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ABSTRACT

Objectives To investigate low-grade inflammation in type 2 diabetes and explore associations to clinical aspects as well as microvascular and macrovascular complications.

Design Cross-sectional analysis.

Setting The outpatient diabetes clinic at the Department of Endocrinology at Aalborg University Hospital, Denmark.

Participants 100 participants with type 2 diabetes confirmed by a haemoglobin A1c (HbA1c)≥6.5% for a minimum of 1 year and 21 healthy controls.

Outcome measures Serum levels of 27 inflammation-related biomarkers measured by immunoassay. Associations with microvascular and macrovascular complications, body weight, glycaemic control, medication and sex were investigated in the diabetes cohort.

Results Serum levels of tumour necrosis factor (TNF)-α and eotaxin were elevated in type 2 diabetes ($p<0.05$), while interleukin (IL)-7 was decreased ($p<0.001$). IL-12/IL-23p40, IL-15, macrophage-derived chemokine (MDC) and C reactive protein (CRP) levels were increased with body weight ($p<0.05$), while eotaxin and TNF-α were increased with elevated HbA1c levels ($p<0.04$). Dipeptidyl peptidase-4 inhibitor therapy was associated with lower levels of induced protein-10, MDC and thymus and activation regulated chemokine ($p<0.02$), while females had higher levels of MDC ($p=0.027$). Individuals with ≥3 diabetic complications had elevated levels of IL-6, IL-10, IL-12/IL-23p40, IL-15 and CRP compared with those with ≤3 ($p<0.05$).

Conclusion The level of low-grade inflammation in type 2 diabetes is associated with obesity, glycaemic regulation, therapeutical management, sex and complications. Our results underline the importance of addressing inflammatory issues in type 2 diabetes, as these may predispose for crippling comorbidities.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Analysis of a broad palette of inflammatory biomarkers in serum in 100 participants with type 2 diabetes and 21 healthy controls.
⇒ High degree of heterogeneity of our cohort, which allows for generalisation to the population of type 2 diabetes.
⇒ Well-characterised cohort in regard to microvascular and macrovascular comorbidities.
⇒ The cross-sectional design is a limitation of the study and hinders any assumptions of causality.
⇒ This study is based on secondary analysis and thus inclusion and exclusion criteria were not designed specifically with the investigation of inflammatory biomarkers in mind.

INTRODUCTION

Tight glycaemic regulation is vital for balancing the existing energy demand in tissues by combining resources originating from the nutritional supply and release from internal storages. Low blood glucose is potentially life threatening, while long-term elevated levels have several metabolic consequences, including sorbitol production, mitochondrial dysfunction, and formation of advanced glycation end products.[1] Chronic hyperglycaemia can be caused either by insulin deficiency, as seen in type 1 diabetes, or by a combination of generalised insulin resistance in peripheral tissues and insufficient insulin production resulting in type 2 diabetes. The latter is the most prevalent diabetes type accounting for up to 90% of the cases.[2]

The pathogenesis of type 2 diabetes is highly complex and multifactorial, and many aspects of the disease require further elucidation. However, it is clear that obesity along with a sedentary lifestyle is a substantial risk factor for development of insulin resistance and type 2 diabetes through stress-induced inflammation in adipose tissue leading to insensitivity of the insulin receptor.[3] In recent years, the previous view on adipose tissue as a mere storage of fat has been disproved, and it is now accepted that especially visceral adipose tissue possesses important endocrine and inflammatory properties. As an example, adipocytes activated by expansion-associated hypoxia secrete cytokines and so-called adipokines, many of which are proinflammatory in nature.[4] As the prevalence of both obesity and type 2 diabetes continue to rise worldwide,[2] a better understanding of the inflammatory link between these lifestyle-associated conditions is crucial.

In addition to obesity-induced inflammation, excess glucose availability in diabetes causes alterations in normal homeostasis, facilitating the progression of proinflammatory cytokine release to the microenvironment. Low-grade systemic inflammation is thus regarded as an accompanying condition in type 2 diabetes.[5] Increased levels of proinflammatory biomarkers such as interleukin (IL) 6 and C reactive protein (CRP) have been shown to be associated with an increased risk of type 2 diabetes development in several prospective studies.[6 7] This suggests that the pathogenetic mechanisms in type 2 diabetes is influenced by systemic low-grade inflammation. It is, however, unclear whether this proinflammatory state remains during the course of the disease or if it increases or diminishes over time. In addition, standard medical treatment in type 2 diabetes such as statins and dipeptidyl peptidase-4 (DPP-4) inhibitors have immunomodulating properties and may thus influence the inflammatory response.[8 9]

The low-grade systemic inflammation in type 2 diabetes is clinically essential, because it is associated with the development and progression of long-term complications such as nephropathy, neuropathy and retinopathy.[10–12] Moreover, low-grade inflammation is associated with cardiovascular disease in diabetes,[13] which is the primary cause of morbidity and mortality in individuals with type 2 diabetes.[14]

The aim of this study was to investigate the level of low-grade systemic inflammation in a cohort of individuals with type 2 diabetes with varying disease duration. We hypothesised that individuals with type 2 diabetes exhibited higher levels of proinflammatory biomarkers than healthy controls, and accordingly, the primary endpoint was differences in circulating inflammatory biomarkers in healthy and people with type 2 diabetes. Furthermore, we hypothesised that levels of proinflammatory biomarkers in type 2 diabetes were associated with disease duration, obesity, glycaemic control, therapeutical management and presence of diabetes-related microvascular and macrovascular complications. The secondary endpoints were thus to investigate associations between inflammatory biomarkers and clinical characteristics of type 2 diabetes.

