Cytokine based intervention strategies in type 1 diabetes

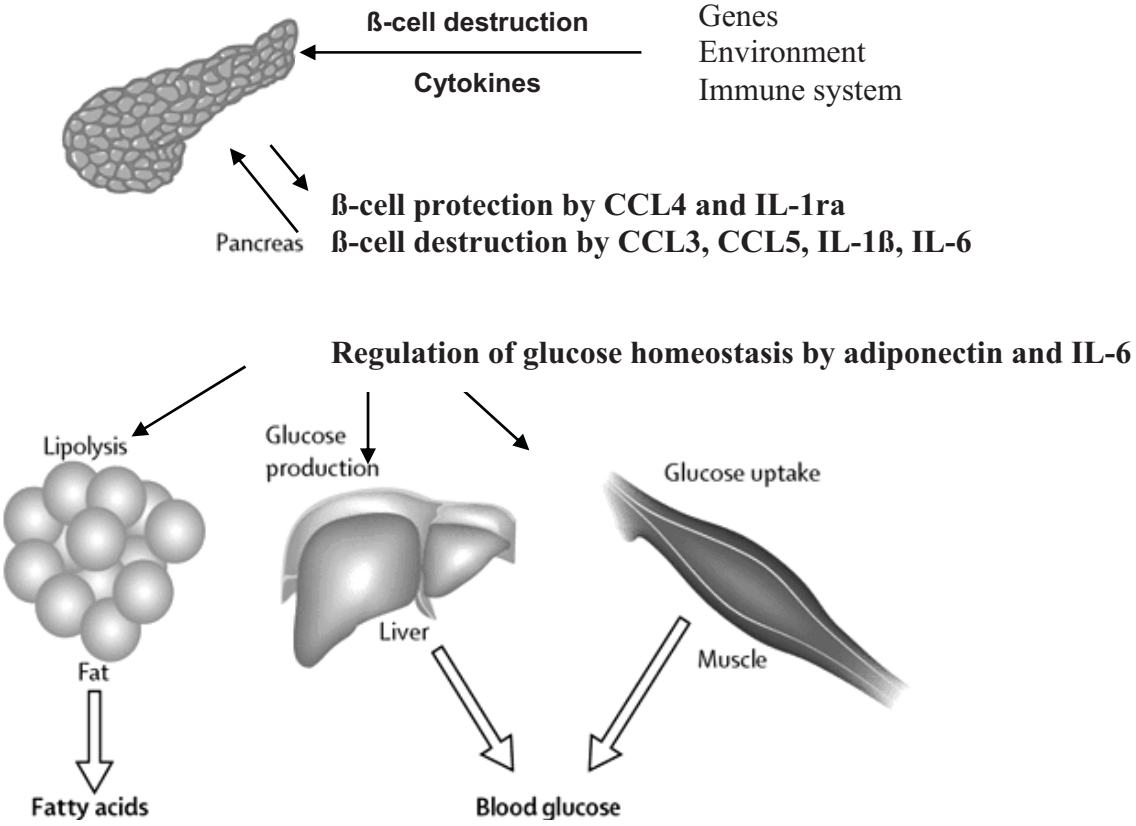
Based on the observation that cytokines and chemokines are important players in type 1 diabetes, cytokine directed intervention studies may help to protect remaining ß-cells after diagnosis of type 1 diabetes. Of note, one intervention study is planned to start in September 2008 using Anakinra, a recombinant IL-1ra protein to ameliorate ß-cell toxicity. This study is headed by Prof. Thomas Mandrup-Poulsen at Steno Diabetes Center in Copenhagen, Denmark, and Prof. Marc Donath at Zürich University hospital in Switzerland. Since we have shown a positive association of IL-1ra and a negative association of IL-1ß with ß-cell function (**Chapter 4**) it would be very interesting to see how peripheral cytokine concentrations are influenced by treatment with IL1ra and how treatment can influence ß-cell function in patients with type 1 diabetes.

