Evaluation of molecular profiling platforms in clinical pharmacology

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SECTION 2

Evaluation of ‘traditional’ versus ‘non-traditional’ biochemical markers in clinical pharmacology

CHAPTER 3

Evaluation of pro-inflammatory cytokines and inflammation markers as biomarkers for the action of thiazolidinediones in type 2 Diabetes Mellitus patients and healthy volunteers

BR J CLIN PHARMACOL 2006; 62:4 391-402

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Abstract

Thiazolidinediones (TZDs) not only enhance cellular glucose transport but are also reported to have potent anti-inflammatory effects. These effects may play an important role in the insulin sensitizing mechanism, and possibly precede the effects on parameters of glucoregulation. We sought to investigate whether these anti-inflammatory effects could yield early responding biomarkers for TZD action in type 2 Diabetes Mellitus (T2DM) patients and healthy volunteers (HVs) that could eventually be used to expedite the early clinical development of novel compounds. We investigated the timing of treatment effects on several pro-inflammatory cytokines and markers of inflammation in comparison with effects on typical measures of glucoregulation in T2DM patients and HVs receiving rosiglitazone 4 mg or placebo twice daily for 6 weeks. We found a significant reduction in Interleukin-6 (-39.4% CI (-60.0, -8.2)) and white blood cell count (-18.4% CI (-30.2, -4.5) after 4 weeks of treatment in the T2DM group. These anti-inflammatory effects did not precede the effects on typical parameters of glucoregulation in the T2DM group and there was no significant anti-inflammatory response in the HVS group. We conclude that we could not identify novel plasma markers that respond earlier to treatment with rosiglitazone than typical parameters of glucoregulation or that have a significant response in HVs. However, the Interleukin-6 response observed in this study indicates a potential role for this cytokine as complementary biomarker in clinical 'proof of concept' studies with novel TZDs.

Introduction

Type 2 Diabetes Mellitus (T2DM) is a heterogeneous disorder characterized by impaired insulin secretion on a background of insulin resistance and is associated with a marked increase in cardiovascular disease (CVD) risk [1, 2].

A growing body of evidence suggests that chronic sub-clinical inflammation may play an important role in the pathogenesis of insulin resistance, T2DM and CVD [3-13]. This is illustrated by recent studies, which showed that several markers of inflammation, in particular plasma levels of high sensitivity C-reactive protein (HS-CRP) and Interleukin-6 (IL-6), are independent predictors of T2DM and CVD risk [6, 8, 10]. Moreover, elevated plasma concentrations of pro-inflammatory cytokines secreted by adipose tissue ('adipokines')- tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1ß) and IL-6 - appear to be associated with insulin resistance and T2DM [14-16].

Thiazolidinediones (TZDs), such as rosiglitazone (RSG), are a class of oral antidiabetic drugs that primarily act as insulin sensitizers, ameliorating insulin resistance with associated improvements in glycaemic control [17, 18]. These drugs are agonists of the peroxisome proliferator activated receptor gamma (PPARγ) which is a nuclear transcription factor controlling the expression of its target genes in various tissues [19,20]. Importantly, results from *in vitro* [21, 22], pre-clinical [23] and recent clinical [24-28] studies suggest that TZDs not only exert their actions by enhancing cellular glucose transport but also possess distinct anti-inflammatory properties. These properties may play an important role in the insulin sensitizing mechanisms and hold the promise of reduced CVD risk. In addition, Xiang *et al.* recently showed the potentially important role of troglitazone, and possibly of other TZDs, in the prevention of T2DM in a population at risk. Furthermore, they demonstrated that amelioration of insulin resistance could preserve pancreatic ß-cell function and stabilize glycaemia at the time T2DM develops [29]. These properties may potentially turn the TZDs into the first class of disease modifying drugs for the treatment of T2DM. However, the limited glucose lowering efficacy (about 20% maximum decrease in fasting plasma glucose (FPG) at the highest approved dose) and side-effect profile (mainly weight gain and fluid retention) limit the use of currently available TZDs. Therefore, novel TZD compounds with enhanced glucose lowering efficacy and potentially less side-effects were created.