METHODS

Study population

All individuals with type 2 diabetes scheduled for regular health visits at the outpatient diabetes clinic at the department of endocrinology at Aalborg University Hospital, Denmark were informed about the study and screened for eligibility after signing of the informed consent form, and 100 participants were included for cross-sectional analysis. Inclusion criteria included Northern European descent, age above 18 years, a verified diagnosis of type 2 diabetes with haemoglobin A1c (HbA1c)≥6.5% for a minimum of 1 year, and stable diabetes treatment. People with other endocrinological or neurological diseases were excluded. The primary outcome of the study was cardiac vagal tone and the results have been published elsewhere.[15] The control cohort consisted of sex-matched healthy volunteers (n=21) recruited for a randomised controlled trial (N-20090008) likewise conducted by our research group.

Blood samples

Morning blood samples were drawn from the cubital vein after a fasting period of minimum 6 hours. For analysis of inflammatory biomarkers, blood was collected in EDTA tubes and centrifuged for 10 min at 1000*g*. Isolated serum was aliquoted in appropriate volumes and stored in a biobank at −80°C until the complete data set was collected. All samples were thawed just prior to analysis. Samples from both cohorts were analysed consecutively to minimise interplate variability. For analysis of HbA1c, blood was collected in lithium heparin tubes and analysed by routine biochemical procedures.

Inflammatory biomarkers

Biomarker concentrations in serum samples were analysed using the V-PLEX Neuroinflammation Panel 1 Human Kit (Meso Scale Diagnostics (MSD), Gaithersburg, Maryland, USA) on a MESO QuickPlex SQ 120 instrument (MSD) according to the manufacturer's specifications. Sample values below the detection limit of the assay were assigned a value of the detection limit divided by $\sqrt{2}$.[16] If more than 30% of the measured samples for any given biomarker were below the detection limit, the biomarker was excluded from the analysis. Likewise, samples with a coefficient of variation>30% between duplicate measurements were excluded from the analysis (online supplemental table 1). Biomarkers on the panel included: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-13, IL-15, IL-16, IL-17A, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, TNF-β, eotaxin, eotaxin-3, IFN-γ-induced protein (IP)-10, monocyte chemoattractant protein (MCP)−1, MCP-4, macrophage-derived chemokine (MDC), macrophage inflammatory protein (MIP)-1α, MIP-1β, thymus and activation regulated chemokine (TARC), and CRP.

Assessment of diabetic comorbidities

All participants in the type 2 diabetes cohort underwent investigations concerning common diabetic comorbidities: (1) *peripheral neuropathy*: signs of peripheral neuropathy was investigated by vibration perception threshold (VPT) at the dorsum of the first phalanx using a biothesiometer (Bio-Medical Instruments). The measurement was done three consecutive times bilaterally, and the final VPT was calculated as the mean value of both feet. Results above 18 V were considered abnormal and thus as signs of diabetic peripheral neuropathy. (2) *Nephropathy*: morning urine samples were collected by participants at home and handed over to study personnel for standard biochemical analysis. Diabetic nephropathy was defined as a urine albumin/creatinine ratio above 30 mg/g, which is a standard cut-off for early diabetic nephropathy and microalbuminuria. (3) *Retinopathy*: participants were asked if they had ever been diagnosed

Table 1 Demographic and clinical characteristics among groups

	Healthy (n=21)	Type 2 diabetes (n=98)	P value
Basic demography			
Age (years)	52 (48–55)	65 (56–71)	**<0.001**
Sex (% of males)	71	63	0.478
BMI (kg/m$^{2)}$	25.6 (23.7–28.0)	31.4 (27.5–34.5)	**<0.001**
Current smokers (%)	19	5	**0.028**
Disease duration (y)	–	10 (5–17)	–
Vital signs			
Systolic BP (mm Hg)	128 (119–137)	137 (128–147)	**0.021**
Diastolic BP (mm Hg)	76±11	77±9	0.720
Pulse (beats/min)	66±7	69.8±10	0.110
Biochemistry			
HbA1c			
(mmol/mol)	33 (33–37)	55 (48–61.5)	**<0.001**
(%)	5.2 (5.2–5.5)	7.2 (6.5–7.8)	**<0.001**
Cholesterol (mmol/L)	5.4±0.9	3.9±0.9	**<0.001**
eGFR (mL/min/1.73 m^2)			
>90 (%)	62	46	0.197
40–90 (%)	38	52	0.264
<40 (%)	0	<1	0.507
HDL (mmol/L)	1.5 (1.3–1.8)	1.2 (1.0–1.5)	**0.006**
LDL (mmol/L)	3.3±0.9	1.9±0.7	**<0.001**
Triglycerides (mmol/L)	1.1 (0.7–1.4)	1.4 (1.0–2.0)	**0.023**
Diabetic comorbidities			
Neuropathy (%)	–	60	–
Nephropathy (%)	–	19	–
Retinopathy (%)	–	8	–
CAN (%)	–	40	–
Medication			
Antihypertensives (%)	–	67	–
DPP-4 inhibitor (%)	–	18	–
Metformin (%)	–	78	–
SGLT-2 inhibitor (%)	–	23	–
GLP-1 receptor agonist (%)	–	23	–
Statins (%)	–	66	–

