Evaluation of molecular profiling platforms in clinical pharmacology

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CHAPTER 4

Evaluation of monocyte chemoattractant protein 1 and tumor necrosis factor alpha as biomarkers for rapid pharmacological effects of fibrates in type 2 Diabetes Mellitus patients and healthy volunteers

SUBMITTED

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Abstract

We investigated the usefulness of monocyte chemoattractant protein 1 (MCP-1) and tumour necrosis factor alpha (TNFα) plasma concentrations as non-lipid 'mechanism-based' biomarkers for the rapid pharmacological effects of peroxisome proliferator activated receptor alpha (PPARα) agonists in early clinical 'proof of concept studies' with type 2 Diabetes Mellitus (T2DM) patients and healthy volunteers (HVs). In addition, we investigated whether ciprofibrate treatment affected the spontaneous and *ex vivo* stimulated whole-blood secretion of these markers. Effects on MCP-1 and TNFα plasma concentrations were compared with effects on traditional lipid / (apo)lipoprotein measures in T2DM patients and HVs receiving ciprofibrate 100 mg or placebo once daily for 3 weeks. *Ex vivo* whole-blood stimulation experiments were performed with lipopolysacharide (LPS) and C-reactive protein (CRP). We did not detect a significant reduction in MCP-1 and TNFα plasma concentrations after 3 weeks treatment with ciprofibrate. However, ciprofibrate treatment did significantly decrease the *ex vivo* whole blood unstimulated (-35%; 95%CI: -55, -4%), LPS (-38%; 95%CI: -56, -12%) and CRP (-39%; 95%CI: -58, -12%) stimulated MCP-1 release in HVs. TNFα release was unaffected by ciprofibrate treatment in T2DM patients and HVs. MCP-1 and TNFα are unsuitable as 'mechanism-based' biomarkers in small clinical 'proof on concept' studies with novel PPARα agonist-class drug candidates. Nonetheless, ciprofibrate decreased both the unstimulated, and the LPS and CRP stimulated MCP-1 release in whole blood in HVs, which suggests a possible role in the modulation of atherosclerosis and inflammation by PPARα agonists in general.

Introduction

Type 2 Diabetes Mellitus (T2DM) is a multifactorial, heterogeneous disorder characterized by impaired insulin secretion on a background of insulin resistance and is typically accompanied by a high triglyceride (TG) - low high density lipoprotein-cholesterol (HDL-c) dyslipidemia [1]. Furthermore, many clinical studies have shown that elevated plasma concentrations of pro-inflammatory cytokines and other markers of inflammation may play an important role in the development of T2DM [2-4]. This dyslipidemic, pro-inflammatory state is a common feature of the altered risk profile in T2DM [5-7], and is associated with a highly accelerated rate of atherogenesis and increased cardiovascular disease (CVD) risk [8-11].

Monocyte homing and infiltration in the subendothelial space of the artery is an important step in the formation of the atherosclerotic plaque [12]. Several pro-inflammatory cytokines and chemokines are directly or indirectly involved in this process.

More specifically, cytokines like tumour necrosis factor alpha (TNFα) and interleukin 1-beta (IL1ß) have been reported to initiate a complex cascade of events ultimately leading to the phosphorylation and degradation of the inhibitor of nuclear factor kappa B (I-κB) and subsequent activation of nuclear factor kappa B (NF-κB) [13]. This in turn will lead to the nuclear transcription of several genes, including those coding for TNFα and IL-1 themselves, as well as those coding for adhesion molecules like vascular cell adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1) in endothelial cells. The latter are molecules that facilitate the adhesion and penetration of mononuclear cells into the arterial wall [14].

Furthermore, these cytokines can induce various other pro-inflammatory molecules, including chemokine-class molecules like monocyte chemoattractant protein 1 (MCP-1) [15]. Chemokines are small chemoattractive molecules responsible for monocyte and T-lymphocyte recruitment and activation [16] and are highly expressed in macrophage-rich areas in human atheromatous plaques [17-19].

A central mediator in these inflammatory processes appears to be C-reactive protein (CRP), which has been shown to induce both types of inflammatory mediators (i.e. cytokines and chemokines) in several types of cells including endothelial cells [20], aortic smooth muscle cells [21], alveolar macrophages [22] and peripheral blood mononuclear cells (PBMCs) [23-25].