To facilitate a more efficient early clinical development program ('proof of concept') for these compounds, there is a high demand for a large array of early responding *in vivo* biomarkers, that are easily assessable in small groups of subjects and are more closely related to the insulin sensitizing mechanism. The traditional FPG concen-tration is a fairly non-specific biomarker, and generally takes about 6-12 weeks to reach its lowest concentration for selected TZDs [30, 31]. In contrast, recent evidence suggests that the potent anti-inflammatory effects of TZDs may play an important role in the insulin sensitizing mechanism [27], and might therefore precede the effects on the FPG. An additional advantage may be that the anti-inflammatory effects, as previously reported for adiponectin [32], may also be observed in healthy volunteers. Hence, studying novel TZD compounds in relatively small groups of healthy volunteers using a broad array of early responding, mechanistically more closely related biomarkers, could circumvent the need for longer and more complicated studies in T2DM patients.

The primary objective of this study was to investigate the timing of treatment effects on several pro-inflammatory cytokines (IL-6, IL-1ß, TNFα) and markers of inflammation (HS-CRP and white blood cell count [WBC]) in comparison with effects on typical measures of glucoregulation in a small group of T2DM patients receiving RSG 4 mg b.i.d. for 6 weeks. As secondary objective we sought to investigate whether any of the effect parameters would respond in healthy volunteers (HVs).

In addition, we investigated the effects of RSG on parameters of lipid metabolism, haemodynamics and haemodilution in both study groups, as well as the differences in effect parameters between the T2DM and HVs groups at baseline.

Methods

Patients

Eight male and eight female T2DM patients uncontrolled by diet alone, aged between 40 and 75 years, with a body mass index (BMI) > 25 kg/m², increased fasting plasma glucose concentrations (>7.0 mm), and C-peptide > 0.17 nmol/l were to be included. Patients were excluded if they had a significant medical history or current symptoms of clinically relevant conditions, or had used any NSAID, thiazolidinedione or insulin preparation within two weeks of the expected study start. In addition, eight male and eight female healthy subjects (as determined by medical history, physical examination and routine laboratory tests), aged between 18 and 45 years, were to be included.

Study design

This was a randomized, double blind, placebo-controlled, multiple oral dose study. The subjects were studied in an 8-week period consisting of 6 Visits (figure 1; p. 203). Within one week after medical screening all subjects started with a two-week single blind placebo run-in period. At the end of the run-in period (baseline) subjects were randomly assigned to a six-week treatment with capsules containing 4 mg RSG or matching placebo twice daily. Treatment allocation took place according to randomly permuted blocks and was stratified by gender and subject type (T2DM patient or HVs).

Blood samples for RSG concentrations and pharmacodynamic parameters were collected on all Visits. At baseline three blood samples were collected; pre-dose, 3.5 and 10 hrs post-dose for RSG pharmacokinetic assessments. Throughout the study, blood and urine samples were collected for standard clinical (safety) laboratory measurements (including urine HCG test for female subjects). Moreover, frequent measurements of vital signs (heart rate and blood pressure) were performed. Finally, an oral glucose tolerance test (OGTT) was performed at baseline and at the end of the active treatment period. 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 and 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), triglycerides (TG), high density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c) (Friedewald formula). TC and TG concentrations were analyzed on a fully automated Hitachi 747 apparatus. HDL-c was measured using a Hitachi 911 apparatus.LDL-c was estimated using the Friedewald equation (LDL-c (mmol/l) = TC-(HDL-c-(TG/2.2)).

GLYCOSYLATED HAEMOGLOBIN (HbA_{1c})

Blood samples were collected in plastic EDTA tubes and plasma HbA_{1c} concentrations were measured by High Performance Liquid Chromatography (Bio-Rad Laboratories, Richmond, CA, USA). The reference range was 4.5%-6.5%.

INSULIN, C-PEPTIDE AND FRUCTOSAMINE

Blood samples were collected in 8.5 ml SST® Gel and Clot Activator tubes and were centrifuged within 45 minutes at 4 °C (10 minutes at 2000g) and stored at -40 °C. Plasma insulin concentrations were measured by Radio Immuno Assay (RIA) (Medgenix Diagnostics, Fleurus, Belgium) with a detection limit of 3 mU/l and an interassay coefficient of variance of 3.8-8.0%. Plasma C-peptide concentrations were measured with a RIA (Biolab, Brussels, Belgium). Plasma fructosamine concentrations were measured on a Hitachi gh (Hitachi, Tokyo, Japan) with a turbidimetric assay.

FREE FATTY ACIDS (FFA)

Blood samples were collected in EDTA tubes, stored on ice-water, centrifuged within 30 min at 4 °C (10 minutes at 2000g) and stored at -70 °C. FFA were measured with an optimized enzymatic colorimetric assay (Roche Diagnostics GmbH, Mannheim, Germany).