Boldface font indicates statistical significance (p<0.05). Antihypertensive medication includes ACE inhibitors, angiotensin II receptor antagonists, calcium channel blockers, beta blockers, diuretics and I1-imidazoline receptor antagonists. Results displayed as either mean±SD or median (1st–3rd quartiles) based on distribution of the data.
BMI, body mass index; CAN, cardiac autonomic neuropathy; DPP-4, dipeptidyl peptidase-4; eGFR, estimated glomerular filtration rate; GLP, glucagon-like peptide; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SGLT, sodium-glucose transport protein.

with proliferative or non-proliferative retinopathy. (4) *Cardiac autonomic neuropathy (CAN)*: electrocardiographic recordings by the VAGUS device (Medicus Engineering Aps, Aarhus, Denmark) described in detail elsewhere[15] were applied for evaluation of cardiac autonomic neuropathy. Recordings were made during rest, postural change, deep breathing and the Valsalva manoeuvre. Age-specific cut-off values were applied,[17] and abnormal results in one or more exercises were considered as signs of cardiac autonomic neuropathy.

Data handling and statistics

Distribution of raw and log-transformed data was evaluated by Shapiro-Wilk test of normality. Pairwise comparisons among groups were achieved by independent

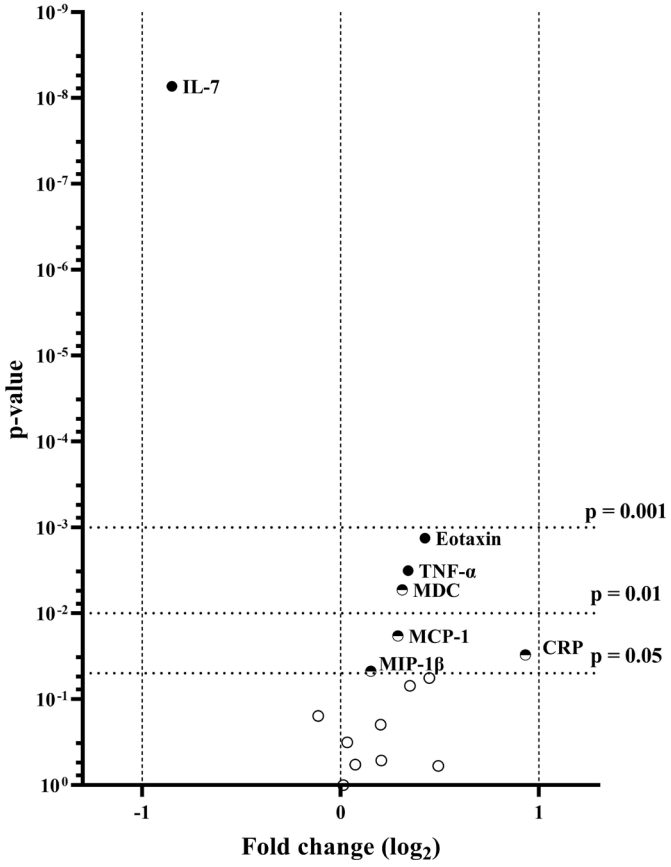

Figure 1 Volcano plot displaying pairwise comparisons of inflammatory factors in type 2 diabetes and healthy controls. Vertical dashed lines indicate threshold for twofold differences among groups. Horizontal dashed lines indicate p value thresholds of 0.05, 0.01 and 0.001, respectively. ●Significantly different after adjustment for age and BMI, ◓significantly different in the unadjusted model,○ above significance threshold in both models. Only significant analytes are labelled. BMI, body mass index; CRP, C reactive protein; IL, interleukin; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; TNF, tumour necrosis factor.

samples t-test or Mann-Whitney U based on data distribution. Differences in inflammatory biomarkers between healthy and type 2 diabetes were investigated first by pairwise comparisons and second by a logistic regression model including age and body mass index (BMI) as confounders, as these factors were different between groups and known to influence systemic low-grade inflammation. For the volcano plot, the fold difference was calculated as the $\log_2$-ratio between two group means. Differences in inflammatory biomarkers between people with short-term and long-term disease duration were likewise investigated by a logistic regression model including age and BMI as confounders. Multiple logistic regression analyses were performed to investigate the association between clinical parameters and inflammatory biomarkers. The independent variables included obesity (BMI<30 vs BMI>30), blood glucose level (HbA1c<55 vs HbA1c>55), DPP-4 inhibitor therapy, glucagon-like peptide (GLP-1) receptor agonist therapy and sex. Additionally, two models were applied in which associations were adjusted for the remaining clinical variables, and total plasma cholesterol or statin therapy, all of which may have an impact on the systemic inflammatory status. Differences in inflammatory biomarkers between people with 0, 1, 2 or ≥3 comorbidities were investigated by a Bonferroni-corrected analysis of variance and subsequently the Dunn's test. An α level of 0.05 was applied for all analyses. The STATA software (StataCorp LLC, V.15.1) was applied for all statistical analyses.