Large clinical studies have shown that fibric acid derivatives (fibrates), which are peroxisome proliferator activated receptor alpha (PPARα) agonists, can reduce CVD risk while inducing only marginal improvements in lipid profile (i.e. the Veterans Affairs HDL Intervention trial – VA-HIT [26;27]), whereas other studies (i.e. Bezafibrate Infarction Prevention study – BIP) have found robust changes in lipid profile (14% increase in HDL-c) without significant reductions in CVD risk [28]. Therefore, it appears that the magnitude of traditionally evaluated changes in lipid / (apo)lipoprotein measures (e.g. TG and HDL-c) is not necessarily relevant for the success of fibrate therapy in reducing CVD risk [29]. This is with the note that the outcome of the BIP study may be particular for bezafibrate, since this fibrate has recently been shown to be a pan-PPAR agonist with a relatively weak PPARα agonist action [30]. Assuming the effects of fibrates are class-specific and mediated through

PPARα, it may be hypothesised that besides lipid lowering, other PPARα-mediated effects (i.e. anti-inflammatory effects) contribute to the observed beneficial effects on CVD risk.

Fibrates have been reported to decrease (amongst others) the plasma concentrations and (CRP-stimulated) production of TNFα and MCP-1 in several cell types including peripheral blood mononuclear cells [31-36], presumably at least in part through inhibition of the NF-κB and activator protein-1 pathways [37]. Therefore, the anti-inflammatory effects of fibrates are proposed to provide complementary, rapid responding 'mechanism-based' biomarkers for the action of PPARα agonists *in vivo* that are potentially more informative than the effects on lipid variables alone.

Consequently, these non-lipid biomarkers could serve as important effect parameters in early clinical 'proof of concept' studies with novel PPARα agonists. Ideally, and assuming elevated levels of these inflammation markers are not required to detect a significant decrease after fibrate treatment, these variables may also be useful biomarkers in small groups of (more easily studied and recruited) healthy volunteers (HVs). Hence, studying the effects of an established PPARα agonist in T2DM patients and HVs on candidate biomarkers that are more closely related to the alleged beneficial mechanisms of drug action, could provide additional, and possibly better decision-making tools for the early clinical evaluation of novel PPARα agonists-class drug candidates.

Therefore, in the present study our primary objective was to investigate whether we could detect significant changes in TNFα and MCP-1 plasma concentrations in T2DM patients and HVs following short-term (3-week) ciprofibrate treatment *vs.* placebo (ciprofibrate being considered as 'prototype' for the PPARα agonist-class drugs). In parallel, and for comparison with the anticipated anti-inflammatory effects, we investigated the effects of ciprofibrate treatment on typical parameters of lipid metabolism, including several (apo)lipoproteins.

As secondary objective, and in order to verify and corroborate previous observations which indicated that PBMCs are target cells for fibrate treatment in humans [38;39], we sought to assess if the action of ciprofibrate on whole-blood PBMCs contributes to its anticipated effects on circulating plasma concentrations of TNFα and MCP-1.

Methods

Patients

Eight male and eight female patients with an established diagnosis of T2DM (i.e. increased fasting plasma glucose (FPG) concentrations >7.0 mm) uncontrolled by diet alone, aged between 18 and 75 years and fasting plasma TG concentrations > 1.5 mm (>133 mg/dl), were included. Patients were excluded if they had a significant medical history or current symptoms of clinically relevant conditions, or had used any NSAID, PPAR agonist (α,ß/∂ or γ) or lipid lowering drugs within two weeks of the expected study start date.

In addition, eight male and eight female HVs (as determined by medical history, physical examination and routine laboratory tests), aged between 18 and 45 years, were included.

Study design

This was a randomized, double blind, placebo-controlled, repeated oral dose study. The study participants were studied in a four-week period consisting of six Visits (figure 1; p. 205). Within one week after medical screening all subjects started with a one-week single blind placebo run-in period. At the end of the run-in period (baseline) subjects were randomly assigned to a three-week treatment with capsules containing 100 mg ciprofibrate or matching placebo once daily. Treatment allocation took place according to randomly permuted blocks and was stratified by gender and subject type (T2DM patient or HV).