TNFα, IL-6, IL-1ß AND HS-CRP

Blood samples were collected in EDTA tubes, centrifuged at 4 °C within 30 min. (10 min. at 2000g) and stored at -80 °C. Plasma IL-1ß, IL-6 and TNFα were measured using high sensitivity ELISAS of R&D Systems (Abington, UK). Plasma HS-CRP concentrations were measured using a custom made validated high sensitive sandwich ELISA with coating and detecting polyclonal antibodies against human CRP (Dako, Glostrup, Denmark).

RSG BIOANALYSIS

Blood samples were collected in 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 human plasma samples were analyzed for RSG by a validated high-performance single quadrupole-liquid chromatographic mass spectrometric (LC-MS) procedure (MDS Pharma Services, St-Laurent (Montreal), Canada).

OGTT Blood samples of 2.5 ml were collected in SST® Double Gel and Clot Activator tubes, centrifuged at 4 °C within 45 min (10 minutes at 2000g) and serum was stored at -40 °C. Glucose concentrations were measured in an automated assay.

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 modelling antidiabetic drug effects. In addition, RSG trough concentrations were measured to support compliance monitoring.

Statistical analysis

INCLUDED DATA

All subjects that dropped out before Visit 4 (three subjects: one male and two female T2DM patients) were replaced with newly recruited subjects receiving the same treatment. Data of subjects dropping out after Visit 2 (baseline) were included in the statistical analysis even when a subject was replaced. Consequently, data of subjects dropping out during the run-in period (one female T2DM patient) were not used in the analysis. Therefore, the statistical analysis at baseline was restricted to: nine male and nine female T2DM patients; eight male and eight female HVs (tables 1a, 1b and 2). Furthermore, for the T2DM patient group, the exact number of subjects in each treatment group during the study (and thus the number of dropouts) is indicated in table 4.

Since the analysis procedures use maximum likelihood based techniques, the results will still be valid even in the presence of missing data, because response for missing measurements is estimated based upon the information contained in the data prior to the discontinuation [33]. The model chosen, with an unstructured covariance matrix, gives as unbiased a prediction as possible for the missing values.

PHARMACOKINETICS

Data at Visit 4, 5 and 6 below the detection limit were set to the detection limit (5 ng/ml). RSG data were log transformed. Log transformed RSG data at Visit 3, 3.5 and 10 hours post-dose, were analysed using ANOVA with group as factor.

Log transformed RSG data at Visit 4, 5 and 6 were analysed with a mixed effect model with group, Visit and group*Visit as fixed factors and subject as random factor.

PHARMACODYNAMICS

The pharmacodynamic measurements were analysed using a repeated measures mixed effect model, with group, treatment, gender, Visit, Visit*treatment, group*treatment, Visit*group*treatment as factors and baseline measurement as covariate, using an unstructured covariance matrix. The results were presented as *p* values, estimate of difference and 95% confidence intervals for the estimated difference. In case of log transformation, the estimate of difference and the confidence intervals were presented as percentages. Least square means were also calculated for change from baseline and graphically represented as % change from baseline in case of log transformation.
To assess the timing of treatment effects, contrasts for RSG and placebo treatment (corrected for baseline) were calculated for Visit 4, 5 and 6. These calculations were performed for parameters showing an overall significant ($p<0.05$) or nearly significant ($p\approx0.05$) treatment effect, and for which multiple measurements (>2) were available.

Groups (T2DM *vs.* HVs) were compared at baseline using ANOVA with treatment as factor, on the log-transformed data. Results were presented as (back transformed) least square means, p-values, estimates of difference in % and 95% confidence intervals in % for the estimated difference.

Since these analyses are of an exploratory nature, no formal correction for multiple comparisons was implemented.

COMPLIANCE

The percentage of the incorrect number of capsules taken per study was compared between groups. Because the data were not normally distrib-

uted, the Wilcoxon two-sample test was used. The percentage of days that an incorrect number of capsules per day were taken 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).

Results

Subjects

Seven T2DM patients were withdrawn: one subject developed a clinically significant elevated triglyceride concentration (12.9 mmol/l; RSG treatment group), five subjects had repetitive measurements of glucose exceeding 15 mmol/l (four subjects in the placebo and one subject in the RSG treatment group) and one subject was hospitalized (severe bronchitis) during the placebo run-in period. Three (placebo treated) patients were replaced because (in the opinion of the investigators) insufficient evaluable data were obtained up to the point of withdrawal (i.e. withdrawal prior to Visit 4; after 2 weeks of active treatment). For all other subjects no clinically significant changes in routine laboratory parameters and vital signs were observed and none of the subjects developed clinical symptoms of oedema.