Patient and public involvement

Patients or members of the public were not included in the design, conduction, reporting or dissemination plans of this project.

RESULTS

Study population

Two subjects in the type 2 diabetes group were excluded due to haemolysis of collected blood samples. Individuals in the diabetes group were older, had higher BMI and higher HbA1c compared with the healthy controls (p<0.001). On the contrary, healthy controls had higher total cholesterol (p<0.001), high-density lipoprotein (p=0.006), and low-density lipoprotein (p<0.001) compared with individuals in the type 2 diabetes cohort of which 66% were on lipid-lowering statin therapy. A full demographic overview can be found in table 1.

Inflammatory biomarkers in type 2 diabetes compared with healthy

Serum levels of 27 inflammatory biomarkers were measured in individuals with type 2 diabetes and healthy controls. Eleven biomarkers were excluded from the statistical analyses due to being undetectable or of insufficient measurement quality due to low levels (online supplemental table 1). The remaining 16 biomarkers (IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-15, IL-16, IFN-γ, TNF-α, eotaxin, IP-10, MCP-1, MDC, MIP-1β, TARC, CRP) were measured in ≥95% of the samples. The concentrations of TNF-α (p=0.003) and CRP (p=0.030) were significantly higher in the type 2 diabetes cohort compared with the control cohort (figure 1). Similarly, four chemokines (eotaxin (p=0.001), MCP-1 (p=0.018), MDC (p=0.005) and MIP-1β (p=0.047)) showed elevated levels in the diabetes cohort. In contrast, the level of cytokine IL-7 was significantly lower in participants with type 2 diabetes compared with healthy controls (p<0.001). After adjustment for age and BMI, only IL-7, eotaxin and TNF-α remained significantly different. Serum concentrations of all measured biomarkers are presented in online supplemental table 2. When subdividing the type 2 diabetes cohort according to disease duration, only IL-10 was significantly different (p=0.008) between groups, even after adjustment for age and BMI, with a modestly increased levels found in subjects with disease

Table 2 OR for associations between serum concentrations of inflammatory factors (cytokines (n=4), chemokines (n=6), proinflammatory cytokines (n=5), vascular injury (n=1)) in type 2 diabetes with short-term disease duration (<10 years, n=44) and long-term disease duration (>10 years, n=50) unadjusted and adjusted for age and BMI

		Unadjusted model		Adjusted model	
		Or (95% CI)	P value	Or (95% CI)	P value
Cytokines	IL-7	1.03 (0.93 to 1.15)	0.565	1.04 (0.93 to 1.18)	0.466
	IL-12 /IL-23p40	1.00 (1.00 to 1.00)	0.446	1.00 (0.99 to 1.01)	0.717
	IL-15	1.51 (0.74 to 3.10)	0.256	1.32 (0.62 to 2.79)	0.471
	IL-16	1.00 (1.00 to 1.00)	0.832	1.00 (1.00 to 1.00)	0.813
Chemokines	Eotaxin	1.00 (1.00 to 1.00)	0.887	1.00 (1.00 to 1.00)	0.687
	IP-10	1.00 (1.00 to 1.00)	0.512	1.00 (1.00 to 1.00)	0.244
	MCP-1	1.00 (1.00 to 1.00)	0.864	1.00 (1.00 to 1.00)	0.523
	MDC	1.00 (1.00 to 1.00)	0.810	1.00 (1.00 to 1.00)	0.319
	MIP-1β	1.00 (0.99 to 1.01	0.992	1.00 (0.99 to 1.01)	0.916
	TARC	1.00 (1.00 to 1.00)	0.719	1.00 (1.00 to 1.00)	0.260
Proinflammatory cytokines	IL-6	1.34 (0.79 to 2.27)	0.271	1.21 (0.70 to 2.09)	0.504
	IL-8	1.00 (0.94 to 1.06)	0.983	1.00 (0.94 to 1.06)	0.904
	IL-10	111.85 (2.86 to 4377.78)	**0.012**	103.97 (2.30 to 4699.58)	**0.017**
	IFN-γ	1.02 (0.97 to 1.08)	0.438	1.02 (0.96 to 1.09)	0.447
	TNF-α	1.69 (0.70 to 4.12)	0.246	1.78 (0.69 to 4.62)	0.234
Vascular injury	CRP	1.00 (1.00 to 1.00)	0.697	1.00 (1.00 to 1.00)	0.713

Boldface font indicates statistical significance (p<0.05).
BMI, body mass index; CRP, C reactive protein; IL, interleukin; TNF, tumour necrosis factor.

duration above 10 years (table 2). Similarly, we investigated whether the presence of CAN (early or manifest) influenced the levels of inflammatory factors; however, none of these reached significant levels (data not shown).

Inflammatory biomarkers in subgroups of type 2 diabetes

Obesity was significantly associated with concentration of five inflammatory biomarkers (IL-12/IL-23p40, IL-15, IFN-γ, MDC and CRP) (table 3—only analytes with p value below 0.05 shown). When adjusting for HbA1c, sex and total plasma cholesterol or statin use, IL-12/IL-23p40, IL-15 and CRP remained statistically significant associated with obesity. HbA1c was significantly associated with eotaxin and IL-12/IL-23p40 levels after adjusting for confounders, and levels of MDC were associated with sex with lower levels found in male subjects compared with females. Lower levels of IL-8, IP-10 and MDC were associated with DPP-4 inhibitor therapy, while higher levels of TNF-α were associated with GLP-1 receptor agonist therapy. Lastly, SGLT2 inhibitor therapy was associated with lower levels of MDC.