Blood samples for ciprofibrate trough concentrations and lipid parameters were collected on all Visits. Samples for the plasma MCP-1 and TNFα concentrations and *ex vivo* whole-blood stimulation experiments were collected at baseline and after 3 weeks of active treatment. At baseline, four blood samples were collected (at 1, 3, 6 and 10 hrs post-dose) for ciprofibrate pharmacokinetic assessments. Throughout the study, blood and urine samples were collected for standard clinical (safety) laboratory measurements (including urine human chorionic gonadotropin test for female subjects). Moreover, frequent measurements of vital signs (heart rate and blood pressure) were performed.

The protocol for this study was approved by the Medical Ethical Committee of the Leiden University Medical Centre and performed according to the principles of ICH-GCP, the Helsinki Declaration and

Dutch law. All subjects gave their written informed consent. This study was part of a larger study in which comprehensive transcriptomic and metabolomic analyses were performed. The results of these analyses will be published separately.

Blood sampling

On each Visit, an intravenous cannula was inserted in a forearm vein while the subject was in a supine position. Blood samples were collected after approximately 30 min of supine rest.

CHOLESTEROL AND TRIGLYCERIDES

Blood samples of 8.5 ml were collected in SST® Gel and Clot Activator tubes for measurements of total cholesterol (TC), TG, HDL-C and fasting plasma glucose (FPG). TC, TG and FPG concentrations were analyzed on a fully automated Hitachi 747 apparatus. HDL-C was measured using a Hitachi 911 apparatus. Low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald equation [40] [LDL-C (mmol/l) = TC - (HDL-C - (TG/2.2)].

APOLIPOPROTEINS

Blood samples were collected for apolipoproteins-B, -CII, -CIII, and Lipoprotein (a) in 8.5 ml SST® Gel and Clot Activator tubes and centrifuged at 4° C (10 minutes at 2000 g) within 30 min and stored at -80° C. Plasma apolipoproteins-B, CII and CIII concentrations were determined on a Roche Modular analytics system using a turbidimetric assay. Plasma Lp(a) concentrations were measured using a high sensitivity ELISA (Biopool AB, Umeå, Sweden). The detection limit for apo-B was 0.3 g/l with an interassay coefficient of variation of 2.2% at a level of 0.9 g/l and 2.7% at a level of 1.8 g/l. The detection limit for apo-CII was 0.5 g/l, with an interassay coefficient of variation of 10% at a level of 300 mg/l. The detection limit for apo-CIII was 0.2 g/l with, an interassay coefficient of variation of 5.0% at a level of 75 mg/l. The detection limit for Lp(a) was 9.0 mg/l, with an interassay coefficient of variation of 5.0% at a level of 337 mg/l.

FREE FATTY ACIDS (FFA)

Blood samples were collected in 2 ml EDTA tubes, immediately stored on ice-water, centrifuged within 30 min at 4° C (10 minutes at 2000 g) and plasma was stored at -80° C until analysis. FFA concentrations were determined using an enzymatic colorimetric assay (Roche Diagnostics GmbH, Mannheim, Germany) with a detection limit of 0.01 mmol/l and an interassay coefficient of variation of 2.0% at a level of 0.63 mmol/l and 6.1% at a level of 0.48 mmol/l.

MCP-1 AND TNFα MEASUREMENTS

Blood samples were collected in 10 ml EDTA tubes, centrifuged within 30 minutes at room temperature (10 minutes at 2000 g) and plasma was stored at -80° C until analysis. TNFα and MCP-1 concentrations were measured using commercially available high sensitivity ELISAS of R&D Systems (Abington, UK) according to the manufacturer's instructions.

In addition, blood samples for the *in vitro* stimulation tests were collected in 10 ml pyrogen free EDTA tubes and kept on 37° C. The whole-blood stimulation experiment was started within 60 minutes after blood collection. Twenty ml of blood was stimulated with lipopolysacharide (LPS; L-3012, Sigma, St. Louis, USA; 5 ng/ml) and 20 ml blood was stimulated with CRP (Trichem Resources, West Chester, USA; 10 µg/ml) in the presence of polymyxin B (5 µg/l). In addition, twenty ml of blood was incubated without intended stimulator. The blood volume was mixed with an equal volume of Hepes buffered RPMI-1640 culture medium. The whole-blood stimulation test was performed for 24 hrs at 37° C under 5% CO_2 airflow. After incubation, the EDTA blood and medium mixture was centrifuged at room temperature for 10 minutes at 1000 g. The supernatant was collected and stored in samples at -80° C. TNFα and MCP-1 concentrations in the supernatant were determined using commercially available assays as mentioned before.