COMPLIANCE

Mean overall compliance was similar in both patient (84.4% of prescribed dose and 85.5% days correct dose regimen) and healthy subject group (79.1% of prescribed dose and 77.8% days correct dose regimen). There was no significant difference [p=0.12] in the mean number of capsules taken during the study period between the T2DM *vs.* HVs group.

RSG EXPOSURE

The results of the single dose pharmacokinetic analysis showed slightly higher mean RSG concentrations in the T2DM *vs.* HVs group at 3.5 and 10 hours post-dosing (23.6% CI -0.3 to 53.4 (non-significant) and 53.7% CI 6.6 to 121.7). There were no significant differences in RSG trough concentrations between the two groups at subsequent Visits (figure 2; p. 204).

BASELINE CHARACTERISTICS

The demographics and baseline characteristics of both study populations are presented in table 1a and 1b.

TABLE 1A **Demographics and baseline characteristics.** This table shows the demographics of both study populations as well as the baseline characteristics for the glycaemic control parameters and lipid profile as measured after the two-week placebo run-in period. The data between parentheses represent the standard deviation. OAD: oral antidiabetic drug; AUE: area under the effect curve; OGTT: oral glucose tolerance test; NA: not applicable.

	T2DM patients		**Healthy volunteers**	
Parameter	*Male*	*Female*	*Male*	*Female*
Gender	N= 9	N= 9	N= 8	N= 8
Age (yrs)	56.8 (10.53)	54.0 (8.56)	22.1 (4.79)	24.4 (7.25)
BMI (kg/m²)	28.8 (2.39)	32.7 (4.61)	24.0 (4.07)	24.6 (5.07)
Waist/hip ratio	1.01 (0.047)	0.92 (0.056)	0.85 (0.061)	0.77 (0.072)
Disease duration (yrs)	5 (2.1)	2 (1.6)	N.A.	N.A.
Prior treatment: No medication	N= 0	N= 0	N= 8	N= 8
OAD Monotherapy	N= 7	N= 5	N= 0	N= 0
OAD Combination	N= 2	N= 4	N= 0	N= 0
Statin	N= 3	N= 3	N= 0	N= 0
Antihypertensive	N= 3	N= 2	N= 0	N= 0
Glucose (mmol/l)	11.8 (3.09)	11.2 (4.96)	4.7 (0.31)	4.6 (0.63)
C-peptide (nmol/l)	1.1 (0.25)	1.0 (0.37)	0.6 (0.33)	0.8 (0.38)
Insulin (mU/l)	11.7 (3.00)	12.6 (6.67)	10.4 (8.03)	9.8 (4.89)
Fructosamine (mmol/l)	294 (50.5)	278 (67.8)	196 (11.5)	192 (10.4)
HbA_{1C} percentage (%)	7.3 (0.84)	6.9 (1.74)	4.6 (0.32)	4.7 (0.27)
AUE glucose (nmol/l) after OGTT	17.2 (3.643)	15.7 (5.155)	7.37 (1.116)	7.21 (0.977)
AUE insulin (mU/l) after OGTT	23.5 (9.831)	33.7 (23.01)	68.7 (56.33)	58.5 (13.93)
Peak glucose (nmol/l) after OGTT	22.8 (4.222)	21.4 (6.679)	8.96 (2.168)	8.71 (1.019)
Peak insulin (mU/l) after OGTT	38.1 (15.42)	66.3 (44.24)	106 (93.11)	91.0 (24.62)
Cholesterol (mmol/l)	5.4 (0.62)	5.4 (0.86)	3.9 (0.54)	4.1 (0.67)
Triglycerides (mmol/l)	3.4 (2.19)	1.8 (0.72)	1.3 (0.44)	1.0 (0.28)
HDL-cholesterol (mmol/l)	1.05 (0.179)	1.20 (0.135)	1.18 (0.324)	1.51 (0.417)
LDL-cholesterol (mmol/l)	2.8 (1.23)	3.3 (0.87)	2.1 (0.58)	2.1 (0.89)
Free Fatty Acids (mmol/l)	0.31 (0.099)	0.41 (0.083)	0.27 (0.073)	0.43 (0.128)

Baseline group comparisons

GLYCAEMIC CONTROL PARAMETERS

As shown in table 2, mean baseline glucose, C-peptide, fructosamine, HbA_{1C}, TC, LDL-C and TG concentrations were significantly higher in the T2DM *vs.* HVs group. In addition, baseline HDL-C concentrations were lower and insulin concentrations higher in the T2DM *vs.* HVs group. However, the latter differences were not statistically significant. Furthermore, baseline area under the effect curve (AUE) insulin and peak insulin concentrations after OGTT were significantly lower and baseline AUE glucose and peak glucose concentrations after OGTT were significantly higher in the T2DM *vs.* HVs group.