Another interventional approach may be based on blocking chemotaxis of autoreactive T-cells to and into ß-cells via CCR5-blockade by Maraviroc. As experiments in animal models have successfully prevented immune-mediated diabetes, this therapy could also support the current treatment of type 1 diabetes in humans. As we showed negative association of CCL3

and CCL5 with ß-cell function but negative association of CCL4 with ß-cell stress (**Chapter 3**) we would assume, that CCR5 blockage would alter disease course also in humans. One question in this context is whether treatment with CCR5 antagonist can still be successful after manifestation of diabetes or has to be started in the prediabetic phase, i.e. before the destruction of the majority on insulin-producing cells.

Both intervention studies have the advantage of using licensed drugs: Anakinra is used during rheumatoid arthritis, Maraviroc in HIV therapy. This can simplify planning and realization of such intervention studies. Still, it seems unlikely that the complex regulated immune process resulting in ß-cell destruction can be stopped completely by treating only one "arm" of the immune system. A combination rather than single treatment may be the key for long-term success.

Figure 1: Hypothesized role of cytokines in the metabolism and ß-cell destruction



T-cell specific cytokine secretion

In contrast to the measurement of circulating cytokines that are the unspecific result of secretion by many cell types throughout the body, ELISPOT technology specifically provides insight in T-cell driven ß-cell antigen specific processes. So far, most studies have addressed the difference between type 1 diabetes patients and healthy controls in a rather qualitative manner and attempted to identify destructive or protective phenotypes of T-cells.

We show in this thesis that cytokines that have been used to characterize T-cell phenotypes also give information about the activity by their secreted ELISPOT responses that are related to ß-cell function (**Chapter 5**). This is of particular interest since T cells were stimulated by autoantigens like GAD65, insulin or heat shock protein 60 that are associated with type 1 diabetes. In addition, we have shown that sex, age and BMI considerably influence these responses. Thereby, analysis of T-cell responses needs to adjust for confounding factors such as sex, age, BMI and HLA. Interestingly, analysis of data of study p520 and p521 with recent onset type 1 diabetes patients showed that secretion of IL-13 and IFN-γ by autoreactive T-cells seems to play a predominant role in both adults and children but with different patterns.

However, ELISPOT is limited to peripheral blood like investigations of circulating cytokines that result in low numbers of ß-cell specific T-cells and low numbers of spots during antigen stimulation in line with a recent study of Arif et al (35).

When comparing the immune monitoring by circulating cytokines or cytokine secretion by stimulated T-cells as performed in the p520/521 study, both circulating cytokines and T-cell secretion are valuable and showed changes due to disease progression or treatment. Interestingly to note, changes due to DiaPep277 treatment were first seen in cytokine secretion of T-cells and later in circulating cytokines. However, whereas circulating cytokines give a complex picture of all ongoing processes and require small volume samples, ELISPOT gives ß-cell specific information but is time consuming and requires high sample volumes.

The recommendation or choice of method to monitor the immunological process depends on the requirements of the study and might even involve both circulating cytokines and T-cell assays as was done in study p520/521.

Remission: threshold or continuous clinical variable?

Adults and adolescents are more likely to enter remission than very young children. This observation probably reflects a more aggressive disease progression at younger age (36). The

underlying mechanisms of remission are not well understood and are object of current discussions. However, it is important to understand why some patients go into remission while other not, because (i) it gives important insights in the pathogenesis of type 1 diabetes; (ii) interventions are thought to be more likely to succeed in patients undergoing remission.

To investigate the association of remission with cytokines, we used different definitions of remission; HbA1c below 7.5% or 6.5% with an insulin requirement less than 0.4 U/kg body weight per day 6 months after diagnosis and improved C-peptide secretion of at least 20% from diagnosis to 6 months after diagnosis. Whereas the more classical definition of remission with HbA1c below 7.5% identified half of the cohort as remitters, the stricter definition of remission, in line with more recent therapy guide lines reduced the proportion of remitters to ¼ of the study group. Strikingly, the classification by improved C-peptide indicated that only 28 patients (~10%) were in remission. Although the different classifications revealed different absolute numbers of remitters, cytokine patterns were similar regardless which classification was used. This is an important observation because it suggests that our findings are valid and cannot be attributed to the choice of definition of remission.