CIPROFIBRATE BIOANALYSIS

Blood samples were collected in 5 ml sodium heparin tubes, immediately stored on ice-water, centrifuged at 4° C within 30 minutes (10 minutes at 2000g) and stored at -20° C. The samples were analyzed for ciprofibrate by a validated high-performance single quadrupole-liquid chromatographic mass spectrometric (LC-MS) procedure (MDS Pharma Services, St-Laurent (Montreal), Canada) with a lower limit of quantitation of 0.25 mg/ml and a coefficient of variation ≤ 5.40 %.

Compliance monitoring

Study medication was delivered to the subjects in vials with automated recording of the time of vial opening (Aardex® electronic drug exposure monitor (eDEM™)). Registered opening times and capsule counting

were used for monitoring of subject compliance, and as input for modeling ciprofibrate drug effects. In addition, ciprofibrate trough concentrations were measured to support compliance monitoring.

Statistical analysis

INCLUDED DATA

The efficacy population was structured as follows: T2DM ciprofibrate group n=8, T2DM placebo group n=8, HVs ciprofibrate group n=10 (including two replacements), HVs placebo group n=8. Data of drop-outs was included in the analysis, even when a subject was replaced.

CIPROFIBRATE EXPOSURE

Ciprofibrate has a long half-life and exhibited accumulation up to the final Visit. For this reason, log-transformed ciprofibrate data at Visit 5 (3 weeks of active treatment) were compared between the T2DM and HVs groups using an unpaired t-test.

PHARMACODYNAMICS

All variables were log transformed to meet requirements for the analysis of variance (ANOVA). The lipid profile variables were analysed using a repeated measures mixed effect model, with group, treatment, gender, Visit, Visit by treatment, group by treatment, Visit by group by treatment as factors and baseline measurement as covariate, using an unstructured covariance (ANCOVA) matrix. The MCP-1 and TNFα variables were analysed using analysis of co-variance with group, treatment, gender and group by treatment as factors and baseline measurement (Visit 2) as covariate.

Groups (T2DM *vs.* HVs) were compared at baseline using ANOVA on the log-transformed data, with treatment as factor.

COMPLIANCE

The percentage of the incorrect number of capsules taken per study was compared between groups. Because the data were not normally distributed, the Wilcoxon two-sample test was used. All calculations were performed using SAS for Windows V8.2 (SAS Institute, Inc., Cary, NC).

MONOCYTE *EX VIVO* EXPERIMENT

To investigate whether the values differed after different types of stimulation, analysis of pre-values (Visit 2, after two weeks placebo run-in) was done, with a repeated measures mixed effect model, repeated over stimulation type, subject as random factor and stimulation type, group (T2DM patients and HVs) and group by stimulation type as fixed factors.

To investigate the treatment effect with stimulation type as a factor, log transformed data was analysed with a repeated mixed effect model, repeated over stimulation type, with subject as random factor and treatment, group, stimulation type, sex, group by stimulation type, group by treatment, treatment by stimulation type and group by treatment by stimulation type as fixed factors.

All calculations were performed using SAS for Windows V9.1.2 (SAS Institute, Inc., Cary, NC).

Results

Subjects

Two female subjects from the HVs (ciprofibrate) group withdrew consent for personal reasons and were replaced with two newly recruited healthy female volunteers. No clinically significant changes in routine laboratory parameters and vital signs were observed.

COMPLIANCE AND CIPROFIBRATE EXPOSURE

Mean overall compliance was similar in both patient (99.7% of prescribed dose) and HVs group (97% of prescribed dose). There was no significant difference in the mean number of capsules taken during the study between the T2DM and HVs group.

There were no significant differences in ciprofibrate trough concentrations between the T2DM (39.5 µg/ml; 95%CI: 33.6, -46.4 µg/ml) and HVs group (41.9 (g/ml; 95%CI: 32.5, -51.7 (g/ml) after 3 weeks of active treatment.

BASELINE CHARACTERISTICS

The demographics of both study populations are presented in table 1.