MARKERS OF INFLAMMATION

Mean baseline HS-CRP and IL-6 concentrations were significantly higher in the T2DM *vs.* HVs group. Differences in mean IL1ß and TNFα concentrations were not statistically significant (table 2).

TABLE 1B **Baseline characteristics (continued).** This table shows the baseline characteristics for pro-inflammatory cytokines and inflammation markers as well as haemodynamic parameters as measured after the two-week placebo run-in period. The data between parentheses represent the standard deviation.

	T2DM patients		Healthy volunteers	
Parameter	*Male*	*Female*	*Male*	*Female*
WBC (10E9/l)	5.3 (1.06)	5.5 (1.32)	5.6 (1.01)	4.8 (0.58)
HS-CRP (mg/l)	1.5 (0.80)	3.2 (2.63)	1.1 (2.23)	1.7 (2.34)
IL-1beta (pg/ml)	0.19 (0.184)	0.08 (0.047)	0.08 (0.089)	0.08 (0.075)
IL-6 (pg/ml)	2.5 (1.58)	2.7 (1.15)	1.9 (1.87)	1.1 (0.50)
TNF alpha (pg/ml)	1.7 (0.65)	3.5 (3.94)	3.4 (2.58)	2.3 (1.66)
Systolic blood pressure (mm Hg)	129 (11.4)	126 (24.0)	119 (9.8)	115 (5.9)
Diastolic blood pressure (mm Hg)	78 (5.5)	75 (8.5)	69 (8.1)	70 (8.5)
Heart rate (bpm)	66 (10.7)	72 (10.8)	69 (12.9)	69 (12.0)

HAEMODYNAMIC PARAMETERS

Systolic and diastolic pressure were significantly higher in the T2DM *vs.* HVs group. Heart rate did not differ between the two groups (table 2).

Overall treatment effects

GLYCAEMIC CONTROL PARAMETERS

In the T2DM active treatment group there was a significant decrease in FPG, C-peptide, insulin and fructosamine compared to placebo (table 3), whereas these parameters showed no significant decrease in the HVs group. In the HVs group there was a significant decrease in mean peak insulin (-25.5% (-43.5 to -1.9) p=0.0375) and peak glucose concentrations (-16.3% (-29.7 to -0.4) after an oral glucose load (OGL) but no significant change in mean AUE insulin and AUE glucose after OGL compared to placebo (data not shown). No significant changes in glucose tolerance were noted in the diabetic group. Furthermore, in both treatment groups there were no significant changes in TC, HDL-C, LDL-C, TG, FFA and HbA_{1c} compared to placebo (data not shown).

MARKERS OF INFLAMMATION

In the T2DM treatment group there was a significant decrease in mean IL-6 concentration and WBC *vs.* placebo. In addition, this group showed a decrease in mean HS-CRP concentration compared to placebo, but this was not statistically significant (table 3). Furthermore, in the T2DM group there were no significant changes in IL-1ß and TNFα concentrations compared to placebo (table 3). In the HVs group there were no significant changes in plasma concentration for any of the inflammation markers (data not shown).

TABLE 2 **Baseline comparisons between T2DM and HVs groups.** This table shows the results of the baseline comparisons (ANOVA; log transformed data) between the T2DM and HVs groups for glycaemic control, lipid profile, inflammation and haemodynamic parameters. AUE: area under the curve; OGTT: oral glucose tolerance test.