We here described that remission is characterized by slightly but significantly increased IL-1ra and IL-6 concentrations in addition to decreased adiponectin concentrations. These findings were seen for the classical and the stricter definition of remission and also for the classification based on C-peptide change. This indicates that there is no clear immunologic threshold that distinguishes clinically defined different grades of remission, but rather suggests that, clinically as well as immunologically, remission and disease progression are part of a continuum.

Taken together, our results indicate that systemic concentrations of cytokines are associated with remission and disease progression.

Conclusion

This thesis presented for the first time associations of circulating cytokines with ß-cell function, ß-cell stress, metabolic control, glucose disposal and clinical remission in type 1 diabetes.

Circulating cytokines may therefore have the potential to serve as biomarkers of disease progression, prediction or assessment of the metabolic situation in type 1 diabetes. Furthermore they may provide new insights in the pathogenesis of the disease that would be of particular interest since most observations from models so far could not be extrapolated to humans.

Anti-inflammatory IL-1ra was associated with preserved endogenous insulin release, whereas Th1 related chemokines CCL3 and CCL5 showed negative association with ß-cell function. However, it needs to be kept in mind that our findings are based on descriptive associations Therefore, it will be interesting to see whether clinical trials can prove our association and provide a causal role of these cytokines in the pathogenesis of type 1 diabetes. In September 2008 a clinical trial is planned to start using Anakinra which is artificial IL-1ra to block ß-cell apoptosis by antagonism of IL-1ß. Antagonism of CCR5 by Maraviroc might by another therapeutical option to delay ß-cell destruction.

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Summary/ Zusammenfassung

Summary

Type 1 diabetes is an immune mediated disease resulting in selective destruction of insulin producing ß-cells. T-lymphocytes and cytokines play an important role in the process of ß-cell destruction. Cytokines and chemokines have been shown to be major contributors in insulitis and ß-cell destruction. *In vitro,* IL-1ß, IFN-γ and TNF-α reveal direct cytotoxic effect on ß-cells whereas anti-inflammatory adiponectin and pro-inflammatory IL-6 beside their role in inflammation have been shown to be involved in metabolic control and glucose disposal. In animal models, blockage of chemokine receptor 5 (CCR5) leads to altered T-cell trafficking and was associated with diabetes prevention and preserved ß-cell function.

The aim of this thesis was to investigate systemic cytokine concentrations and cytokine secretion of T-cells and their relation to the pathogenic processes of type 1 diabetes determined by ß-cell function, ß-cell stress and metabolic control. In collaboration with the Hvidøre study group and the p520/p521- study group, we had access to blood samples and metabolic data of patients with recent onset type 1 diabetes.

First, we optimized the protocol for measuring circulating cytokines and chemokines in the peripheral blood by defining an optimized diluent for the multiplex bead based immunoassay.

Second, we investigated CCR5 ligands CCL3, CCL4 and CCL5. Both Th1 related chemokines CCL3 and CCL5 were associated with decreased ß-cell function confirming previous work that suggested a deleterious role of these chemokines. However, differential associations with remission and metabolic parameters suggest a differential role in disease progression of these two chemokines. The Th2 related CCL4 was negatively related to ß-cell stress which was determined by proinsulin. This finding suggests a potential protective role of CCL4. Since all these chemokines bind to the same receptor, the balanced reaction of theses three chemokines may influence the disease process of type 1 diabetes. As blockage of chemokines or antagonism of CCR5 by therapeutic agents such as maraviroc is available, they may provide a new therapeutic target to ameliorate disease progression in type 1 diabetes.

Third, we determined the relation of pro-inflammatory IL-1ß and its anti-inflammatory receptor antagonist IL-1ra with ß-cell function. IL-1ra was positively associated with stimulated C-peptide whereas IL-1ß was negatively associated with endogenous insulin production. This novel finding is suggestive of a protective role of IL-1ra by the direct

antagonism of IL-1ß. Interestingly enough, IL-1ra administration successfully improved ß-cell function in type 2 diabetes, and may also be a new therapeutic approach in type 1 diabetes. Indeed, in September 2008, a trial named AIDA is going to be conducted applying IL-1ra (Anakinra) to newly diagnosed patients with type 1 diabetes.