Diabetic comorbidities

When subdividing the type 2 diabetes cohort into groups according to number of diabetic comorbidities, five biomarkers (IL-6, IL-10, IL12/IL-23p40, IL-15 and CRP) were significantly elevated in participants with three or more comorbidities compared with those with fewer or none (figure 2—only analytes with p values below 0.05 shown).

DISCUSSION

In this study, we investigated the level of systemic low-grade inflammation in a cohort of individuals diagnosed with type 2 diabetes. Elevated levels of several inflammatory biomarkers were found in comparison to healthy controls, evident in both short-term and long-term disease duration. Moreover, in the type 2 diabetes cohort, obesity, hyperglycaemia and female sex were found to be associated with elevated levels of various inflammatory biomarkers. Lastly, we were able to establish a connection between the number of common diabetic comorbidities and elevated levels of inflammatory biomarkers.

Inflammatory biomarkers in type 2 diabetes compared with healthy

After adjustment for age and BMI, we showed that IL-7 was significantly decreased, while eotaxin and TNF-α was significantly increased in type 2 diabetes compared with healthy. The majority of research regarding IL-7 has been conducted in type 1 diabetes, where elevated levels are shown compared with healthy.[18] IL-7 is highly involved in T cell function and proliferation, and a role of this cytokine in mediating expansion of insulin-producing

Table 3 Multiple logistic regression analysis of serum concentrations between (A) type 2 diabetes+BMI<30 (n=40) and type 2 diabetes+BMI>30 (n=58), (B) type 2 diabetes with HbA1c<55(n=47) and type 2 diabetes with HbA1c>55 (n=51), (C) male type 2 diabetes (n=62) and female type 2 diabetes (n=36), (D) type 2 diabetes (n=80) and type 2 diabetes treated with DPP-4 inhibitors (n=18), (E) type 2 diabetes (n=75) and type 2 diabetes treated with GLP-1 receptor agonists (n=23), and (F) type 2 diabetes (n=75) and type 2 diabetes treated with SGLT2 inhibitor therapy (n=23) with overall R-squared value and effect size (95% CI) of BMI, HbA1c, sex, DPP-4 inhibitor therapy, GLP-1 receptor agonist therapy, or SGLT2 inhibitor therapy displayed

	Unadjusted model		Adjusted model 1		Adjusted model 2	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
A. Obesity						
IL-12/IL-23p40	1.01 (1.00 to 1.02)	**0.003**	1.01 (1.00 to 1.02)	**0.007**	1.01 (1.00 to 1.02)	**0.007**
IL-15	2.32 (1.05 to 5.11)	**0.038**	2.30 (1.02 to 5.15)	**0.043**	2.30 (1.02 to 5.16)	**0.043**
IFN-γ	1.12 (1.00 to 1.25)	**0.041**	1.10 (0.99 to 1.23)	0.087	1.10 (0.99 to 1.22)	0.091
MDC	1.00 (1.00 to 1.00)	**0.029**	1.00 (1.00 to 1.00)	0.051	1.00 (1.00 to 1.00)	**0.049**
CRP	1.00 (1.00 to 1.00)	**0.001**	1.00 (1.00 to 1.00)	**0.001**	1.00 (1.00 to 1.00)	**0.001**
B. Blood glucose						
IL-8	1.08 (1.00 to 1.15)	**0.037**	1.07 (0.99 to 1.14)	0.076	1.07 (1.00 to 1.15)	0.055
TNF-α	3.25 (1.21 to 8.73)	**0.019**	2.64 (0.95 to 7.34)	0.062	3.09 (1.11 to 8.58)	**0.031**
Eotaxin	1.00 (1.00 to 1.01)	**0.031**	1.00 (1.00 to 1.01)	**0.027**	1.00 (1.00 to 1.01)	**0.025**
C. Sex						
MDC	1.00 (1.00 to 1.00)	**0.009**	1.00 (1.00 to 1.00)	**0.021**	1.00 (1.00 to 1.00)	**0.027**
D. DPP-4 inhibitor therapy						
IL-8	0.88 (0.79 to 0.99)	**0.040**	0.89 (0.79 to 1.00)	0.052	0.89 (0.79 to 1.00)	0.051
IP-10	1.00 (1.00 to 1.00)	**0.013**	0.99 (0.99 to 1.00)	**0.008**	0.99 (0.99 to 1.00)	**0.008**
MDC	1.00 (1.00 to 1.00)	**0.027**	1.00 (1.00 to 1.00)	**0.011**	1.00 (1.00 to 1.00)	**0.011**
TARC	1.00 (1.00 to 1.01)	**0.004**	1.00 (1.00 to 1.01)	**0.005**	1.00 (1.00 to 1.01)	**0.005**
E. GLP-1 receptor agonist therapy						
IL-8	1.08 (1.01 to 1.15)	**0.025**	1.08 (0.99 to 1.17)	0.069	1.08 (1.00 to 1.18)	0.058
IL-15	0.57 (0.23 to 1.41)	0.226	0.29 (0.09 to 0.95)	**0.042**	0.27 (0.08 to 0.92)	**0.036**
IL-16	1.00 (0.98 to 1.00)	**0.042**	1.00 (0.98 to 1.00)	0.077	1.00 (0.98 to 1.00)	0.068
TNF-α	6.50 (2.07 to 20.42)	**0.001**	4.60 (1.26 to 16.76)	**0.021**	4.70 (1.25 to 17.69)	**0.022**
F. SGLT2 inhibitor therapy						
MDC	1.00 (1.00 to 1.00)	**0.014**	1.00 (1.00 to 1.00)	**0.027**	1.00 (1.00 to 1.00)	**0.033**