	Back transformed least square means			
Parameter	**Diabetic (n=18)**	**Healthy (n=16)**	**Estimate of difference Diabetic – Healthy in % (95% CI)**	**p-value**
Glucose (mmol/l)	10.871	4.596	136.5 (97.0, 184.0)	<.0001
C-peptide (nmol/l)	1.014	0.635	59.6 (23.5, 106.2)	0.0008
Insulin (mU/l)	11.367	8.936	27.2 (-3.8, 68.3)	0.0891
Fructosamine (mmol/l)	280.58	193.84	44.7 (29.7, 61.6)	<.0001
HbA_{1c} percentage (%)	6.977	4.652	50.0 (35.3, 66.3)	<.0001
AUE insulin (mU/l) after OGTT	22.646	45.165	-49.9 (-64.9, -28.3)	0.0004
Peak insulin (mU/l) after OGTT	42.293	86.917	-51.3 (-68.0, -26.1)	0.0014
AUE glucose (nmol/l) after OGTT	15.566	7.064	120.4 (88.8, 157.2)	<.0001
Peak glucose (nmol/l) after OGTT	21.377	8.696	145.8 (107.2, 191.7)	<.0001
Cholesterol (mmol/l)	5.361	3.971	35.0 (22.1, 49.3)	<.0001
Triglycerides (mmol/l)	2.197	1.095	100.7 (42.4, 182.9)	0.0002
HDL-cholesterol (mmol/l)	1.110	1.306	-15.0 (-28.2, 0.5)	0.0565
LDL-cholesterol (mmol/l)	2.841	1.973	44.0 (6.4, 95.0)	0.0200
Free Fatty Acids (mmol/l)	0.345	0.327	5.3 (-17.9, 35.0)	0.6748
WBC (10E9/l)	5.245	5.158	1.7 (-11.8, 17.3)	0.8115
HS-CRP (mg/l)	1.625	0.572	184.2 (28.2, 529.8)	0.0117
IL-1beta (pg/ml)	0.088	0.055	59.7 (-14.4, 197.9)	0.1358
IL-6 (pg/ml)	2.354	1.203	95.7 (32.8, 188.4)	0.0013
TNF alpha (pg/ml)	1.897	2.202	-13.9 (-47.5, 41.3)	0.5436
Heart rate (bpm)	68.833	68.750	0.1 (-7.9, 8.1)	0.9832
Systolic blood pressure (mm Hg)	127.39	117.00	10.4 (0.3, 20.5)	0.0446
Diastolic blood pressure (mm Hg)	76.889	69.500	7.4 (2.1, 12.7)	0.0077

HAEMODYNAMIC / HAEMODILUTION PARAMETERS

In both treatment groups there were no significant changes in blood pressure (systolic and diastolic), heart rate and hematocrit (as measure for haemodilution) compared to placebo (data not shown).

Timing of treatment effects

Since there were no significant overall treatment effects in the HVs group for parameters with multiple (>2) measurements, this analysis was limited to the T2DM group. Results, including average relative change from baseline, 95% confidence intervals and p-values, are presented in table 4 and summarized for selected parameters in figure 3 (p. 205).

Briefly, significant effects on glycaemic control parameters appeared to precede or parallel the effects on inflammation markers. This is illustrated by a significant decrease in mean fasting insulin, and a *near* significant decrease in FPG concentration after 2 weeks of RSG *vs.*

TABLE 3 **RSG treatment effects in T2DM group.** This table shows the back transformed least square means for RSG and placebo treatments as well as the average (overall) % change in selected effect parameters for RSG *vs.* placebo in the T2DM patients group with corresponding 95% confidence intervals and p-values. All subjects with data points up to (and including) Visit 4 or beyond were included in the statistical analyses.

	RSG *vs.* Placebo Back transformed least square means			
Parameter	**RSG**	**Placebo**	**Estimate of difference RSG - placebo in % (95% CI)**	**p-value**
Parameters of glucoregulation				
Glucose (mmol/l)	6.04	7.20	-16.2 (-26.1, -4.8)	0.0082
C-peptide (nmol/l)	0.63	0.78	-19.5 (-31.8, -4.9)	0.0125
Insulin (mU/l)	7.59	10.49	-27.7 (-42.6, -8.8)	0.0080
Fructosamine (mmol/l)	216.03	247.23	-12.6 (-20.7, -3.7)	0.0085
HbA_{1c} percentage (%)	5.92	5.90	0.4 (-7.7, 9.2)	0.9328
Inflammation markers				
WBC (10E9/l)	4.50	5.17	-13.0 (-23.7, -0.8)	0.0381
HS-CRP (mg/l)	0.59	1.06	-44.0 (-69.2, 1.9)	0.0572
IL-1beta (pg/ml)	0.07	0.05	30.3 (-22.9, 120.3)	0.3083
IL-6 (pg/ml)	1.29	1.89	-31.8 (-48.9, -9.1)	0.0109
TNF alpha (pg/ml)	1.79	1.77	1.1 (-16.8, 23.0)	0.9068

placebo. At this point, no significant effect on IL-6 or WBC was present. After 4 weeks of active treatment all glycaemic control parameters considered in this analysis (FPG, fructosamine, insulin and C-peptide) had significantly decreased compared to placebo treatment. This decrease was also observed for IL-6 and WBC. After 6 weeks of RSG treatment mean FPG, fructosamine and IL-6 concentrations as well as the WBC showed a significant decrease, coinciding with a *near* significant decrease in HS-CRP concentration compared to placebo. The decrease in mean fasting C-peptide and insulin concentration in the RSG treated group was not significant compared to placebo at 6 weeks of treatment (figure 3; p. 205).