Baseline group comparisons of pharmacodynamic variables

As shown in table 2, mean baseline FPG, TC, TG, LDL-C, FFA, apolipoprotein B, apolipoprotein CII, apolipoprotein CIII and MCP-1 concentrations were significantly higher in the T2DM patients *vs.* HVs group. There were no significant differences between the T2DM patients and HVs group in mean HDL-C, lipoprotein (a) and TNFα concentrations.

TABLE 1 **Demographics.** Overview of the demographics of both study populations as measured at pre-study medical screening. The data between parentheses represent the standard deviation. N.A.: not applicable. T2DM: type 2 Diabetes Mellitus; HVs: healthy volunteers; OAD: oral anti-diabetic drug.

Parameter	T2DM (n=16)	HVs (n=18)
Age (years)	59 (7.8)	27 (7.8)
Body Mass Index (kg/m²)	30.4 (5.22)	24.0 (2.62)
Waist/Hip ratio	1.0 (0.07)	0.8 (0.05)
Disease duration (years)	6 (4.2)	N.A.
Current treatment: No medication	N= 1	N= 18
OAD Monotherapy	N= 8	N.A.
OAD Combination	N= 3	N.A.
Insulin	N= 2	N.A.
OAD and Insulin	N= 2	N.A.
Antihypertensive	N= 6	N.A.
Heart rate (beats per minute)	74 (14.5)	71 (10.2)
Systolic blood pressure (mm Hg)	145 (19.0)	116 (12.9)
Diastolic blood pressure (mm Hg)	82 (8.5)	70 (10.0)

TABLE 2 **Baseline group comparisons.** Overview of the back transformed least square means (LSMs) as well as the % difference in mean baseline lipid /(apo)lipoprotein and candidate marker concentrations between the T2DM patient and HVs groups with corresponding p-values and 95% confidence intervals (ANOVA; log-transformed data). Baseline concentrations were measured after the one-week placebo run-in period.

	Back transformed LSMs				95% CI (%)	
Parameter	**Diabetic (n=16)**	**Healthy (n=18)**	**p-value**	**Estimate of difference (%)**	**Lower**	**Upper**
Glucose (mmol/l)	9.1	4.7	<0.0001	91.1	68.4	117.0
Cholesterol (mmol/l)	5.5	4.0	0.0003	35.9	16.4	58.6
Triglycerides (mmol/l)	2.2	0.9	<0.0001	133.8	78.2	206.7
HDL-cholesterol (mmol/l)	1.1	1.3	0.1259	-11.1	-23.8	3.6
LDL-cholesterol (mmol/l)	3.2	2.2	0.0061	42.3	11.4	81.8
Free Fatty Acids (mmol/l)	0.49	0.35	0.0129	41.6	8.2	85.4
Apolipoprotein B (mmol/l)	1.02	0.68	0.0007	50.5	20.5	88.0
Apolipoprotein CII (mmol/l)	54	23	<0.0001	133.0	61.9	235.5
Apolipoprotein CIII (mmol/l)	102	80	0.0045	26.9	8.3	48.7
Lipoprotein (a) (mmol/l)	60	56	0.8616	8.9	-59.6	193.3
TNFα (pg/ml)	0.97	0.84	0.1526	15.7	-5.5	41.7
MCP-1 (pg/ml)	182	146	0.0079	24.9	6.5	46.5

Overall treatment effects

LIPIDS AND (APO)LIPOPROTEINS

As shown in table 3, in the T2DM patients group there was a significant decrease in TC, TG, apolipoprotein B and apolipoprotein CIII plasma concentrations after 3 weeks of treatment with ciprofibrate *vs.* placebo in the T2DM group. There was no significant change in HDL-c, LDL-c, apolipoprotein-CII, Lipoprotein(a), FFA, and FPG. In the HVs group, there was a significant decrease in mean TC, HDL-c, FFA, apolipoprotein-B and apolipoprotein-CIII (table 4). There was no significant change in mean TG, LDL-c, apolipoprotein-CII, Lipoprotein(a), and FPG after 3 weeks of treatment with ciprofibrate *vs.* placebo (table 4).

TNFα AND MCP-1

Although there was a significantly higher mean MCP-1 concentration in the T2DM *vs.* HVs group at baseline (table 2), we could not detect a significant decrease in the mean MCP-1 plasma concentrations after 3 weeks of ciprofibrate treatment *vs.* placebo in the T2DM patients and HVs group (tables 3 and 4). In addition, there was no significant treatment-induced change in the mean TNFα plasma concentration in either group (tables 3 and 4).