Results presented as OR and 95% CI. Total plasma cholesterol, BMI, HbA1c, and sex were included in the adjusted model 1 as appropriate, while statin use, BMI, HbA1c and sex were included in the adjusted model 2 as appropriate. For simplicity, only analytes with p values below 0.05 in either model are shown. Bold font indicated statistical significance after Bonferroni adjustment (p<0.003).
BMI, body mass index; CRP, C reactive protein; DPP-4, dipeptidyl peptidase-4; GLP, glucagon-like peptide; HbA1c, haemoglobin A1c; IFN, interferon; IL, interleukin; IP, induced protein; MDC, macrophage-derived chemokine; TARC, thymus and activation regulated chemokine.

β-cell-autoreactive T cells has been proposed thus implicating IL-7 in the pathogenesis of type 1 diabetes.[19] The decreased levels in type 2 diabetes compared with healthy controls found in this study were somewhat surprising but may reflect the lack of T-cell activation the pathology of type 2 diabetes. Eotaxin has been linked to the development of atherosclerosis by facilitating monocyte infiltration in smooth muscle cells under the influence of proinflammatory mediators,[20] and elevated levels of this chemokine have previously been reported in type 1 diabetes individuals with complications compared with individuals with no diabetic complications as well as healthy controls.[21] Increased levels of CRP have previously been reported in adults with type 2 diabetes,[13 22] but in our cohorts, the difference could be attributed to a skewed distribution of age and BMI in the two cohorts.

IL-10 is generally regarded as an anti-inflammatory cytokine with the ability to dampen the immune response, and previous data have shown downregulation of IL-10 in both type 2 diabetes and obesity per se.[23] This contrasts our findings, which showed no differences in the overall diabetes cohort but an increase in individuals with long disease duration. This observation could reflect manifestations of compensatory mechanisms towards a long-term elevated inflammatory environment attempting to elicit an anti-inflammatory response. However, proinflammatory factors (eg, TNF-α) were elevated regardless of disease duration suggesting that any attempt of balancing