Interestingly, other anti- and pro-inflammatory cytokines such as adiponectin and IL-6 showed no relation to ß-cell function but to remission, metabolic control and glucose disposal. The positive association of adiponectin with metabolic control and the relation with remission may reflect a compensatory effort to improve the metabolic situation. In addition, association of proinflammatory IL-6 with remission and glucose disposal indicated also a benign role of IL-6 in type 1 diabetes.

Fourth, we extended our study to an independent cohort of paediatric and adult patients with type 1 diabetes. We reproduced our findings on IL-1ra and CCL4 with ß-cell function in the p520 cohort and demonstrated that antigen-stimulated cytokine secretion of T-cells as determined by ELISPOT activity is influenced by anthropometric and genetic variables and independently related to ß-cell function.

However, it needs to be kept in mind that our findings are descriptive, the outcome of associations and based on cohort studies in which cytokine concentrations largely overlap between groups. Therefore, individual cytokine measurements are presently not of help to define the individual disease stage or progress. Hopefully, pattern analysis will extend those findings to an individual basis.

In summary, results of this thesis show for the first time, that circulating cytokines are associated with different stages of type 1 diabetes and relate to metabolic status, ß-cell function and clinical status. In addition, these results suggest that circulating cytokines can be used as a mirror the complex interactions of immune system and metabolism and indicate circulating cytokines as valuable parameter to get insights in the pathogenesis of type 1 diabetes.

Zusammenfassung

Der Typ 1 Diabetes ist eine immun-mediierte Erkrankung, welche selektiv die Insulin produzierenden ß-Zellen zerstört. T-Lymphozyten, Zytokine und Chemokine spielen eine wichtige Rolle bei der Insulitis und ß-Zell Zerstörung. Interleukin (IL)-1ß, Interferon (IFN)-γ und Tumor Nekrose Faktor (TNF)-α zeigen *in vitro* direkte zytotoxische Effekte auf die ß-Zelle, während das anti-inflammatorische Adiponektin und das pro-inflammatorische IL-6 neben ihrer inflammatorischen Rolle auch eine Beteidigung im Metabolismus und in der Glukoseaufnahme zeigen. Im Tiermodell moduliert die Blockade des Chemokinrezeptors 5 (CCR5) die T-Zell Migration, und führte zu einem Schutz der ß-Zellen.

Das Ziel dieser Arbeit war die Untersuchung von systemischen Zytokinkonzentrationen und die Zytokinsekretion von T-Zellen und deren Verhältnis zu pathogenetischen Prozessen beim Typ 1 Diabetes, welche definiert wurde durch ß-Zellfunktion, ß-Zellstress und metabolische Kontrolle. In Zusammenarbeit mit der Hvidøre Studiengruppe sowie der p520/p521-Studiengruppe hatten wir Zugang zu Blutproben und metabolischen Daten von Patienten mit neu diagnostiziertem Type 1 Diabetes.

Im ersten Schritt optimierten wir die Messung der Zytokine in Serum durch Auswahl eines geeigneten Diluents für den Multiplex-Bead-Based-Immunoassay. Danach untersuchten wir die CCR5 Liganden CCL3, CCL4 und CCL5. Die Th1 Chemokine CCL3 und CCL5 waren mit verminderten ß-Zellfunktion assoziert, was vorherige Arbeiten, die eine destruktive Rolle dieser Chemokine im Tiermodell beschrieben, bestätigte. Die unterschiedlichen Assoziationen von CCL3 und CCL5 mit Remission und metabolischen Parametern legen aber eine differentielle Rolle der beiden Chemokine im Krankheitsverlauf nahe. Das Th2 Chemokin CCL4 war negativ assoziert mit ß-Zellstress, welcher durch die Bestimmung von Proinsulin festgestellt wurde, und weist auf eine protektive Rolle von CCL4 hin. Die Antagonisierung von Chemokinen oder CCR5 durch Medikamente wie Maraviroc könnte ein neues therapeutisches Prinzip sein, um den Krankheitsverlauf des Typ 1 Diabetes zu verlangsamen.