TABLE 3 **Ciprofibrate treatment effects in T2DM group.** Overview of the back transformed least square means for ciprofibrate and placebo treatments as well as the average (overall) % change in effect parameters for ciprofibrate *vs.* placebo in the T2DM group with corresponding p-values and 95% confidence intervals (ANCOVA; log transformed data).

T2DM patients group : Ciprofibrate *vs.* placebo (n=16)			95% CI (%)	
Parameter	**p-value**	**Estimate of difference (%)**	**Lower**	**Upper**
Glucose (mmol/l)	0.9620	0.2	-8.6	10.3
Cholesterol (mmol/l)	0.0061	-13.6	-22.3	-4.5
Triglycerides (mmol/l)	0.0004	-30.5	-42.3	-16.2
HDL-cholesterol (mmol/l)	0.7756	1.1	-6.7	9.6
LDL-cholesterol (mmol/l)	0.1327	-13.0	-27.6	4.6
Free Fatty Acids (mmol/l)	0.1541	-18.7	-39.2	8.7
Apolipoprotein B (mmol/l)	0.0123	-16.1	-26.7	-4.0
Apolipoprotein CII (mmol/l)	0.5844	-11.9	-44.8	40
Apolipoprotein CIII (mmol/l)	0.0007	-17.7	-25.9	-8.6
Lipoproteine (a) (mmol/l)	0.6563	8.0	-24.0	53.5
TNFα (pg/ml)	0.3372	8.2	-8.4	27.9
MCP-1 (pg/ml)	0.7835	2.5	-14.9	23.5

Ex vivo whole-blood stimulation experiments

BASELINE GROUP COMPARISONS

The mean unstimulated MCP-1 concentration (cytokine concentration after 24h incubation without stimulating agent) was non-significantly higher in the T2DM *vs.* HVs group (15%; 95%CI: -10, 48%, figure 2a; p.206).

TABLE 4 Ciprofibrate treatment effects in HVs group. Overview of the back transformed least square means for ciprofibrate and placebo treatments as well as the average (overall) % change in effect parameters for ciprofibrate *vs.* placebo in the HVs group with corresponding p-values and 95% confidence intervals (ANCOVA; log transformed data).

Healthy volunteers group: Ciprofibrate *vs.* placebo (n=18)			95% CI (%)	
Parameter	**p-value**	**Estimate of difference (%)**	**Lower**	**Upper**
Glucose (mmol/l)	0.9620	0.2	-8.2	9.8
Cholesterol (mmol/l)	0.0226	-11.1	-19.5	-1.8
Triglycerides (mmol/l)	0.0636	-14.8	-28.1	1.0
HDL-cholesterol (mmol/l)	0.0049	-10.6	-17.4	-3.7
LDL-cholesterol (mmol/l)	0.1395	-12.1	-26.1	4.6
Free Fatty Acids (mmol/l)	0.0304	-28.6	-47.3	-3.4
Apolipoprotein B (mmol/l)	0.0397	-12.8	-23.5	-0.7
Apolipoprotein CII (mmol/l)	0.9853	0.4	-35.9	57.3
Apolipoprotein CIII (mmol/l)	0.0008	-17.0	-24.9	-8.2
Lipoprotein (a) (mmol/l)	0.8233	3.5	-24.3	41.6
TNFα (pg/ml)	0.4523	-6.1	-20.8	11.3
MCP-1(pg/ml)	0.5758	-5.1	-21.4	14.7

After 24h incubation (stimulation) with either LPS or CRP, there was a significant increase in mean MCP-1 concentration compared to no stimulation in the HVs group (24%; 95%CI: 11, 37% and 17%; 95%CI: 5, 30%, respectively; figure 2a; p. 206). No significant increase in LPS or CRP stimulated mean MCP-1 concentration *vs.* no stimulation was observed in the T2DM group (3%; 95%CI: -7, 14% and 4%; 95%CI: -7, 15%, respectively; figure 2a; p. 206).