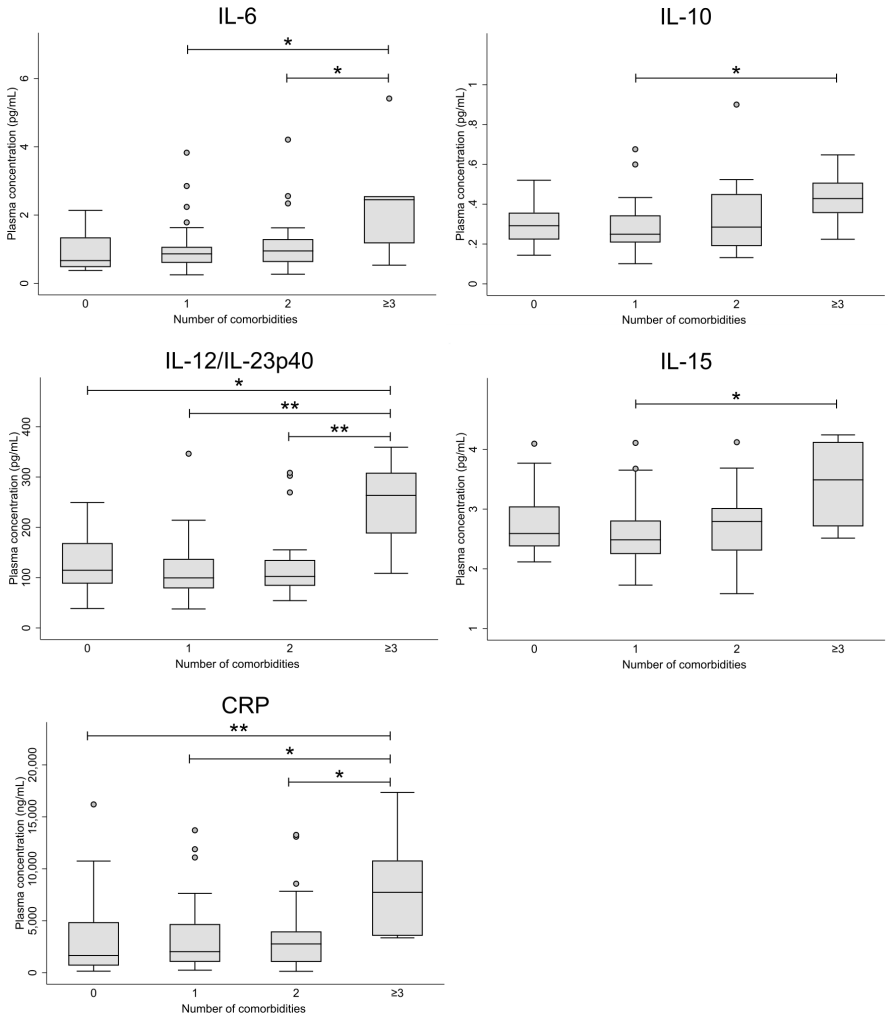

Figure 2 Box plots displaying plasma concentrations of biomarkers in individuals with type 2 diabetes and 0 (n=20), 1 (n=43), 2 (n=28), or 3 or more (n=7) diabetic comorbidities (retinopathy, nephropathy, neuropathy, cardiac autonomic neuropathy). Only analytes with p values below 0.05 are shown. *P<0.05, **p<0.01. CRP, C reactive protein; IL, interleukin.

the immune response remain challenging in the presence of type 2 diabetes.

Inflammatory biomarkers in subgroups of type 2 diabetes

Obesity and blood glucose regulation

In our type 2 diabetes cohort, obesity (BMI>30) was significantly associated with the levels of IL-12/IL-23p40 and CRP, while eotaxin and TNF-α levels were associated with glycaemic regulation (HbA1c). Previously it has been shown that TNF-α release is upregulated in connection with obesity and has been linked to the progression of insulin resistance.[24 25] The fact that TNF-α was not associated with by obesity in our cohort is thus surprising. However, elevated levels of TNF-α in adipose tissue, but not in serum have previously been reported,[26] which could also be the case in our cohort. In animal models, TNF-α antagonist treatment improves insulin resistance in obesity.[27] A clinical study, however, failed to show the same effect in humans.[28] Regarding eotaxin, this chemokine has been linked to the development of cardiovascular disease, which is likewise a complication to long-term hyperglycaemia, and our findings of increased levels in dysregulated individuals could therefore be a possible sign of atherosclerosis.[20]

Sex

We showed that the level of the chemokine MDC was associated with sex with higher levels seen in females compared with males. Different obesity-related inflammatory pathways between men and women with metabolic syndrome have previously been shown. Increased levels of proinflammatory mediators seem to facilitate low-grade systemic inflammation in males, while an insufficient anti-inflammatory milieu appears to be dominant in females.[29] These findings suggest that any inflammation-modulating therapy in obesity should be differentiated according to sex and underlying mechanisms. In our type 2 diabetes cohort, however, this pattern was not recreated, indicating that the crucial factor may be aspects related to the metabolic syndrome rather than hyperglycaemia.

Therapeutical management

Lower levels of three chemokines (IL-8, IP-10 and MDC) were all associated with DPP-4 inhibitor therapy. DPP-4

inhibitor therapy is known to improve glycaemic control via prevention of breakdown of the incretin hormone GLP-1. In addition, several cytokines and chemokines are also substrates of the DPP-4 enzyme, and DPP-4 inhibitor therapy thus possesses immunomodulating properties possibly facilitating low-grade systemic inflammation in diabetes.[9] Potentially this could explain why promising in vitro anti-inflammatory actions of DPP-4 inhibitors have failed to show convincingly results in humans.[30] Surprisingly, we found lower levels of three DPP-4 substrates (IL-8, IP-10 and MDC) in connection with DPP-4 inhibitor therapy. Though seemingly in contrast with the expected result, similar observations have previously been reported e.g. lower levels of eotaxin in type 2 diabetes during DPP-4 inhibitor therapy.[31] In our study, however, eotaxin levels were unaffected by DPP-4 inhibitor therapy, underlining the need for further research in the immunomodulating effects of these compounds.

GLP-1 receptor agonist therapy, which share the same pharmacodynamic endpoint as DPP-4 inhibitor therapy, is known to possess anti-inflammatory properties independent of improved glycaemic control.[32] However, our results showed an approximately 25% increase in proinflammatory TNF-α levels in connection with GLP-1 receptor agonist therapy. This finding is unexpected and in contrast with a previous pilot study showing that liraglutide significantly decreased TNF-α levels in a type 2 diabetes cohort.[33] Preclinical studies have likewise shown inhibitory effects of liraglutide on TNF-α expression.[34] Other preclinical studies, however, have reported decreased proinflammatory effects of TNF-α through inhibition of the NK-κB pathway after GLP-1 receptor agonist therapy.[35] If this is the case, this would neutralise the proinflammatory pathways caused by increased TNF-α levels seen in this study.