Als nächstes untersuchten wir das Verhältnis des pro-inflammatorischen IL-1ß und seines anti-inflammatorischen Rezeptorantagonisten IL-1ra mit der ß-Zellfunktion. IL-1ra war positiv mit der ß-Zellfunktion assoziert, während IL-1ß eine negative Assoziation mit der endogenen Insulinsekretion zeigte. Diese hier erstmals beschriebene Beobachtung legt nahe,

dass die direkte Antagonisierung IL-1ß's durch IL-1ra ß-Zell protektiv sein könnte. Interessant ist, dass eine vorangehende Studie am Menschen zeigen konnte, dass die Applikation von IL-1ra im Typ 2 Diabetes erfolgreich die ß-Zellfunktion verbessert, und somit auch eine therapeutische Option für den Typ 1 Diabetes sein könnte. Im September 2008 startet die Interventionsstudie AIDA, in der frisch diagnostizierten Typ 1 Diabetikern IL-1ra (Anakinra) verabreicht werden wird.

Darüberhinaus zeigten andere anti- und pro-inflammatorische Zytokine wie Adiponektin und IL-6 keine Assoziation mit ß-Zellfunktion, aber mit Remission, metabolischer Kontrolle und Glukoseaufnahme. Die positive Assoziation von Adiponektin mit metabolischer Kontrolle und das Verhältnis mit Remission scheint eher auf einen kompensatorischen Versuch hinzuweisen die metabolische Situation zu verbessern. Die Assoziation von proinflammatorischem IL-6 mit Remission und Glukoseaufnahme deutet auch auf eine gutartige Rolle von IL-6 in Typ 1 Diabetes.

Im vierten Schritt erweiterten wir unsere Untersuchungen auf eine unabhängige Kohorte von Kindern und Erwachsenen mit Typ 1 Diabetes. Wir konnten unsere Ergebnisse von IL-1ra und CCL4 mit ß-Zellfunktionin in der p520 Kohorte bestätigen und zeigen, dass Antigen stimulierte Zytokinsekretion von T-Zellen gemessen durch ELISPOT-Aktivität assoziert ist mit ß-Zellfunktion.

Kritisch muss darauf aufmerksam gemacht werden, dass unsere Ergebnisse deskriptiv und das Resultat von Assoziationen sind, da sie das Ergebnis von Kohortenstudien sind, in welchen die Zytokinkonzentrationen zwischen Gruppen zum großen Teil überlappen. Deshalb kann zum gegenwärtigen Zeitpunkt anhand der Zytokinmessung keine Aussage getroffen werden über den individuellen Krankheitsstatus oder Verlauf. Es ist wahrscheinlich, dass weniger Einzelmessungen von Zytokinen als Zytokinmusteranalysen ein individuelles Profil erkennen lassen, mit dem auch Vorhersagen für einzelne Personen möglich sind.

Zusammenfassend zeigen die Ergebnisse dieser Arbeit zum ersten Mal, dass systemische Zytokine assoziert sind mit verschiedenen Stadien des Typ 1 Diabetes und mit dem metabolischen Status, ß-Zellfunktion und klinischem Status zusammenhängen. Weiterhin lassen die Ergebnisse vermuten, dass systemische Zytokine genutzt werden können, um die komplexen Interaktionen des Immunsystems und des Metabolismus wiederzuspiegeln und zeigen systemische Zytokine als wertvolle Marker, um neue Erkenntnisse in der Pathogenese des Typ 1 Diabetes zu erlangen.