In the T2DM group there was a significant increase in mean TNFα concentration after LPS and CRP stimulation when compared with no stimulation (134%; 95%CI: 66, 229% and 87%; 95%CI: 30, 171%, respectively; figure 2b; p. 206). A similar increase in TNFα concentration after LPS and CRP stimulation was also observed in the HVs group (222%; 95%CI: 129, 354% and 201%; 95%CI: 108, 335%, respectively; figure 2b; p. 206). The increase in TNFα secretion was significantly lower for CRP stimulation (-42%; 95%CI: -62, -11%) and borderline significantly lower for LPS stimulation (-32%; 95%CI: -55, 2%) in the T2DM group compared to the HVs.

TREATMENT EFFECTS

In the T2DM patients group there was a non-significant decrease in the unstimulated, LPS and CRP stimulated MCP-1 secretion (-22%; 95%CI: -46, 11% and -13%; 95%CI: -37, 20% and -14%; 95%CI: -39, 23%, respectively), after 3 weeks of ciprofibrate treatment *vs.* placebo (figure 3a; p. 207). In addition, there was a significant decrease in the unstimulated, LPS and CRP stimulated MCP-1 secretion in the HVs ciprofibrate treatment group *vs.* placebo (-35%; 95%CI: -55, -4% and -38%; 95%CI: -56, -12% and -39%; 95%CI: -58, -12%, respectively) (figure 3b; p. 207).

There was no significant effect of ciprofibrate treatment *vs.* placebo on the unstimulated, LPS or CRP stimulated mean TNFα concentration in the T2DM group (17%; 95%CI: -17, 65% and 13%; 95%CI: -29, 79% and 9%; 95%CI: -27, 62%, respectively) nor in the HVs group (-2%; 95%CI: -31, 38% and -16.3%; 95%CI: -47, 33% and -25%; 95%CI: -49, 11%, respectively).

Discussion

The main finding of this study was that we could not detect a significant decrease in mean MCP-1 or TNFα plasma concentration after three weeks of ciprofibrate *vs.* placebo treatment in neither T2DM patients nor HVs (tables 3 and 4). However, we did observe a significant treatment induced decrease in (unstimulated, LPS or CRP stimulated) MCP-1 production in whole blood of HVs (figure 3b; p. 207). Furthermore, there was virtually no treatment induced change in mean TNFα whole-blood production in either study group.

The strength of this randomized placebo-controlled study lies within its key research questions: 'Is it possible to find (rapidly responding) non-lipid biomarkers for action of ciprofibrate that are more closely related to the pharmacological effects on the pathophysiology of atherosclerosis in T2DM patients that also respond in small groups of HVs?', and, 'Do whole-blood resident cytokine/chemokine-secreting cells contribute to the anticipated decrease in plasma levels of these markers?'. Finding novel 'mechanism-based' markers and increasing our knowledge of potential targets and mechanisms of PPARα agonist action would be very helpful to expedite the (early clinical) development of novel fibrate-class drug candidates.

In this study we observed a significant effect on most of the lipid profile variables in the T2DM patients and HVs groups after short-term (3-week) treatment with ciprofibrate *vs.* placebo (tables 3 and 4, respectively). However, we could not detect a significant change in mean MCP-1 or TNFα concentrations in either the T2DM patients or HVs group. This could be related to the typical Phase I study design, which usually entails a relatively short-term treatment period and limited number of subjects. We chose this relatively short treatment

period since the primary objective of this study was to identify new, 'mechanism-based', non-lipid measures that would rapidly respond to ciprofibrate treatment, analogous to the traditionally used lipid / (apo)lipoprotein markers.
Although we could not detect significant changes in mean MCP-1 and TNFα concentrations in either study group, the *ex vivo* experiments revealed that ciprofibrate *vs.* placebo treatment induced a decrease in unstimulated, CRP and LPS stimulated whole-blood MCP-1 release in HVs (significant) and T2DM patients (non-significant; figure 3; p. 207). The fact that we did not observe significant changes in the T2DM patients group may be related to more (disease related) variability and a sub-optimal (attenuated) response to the stimulation tests in the T2DM group.

In fact, the results of the baseline comparisons indicated that whole blood PBMCs of T2DM patients released significantly less MCP-1 than their HVs counterparts after stimulation with either LPS or in the more physiological stimulation with CRP. Actually, there was no significant increase in MCP-1 secretion after stimulation with LPS or CRP *vs.* no stimulation in the T2DM group at baseline (figure 2a; p. 206). A similar pattern of attenuated cytokine secretion was observed for TNFα when comparing the T2DM patients with HVs at baseline (figure 2b; p. 206). Nonetheless, a significant increase in TNFα concentration after either LPS or CRP stimulation *vs.* no stimulation in the T2DM group was reached at the chosen LPS and CRP concentrations.