In our cohort, SGLT2 inhibitor therapy was associated with a decrease in MDC, known to facilitate and amplify type II immune response.[36] The antidiabetic effects of SGLT2 inhibitors rely on the inhibition of renal reabsorption of glucose, but anti-inflammatory effects have also been reported including attenuation of IL-6 production[37] and modulation of macrophage polarisation.[38] The prospect of using the anti-inflammatory potential of SGLT2 inhibitors in various pathologies is currently receiving much attention.[39]

Additional subgroups

Apart from obesity, hyperglycaemia and sex, other factors such as current smoking status and specific medical therapy may likewise influence the level of inflammation in type two diabetes.[40 41] In our cohort, only 5% were smokers, which is surprisingly low, giving the fact that smoking is a substantial risk factor for development of type 2 diabetes.[40] The low number of current smokers may reflect selection or reporting bias or perhaps successful free smoking cessation programmes, as 40% of our participants reported to be previous smokers. This is, however, highly speculative. Nonetheless, the degree of a persistent proinflammatory effect of nicotine following smoking cessation is debated,[42] and could potentially be influencing the results in the current study. Moreover, the high proportion of previous smokers could indicate that our cohort consisted of individuals with a high degree of determination and self-efficacy. Such selection bias is potentially also reflected in the median HbA1c of 55 mmol/mol, which is lower in comparison to other cohorts.[13 43]

In our cohort, 66% received lipid-lowering statin therapy, which is known to possess anti-inflammatory properties,[8] which again could impact the level of investigated inflammatory biomarkers. Consequently, the reported elevated levels of several biomarkers compared with the healthy control cohort could be artificially low due to the anti-inflammatory effect of statins. Potentially this could explain why no proinflammatory biomarkers were increased in individuals with longer disease duration as these individuals were more likely to be on statin therapy.

Diabetic comorbidities

It has previously been established that low-grade systemic inflammation plays a role in progression of diabetic complications.[10–12] We found that IL-6, IL-10, IL-12/IL-23p40, IL-15 and CRP were elevated in individuals with multiple diabetic comorbidities compared with those with fewer or none. In the literature, IL-6 elevation has in particular been associated with diabetic complications.[44–47] Likewise, increased levels of CRP has previously been linked to development and severity of diabetic complications.[45 48] In addition, the observed elevated levels of IL-10 were primarily found in subjects with longer disease duration, which could reflect that diabetes comorbidities typically become more prevalent with increasing exposure to glycaemic fluctuations and disease duration.[11] Furthermore, IL-12 has previously been shown to be involved in the pathogenesis of several diabetic microvascular and macrovascular comorbidities.[49] Interestingly, a study in obese and insulin-resistant IL-12 knockout mice showed that IL-12 disruption increased angiogenesis and restored peripheral blood flow perfusion through attenuation of oxidative stress and increased levels of angiogenic factors.[50] In humans, a monoclonal antibody (Ustekinumab) targeting IL-12/IL-23p40 is currently used as a safe and effective treatment of psoriasis.[51] Our data raise the intriguing possibility of applying this drug as a novel treatment option for diabetic microvascular and macrovascular complications but needs to be investigated in future randomised controlled trials. Finally, circulating levels of IL-15 have been shown to be influenced by fat mass and physical activity.[52] Furthermore, IL-15 improve lipid deposition and insulin sensitivity by activation of the GLUT-4 transporter in skeletal muscles. Hence, IL-15 has been proposed as a novel therapeutic option for treating obesity and type 2 diabetes.[53] The increased levels of IL-15 in individuals with three or more comorbidities found in this study seem to contradict the

beneficial effects normally attributed to this cytokine, but as this is a cross-sectional study no conclusions of causality can be made.

Strengths and limitations

A major limitation of this study is the cross-sectional study design, which hinders any assumptions of the predictive potential of low-grade inflammation and clinical characteristics of type 2 diabetes. On this dataset, we tested for association between low grade inflammation in type 2 diabetes, and we selected á priori the anti-inflammatory markers, as they are part of the underlying pathogenesis. According to the study design, each of the serum markers were tested individually, and based on our unadjusted and adjusted models we suggest an association to the specific marker IL-10. As the manufacturer of the multiplex assay had defined division of serum markers into cytokines (n=4), chemokines (n=6), proinflammatory cytokines (n=5), vascular injury (n=1), we believe that Bonferroni's correction is too conservative. The major strength of this study is the high degree of heterogeneity of our cohort, obtained by systematically screening all people in our outpatient diabetes clinic, thereby facilitating generalisation to the larger population of type 2 diabetes. However, selection bias in which individuals with low symptom burdens are more likely to participate cannot be ruled out. Contrary, a majority of patients with complications, who regard participation in a clinical trial as a possibility to receive extra attention from healthcare professionals, is likewise conceivable. It should also be noted that because this study is based on secondary analyses, the inclusion and exclusion criteria were not designed to exclude participants with comorbidities or medication use, which could impact the levels of the investigated inflammatory factors. Lastly, registration of retinopathy was restricted to participant recollection and reporting. Objective measures or consultation in patient records would have improved the validity of this outcome.

CONCLUSION

We showed that individuals with type 2 diabetes exhibit higher degrees of various inflammatory factors in serum, and that obesity and glycaemic dysregulation are associated with the level of specific inflammatory factors. Furthermore, a considerable increase in several inflammatory factors was seen in people with multiple diabetic comorbidities. Regarding medication, DPP-4 inhibitor therapy was associated with decreased levels of several chemokines, while increased TNF-α levels were observed in association with GLP-1 receptor agonist therapy. Taken together, our results show that individuals with type 2 diabetes have systemic low-grade inflammation. Although the cross-sectional nature of our study hinders the ability to look at the causality between systemic low-grade inflammation and diabetic complications, it is intriguing to speculate whether dampening of the inflammatory state could protect against development of comorbidities in type 2 diabetes.

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