Discussion

The main finding of this study was that none of the pro-inflammatory cytokines and inflammation markers investigated in this study demonstrated an earlier response to treatment with RSG than typical parameters of glucoregulation in the T2DM group (table 4). Also, we did not find significant treatment effects on any of the pharmacodynamic parameters in the HVs group, apart from a slight reduction in peak glucose and peak insulin following an oral glucose load.

TABLE 4 **Timing of RSG treatment effects.** This table shows the estimates of the average % change in effect parameters in the T2DM group per Visit, for RSG *vs.* placebo with corresponding 95% confidence intervals and p-values. This analysis was restricted to parameters, which were (nearly) significant for the overall RSG treatment.

	Estimate of % change; 95% confidence interval and p-value			
Parameter	**Overall**	**2 weeks** placebo: n=9 RSG: n=8	**4 weeks** placebo: n=6 RSG: n=7	**6 weeks** placebo: n=5 RSG: n=6
FPG	-16.2% (-26.2,-4.8) p=0.008	-12.0% (-22.9,0.5) p=0.058	-18.8% (-30.4,-5.2) p=0.010	-17.5% (-27.5,-6.1) p=0.010
Fructosamine	-12.6% (-20.7,-3.7) p=0.009	-6.3% (-13.4,1.4) p=0.101	-14.4% (-23.3,-4.5) p=0.007	-16.8% (-26.5,-5.9) p=0.005
Insulin	-27.7% (-42.6,-8.8) p=0.008	-26.9% (-45.3,-2.3) p=0.035	-33.5% (-50.5,-10.5) p=0.010	-22.1% (-44.4,9.0) p=0.137
C-peptide	-19.5% (-31.8,-4.9) p=0.013	-15.2% (-31.0,-4.1) p=0.111	-24.3% (-38.4,-7.0) p=0.010	-18.6% (-37.0,5.0) p=0.109
IL-6	-31.8% (-48.9,-9.1) p=0.011	-17.7% (-44.7,22.3) p=0.321	-39.4% (-60.0,-8.2) p=0.020	-36.4% (-54.9,-10.3) p=0.012
HS-CRP	-44.0% (-69.2,1.9) p=0.058	-31.6% (-72.6,71.1) p=0.404	-45.4% (-84.4,69.6) p=0.282	-52.9% (-78.3,2.2) p=0.056
WBC	-13.0% (-23.7,-0.8) p=0.038	0% (-31.0,-4.1) p=0.996	-18.4% (-30.2,-4.5) p=0.013	-19.4% (-33.7,-2.1) p=0.031

The main question of the study was if (inflammatory) markers could be identified that precede or predict glycaemic response in T2DM patients that may also respond in HVs. If found, these markers would be very helpful to expedite early clinical development of novel glitazones. We have been unable to identify such parameters. Although the duration of our study was short in comparison with the typical treatment periods in therapeutic T2DM trials, we chose this duration for practical purposes. It was our intention to find measures that would respond to treatment with TZDs rapidly. In early drug development many variables like dose and dosing frequency need to be evaluated, and this cannot be done easily with relatively long treatment periods.

A potential interpretation issue of this study could lie within the dropout rate of subjects in the T2DM placebo group. Unfortunately, dropouts are inevitable in this type of study design, in which regular oral hypoglycaemic medication of T2DM patients was withdrawn. We chose to withdraw regular hypoglycaemic therapy since concerns were raised with regard to the glucose lowering potential of RSG as add-on therapy. However, since this potential (drop-out rate) issue was anticipated, an appropriate statistical method was chosen (repeated measures mixed effect model) to provide an as unbiased a prediction as possible for the missing values encountered [33] and thus allow valid conclusions. The elevated IL-6 and HS-CRP baseline concentrations in the T2DM