The CRP and LPS concentrations used in this study were chosen based on a previous pilot study (unpublished data) that showed a satisfactory, clear response (i.e. TNFα and MCP-1 release) in healthy volunteers after LPS or CRP stimulation at the concentrations 5 ng/ml and 10 μg/ml, respectively. In addition, since previous studies showed that PBMCs of patients with mixed dyslipidemia or impaired glucose tolerance produced significantly more TNFα and MCP-1 when compared with matched healthy controls [41;42], the current design of the whole-blood stimulation experiments at the chosen concentrations in our T2DM patients group was expected to be appropriate.

Our baseline findings could be explained by *in vivo* adaptation (i.e. blunted response) of cytokine/chemokine secreting cells that are residing in a pro-inflammatory environment, which could be greatly amplified when progressing from a glucose intolerant to a full blown T2DM state (as reflected by the elevated MCP-1 concentrations; table 2). On the other hand, as the T2DM patients and HVs in our study were not matched for age we cannot exclude that these findings may be partly attributable to differences in age. We chose to study unmatched groups since the primary objective of the study was to identify treatment responsive biomarkers in T2DM patients that would also respond in more easily recruited healthy volunteers, who are usually between 18 and 30 years of age. Therefore, to rule out any confounding effects of age-related differences in cytokine/chemokine release, further studies with matched controls must be performed to verify our findings.

In this study we chose to analyse cytokine production in whole-blood samples. This precludes the identification of specific cells (i.e. peripheral blood monocytes and T-lymphocytes) that are the major sources of MCP-1 and TNFα production and also the identification of any potential interaction between the cellular components of whole blood. However, this study design has been used previously by other groups [43;44], and the results for cytokine production are in good agreement with studies in cell-culture experiments[45]. An advantage of this method is that the measurement of cytokine/chemokine secretion can take place in an environment in which these cellular intermediaries are produced [46].

Notably, recently some issues have arisen with regard to the use of purified commercial CRP (CCRP) as stimulatory agent in the *ex vivo* experiments. We chose to stimulate the whole-blood samples not only with LPS, but also with CRP since we believe CRP represents a more physiological stimulus. In healthy volunteers, the median concentration of CRP is 0.8 mg/l; the 90th percentile is 3 mg/l [47]. There is no absolute concentration threshold for CRP to predict future CVD in patients with angina, but ≥ 8.7 μg/ml appeared to be a reliable reference value for predicting future coronary heart events [48]. Consequently, in our study the concentration of 10 μg/ml that was used as triggering stimulus for the release of MCP-1 and TNFα from whole-blood resident immune cells was considered to be in the clinically relevant range. However, a recent report by Taylor et al. suggests that the stimulatory effects of CRP on cytokine secretion (by endothelial cells) may relate to low molecular weight contaminants in CCRP preparations, and thus are not genuine CRP effects [49]. We performed the CRP stimulation experiments in the presence of polymyxin B in order to eradicate possible bacterial contaminants in the CCRP preparation.

In summary, our results indicate that MCP-1 and TNFα levels did not change significantly after a short-term intervention with the PPARα agonist ciprofibrate when compared with placebo treatment. Consequently, these markers are unsuitable as 'mechanism-based' biomarkers for the action of PPARα-agonists in early clinical 'proof

of concept' studies. Although we did not observe changes in cytokine/chemokine plasma levels, the treatment induced decreased MCP-1 release of PBMCs (after stimulation with a clinically relevant concentration of CRP) in HVs, shows that PBMCs are targets for ciprofibrate action (in HVs) and possibly PPARα-agonists in general. Finally, the attenuated MCP-1 and TNFα release after LPS and CRP stimulation in the T2DM patients *vs.* HVs may represent a specific T2DM trait, and needs to be taken into account when performing future *ex vivo* whole-blood stimulation experiments in T2DM patients. In addition, to rule out the confounding effects of age-related differences in cytokine/chemokine release, further studies with matched controls need to be performed to verify if these are in fact disease related findings.

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