Curriculum vitae

Curriculum Vitae

Last name	**Pfleger**
First name	Christian
Date of birth	23. Mai 1974
Place of birth	Düsseldorf

Education

1984-1993	Quirinus-Gymnasium, Neuss
1993	Abitur
04/1994-04/1998	Study Pharmacy at Heinrich-Heine-Universität, Düsseldorf
04/1998	Second exam (zweites Staatsexamen)
04/1998-04/1999	Practical year, Punkt Apotheke, Düsseldorf
05/1999	Final exam (drittes Staatsexamen) and approbation as Pharmacist

Working experience

06/1999-08/2000	Pharmacist in charge in several public pharmacies
07/2001-01/2004	Chief Pharmacist, Erft Apotheke, Grevenbroich
04/2004-04/2005	Pharmacist, part time, Heine Apotheke, Düsseldorf
since 04/2005	Scientific employee at the German Diabetes Center at Heinrich-Heine-Universität, Düsseldorf

Scientific experience

02/2004	Ph.D. student, German Diabetes Center at Heinrich-Heine-Universität, Düsseldorf under supervision of Priv.-Doz. Dr. N.C. Schloot and Prof. Dr. H.-D. Höltje, taken over by Prof. Dr. M. Kassack in 2008

Experience abroad

09/2000-06/2001	Australia (social farm for school- and handicapped children; hospital ward for koalas, kangaroos, and wombats; organic gardening

List of scientific publications, oral presentations and posters and awards and price

Scientific publications

Effect of serum content and diluent selection on assay sensitivity and signal intensity in multiplex bead-based immunoassays.
C Pfleger, NC Schloot, F ter Veld.
J Immunol Methods, 2008 Jan 1;329(1-2):214-8.

Association of IL-1ra and adiponectin with C-peptide and remission in patients with type 1 diabetes.
C Pfleger, HB Mortensen, L Hansen, C Herder, BO Roep, H Hoey, HJ Aanstoot, M Kocova, NC Schloot.
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Circulating Chemokines in Patients with Autoimmune Thyroid Diseases.
J Domberg, L Chao, C Papewalis, **C Pfleger**, K Xu, HS Willenberg, D Hermsen, WA Scherbaum, NC Schloot, M Schott.
Horm Metab Res. 2008 Apr; 14. (Epub ahead of print)

Relation of circulating concentrations of chemokine receptor CCR5 ligands to C-peptide, proinsulin and HbA1c and disease progression in type 1 diabetes.
C Pfleger, A Kaas, L Hansen, B Alizadeh, P Hougaard, R Holl, H Kolb, BO Roep, HB Mortensen, NC Schloot.
Clin Immunol. 2008 Apr 21. (Epub ahead of print)

Association of ß-cell function with T-cell reactivity to islet antigens in recent onset type 1 diabetes
C Pfleger, G Meierhoff, H Kolb, NC. Schloot
Manuscript in preparation

Oral presentations and posters

- 41st Annual Meeting of the European Association for the Study of Diabetes (EASD), September 12-15, 2005 (Poster)
- 50th Annual Meeting of the German Society of Endocrinology (DGE) & 23rd Meeting of the Dutch Endocrine Society, March 1-4, 2006 (Poster)
- 41st Annual Meeting of the German Diabetes Society (DDG), May 24-27, 2006 (Talk)
- 66th Scientific Sessions, American Diabetes Association (ADA), June 9-13, 2006 (3 Posters)
- 42nd Annual Meeting of the European Association for the Study of Diabetes (EASD), September 14-17, 2006 (Poster)
- 19th World Diabetes Congress (IDF), December 3-7, 2006 (Poster)
- 67th Scientific Sessions, American Diabetes Association (ADA), June 22-27, 2007 (Poster)
- 43rd Annual Meeting of the European Association for the Study of Diabetes (EASD), September 18-21, 2007 (Talk + Poster)
- 43rd Annual Meeting of the German Diabetes Society (DDG), April 30-May 3, 2008 (Poster)
- 8th Annual Meeting of the Federation of Clinical Immunology Societies (FOCIS), June 5-9, 2008 (Talk)
- 13th EASD/JDRF Oxford Workshop, 8-11 August, 2008 (Talk)

Awards and price

- IPSEN PHARMA Poster Price for exceeding work, Meeting of the German Society of Endocrinology (DGE), Essen, Germany, 2006
- Travel award, Meeting of the German Diabetes Society (DDG), München, Germany, 2008
- Travel award, Meeting of the Federation of Clinical Immunology Societies (FOCIS), Boston, USA, 2008
- Travel award, EASD/JDRF Oxford Workshop, UK, 2008

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Danksagung

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