group are consistent with previous observations [34] and corroborate the notion that T2DM reflects a state of chronic inflammation [35]. However, as both groups were not matched for BMI and age these findings cannot solely be attributed to the T2DM state and may partly reflect age related effects. The significant decrease in WBC coinciding with a borderline significant decrease in HS-CRP concentration in the present study is similar to observations made in previous studies [28, 24-27], and illustrates the anti-inflammatory properties of RSG *in vivo*. Furthermore, *in vitro* studies with cultured hepatocytes have shown strong decreases in IL-6 expression and secretion after treatment with RSG [36]. Nonetheless, a significant decrease in IL-6 concentrations with TZD treatment *in vivo* has not been described before. Two previous studies investigating TZD effects in T2DM patients did not report significant effects on plasma IL-6 concentrations [26,37]. Haffner *et al.* reported no significant effects after 26 weeks of treatment with RSG 4 or 8 mg/day [26]. Since adipose tissue is a significant source of IL-6 expression, the authors attribute their negative findings to a decrease in weight (and therefore probably loss of adipose tissue mass) in the placebo group coinciding with an increase in weight in the RSG treated group. Subsequently, Tonelli *et al.* reported no significant effect on the plasma IL-6 concentration after 3 weeks of treatment with pioglitazone [37]. In this study we observed a significant decrease after 4 weeks of treatment with RSG, which was still present after 6 weeks. Therefore, it is possible Tonelli *et al.* failed to show a significant effect because a longer treatment period is required.

Interestingly, the two study groups of HVs and T2DM patients did not differ significantly in IL-1SS and TNFα plasma concentrations. IL-1ß was mainly selected as candidate biomarker based on pre-clinical data, suggesting that plasma IL-1ß is implicated in IL-6 induced HS-CRP production [38], and elevated plasma concentrations might therefore be present in T2DM. However, elevated IL-1ß concentrations could not be confirmed in this clinical study. This may be explained by more recent data suggesting that IL-1ß is mainly an autocrine / paracrine stimulator of IL-6 release from both human PBC and adipocytes, rather than an endocrine signal from adipose tissue [39]. As such, our data are in keeping with observations form a large recent study on inflammatory cytokines in T2DM patients in which no differences with non-diabetic controls could be demonstrated [16]. Furthermore, the normal TNFα plasma concentrations in the T2DM group are consistent with some [14, 40, 41], but not with other previous studies [16, 42-44]. In addition, other studies have shown that its perilous effect on insulin signalling is mostly a consequence of paracrine / autocrine action [14, 40, 41, 45]. Therefore, our observations do not support the notion that elevated plasma TNFα concentrations play a major role in the pathophysiology of T2DM. Nonetheless, there may still be important local tissue effects of this cytokine. Alternatively, since T2DM is a multifactorial and highly heterogeneous disorder, there is a possibility that plasma concentrations of particular cytokines are elevated in distinct sub-populations of T2DM patients, but not in others.

Some of the traditional glycaemic control markers responded earlier than expected from the results of previous studies with TZDs [30, 31]. This illustrates that (although no formal calculation was performed) the present study was properly powered to detect changes in parameters of glucoregulation. A significant effect on insulin and borderline significant effect on FPG was already present after 2 weeks of RSG treatment in the T2DM group (table 4). In addition, the maximum (and significant) decrease in both insulin (34%) and FPG (19%) levels was reached after 4 weeks of treatment. In contrast, the first occurrence of a significant anti-inflammatory effect, illustrated by a decrease in IL-6 concentration and WBC, was observed after 4 weeks of RSG treatment. Our findings appear to differ from the previously reported timing of anti-inflammatory and metabolic effects of RSG *in vivo*. Mohanty *et al.* reported a significant decrease in CRP concentration from baseline after only 1 week of treatment with RSG 4 mg whereas effects on glucose and insulin were not significant after 6 weeks using ANOVA on ranks [27]. These contrasting findings may relate to differences in study design, including the non-randomized design and lack of the use of placebo in the study by Mohanty *et al.*

Finally, the metabolic effects of RSG were independent from changes in fasting FFA and TG concentrations. This observation is in keeping with results from a recent study by Tan *et al.* showing that the late post-prandial fasting FFA and TG concentrations are decreased after RSG treatment, but fasting concentrations remain unaffected [46].

In conclusion, we could not identify novel plasma markers that respond earlier to treatment with rosiglitazone than typical parameters of glucoregulation or that have a significant response in HVs. Nonetheless, the significant IL-6 response observed in this study suggests a place for this cytokine as non-traditional complementary biomarker in clinical 'proof of concept' studies with novel TZDs.

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