Evaluation of metabolite profiles as biomarkers for the pharmacological effects of thiazolidinediones in Type 2 diabetes mellitus patients and healthy volunteers

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What is already known about this subject

- Many studies have investigated the effects of thiazolidinediones on isolated biochemical markers (biomarkers) or sets of markers in Type 2 diabetes mellitus (T2DM) patients and healthy volunteers.
- However, a limited number of parameters is not capable of capturing the broad response to pharmacological intervention with these types of (pleiotropic) drugs, which are known to activate the nuclear transcription factor peroxisome proliferator activated receptor gamma (PPAR γ).
- Our study tested the new hypothesis (primary objective) that nuclear magnetic resonance (NMR)-based metabolomics, capable of providing a readout of global metabolite concentrations in biofluids, could provide a better (more holistic) picture of the the multiparametric response to pharmacological intervention with a PPAR γ agonist and thus yield a broad array of biomarkers ('fingerprint') that could be used to support and expedite clinical development of novel thiazolidinediones.

What this study adds

- NMR-based metabolomics coupled with sophisticated bioinformatics is indeed capable of identifying rapid changes in global metabolite profiles in urine and plasma (treatment 'fingerprints'), which may be linked to the well-documented early changes in hepatic insulin sensitivity following thiazolidinedione intervention in T2DM patients.
- Consequently, this approach (upon proper validation) comprises an important new addition to the early clinical development 'proof of concept' toolbox for thiazolidinediones, and may also be applicable to other classes of drugs.

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Aims

To explore the usefulness of metabolomics as a method to obtain a broad array of biomarkers for the pharmacological effects of rosiglitazone (RSG) in plasma and urine samples from patients with type 2 diabetes mellitus (T2DM) and healthy volunteers (HVs). Additionally, we explored the differences in metabolite concentrations between T2DM patients and HVs to identify a putative metabolic disease fingerprint for T2DM.

Methods

¹H nuclear magnetic resonance (NMR) spectroscopy was used to profile blood plasma and urine samples of 16 T2DM patients and 16 HVs receiving RSG 4 mg or placebo twice daily for 6 weeks. Multivariate analyses were employed to identify treatment- and disease-related effects on global endogenous metabolite profiles.

Results

RSG treatment led to a rapid relative reduction in urinary hippurate and aromatic amino acids as well as an increase in plasma branched chain amino acids and alanine, glutamine and glutamate in the T2DM group. No RSG treatment effects were noted in the HV group. Exploratory baseline analyses showed that urine and plasma metabolites discriminated between genders and disease state. T2DM patients showed a relative increase in urinary concentrations of several amino acids, citrate, phospho(enol)pyruvate and hippurate. Putative T2DM-related changes in plasma were largely attributable to increased plasma lipids.

Conclusion

The results of this study indicate that NMR-based metabolomics of urine and blood plasma samples can yield a broad array of early responding biomarkers for the effects of RSG in T2DM patients, as well as nonglucose biomarkers that may reflect the T2DM state.

Introduction

The bottleneck in delivering new or improved pharmacological treatments to the patient appears to have shifted from finding new targets and producing candidate compounds (i.e. 'drug discovery'), to choosing which candidates warrant follow-up and vital resource allocation by the pharmaceutical industry (i.e. 'drug evaluation') [1]. A pivotal element which may expedite the drug evaluation process is the identification of sensitive and specific, early responding biomarkers that are closely related to the mechanism of drug action. These biomarkers could be very useful in supporting and expediting early clinical development 'proof of concept' studies. A potential source for these biomarkers could be the early change in metabolite profiles in plasma and urine samples of human subjects receiving a pharmacological intervention.

It has been shown that when foreign compounds interact with cells and tissues they alter the ratios, concentrations and fluxes of endogenous compounds in or through key intermediary metabolic pathways [2, 3]. Under mild stress, cells attempt to maintain homeostasis and metabolic control by varying the composition of the body fluids that either enter these cells or are secreted by them. Consequently, following metabolic perturbation, alterations in biofluid composition can be observed [4]. To investigate these complex metabolic consequences of metabolic stresses, such as disease or pharmacological interventions, nonselective, but specific 'information-rich' analytical approaches are required [3, 5].

Nuclear magnetic resonance (NMR) spectroscopy coupled with appropriate bioinformatics is a powerful tool for 'global' profiling of relatively high-abundance (low μM and above) metabolites in biofluids such as plasma or serum [6–11] and urine [12–17] which are easy to obtain in a minimally invasive manner.

Rosiglitazone (RSG) is a member of the thiazolidinedione (TZD) class of antidiabetic agents, a group of pharmaceuticals that has proven successful in the treatment of Type 2 diabetes mellitus (T2DM) in humans [18], improving glycaemic control by enhancing insulin sensitivity. RSG is a selective agonist for the peroxisome proliferator activated receptor gamma (PPAR γ), which is a member of the nuclear hormone receptor family of ligand-activated transcription factors and which controls the expression of many target genes in various tissues [18–20].

Measuring plasma concentrations of biochemical markers (such as glucose and HbA $_{1c}$) is currently the method of choice to evaluate novel TZDs in early clinical development 'proof of concept' studies. However, because it is expected that activation of PPAR γ nuclear

receptors regulates the transcription of several known, but also many as yet unknown genes, it is likely that significant downstream metabolic effects are overlooked when measuring a limited panel of conventional biochemical markers. Thus, NMR-based metabolic profiling could provide a more comprehensive picture of metabolic changes induced by the study drug (e.g. system response profiling or 'systems pharmacology' [21]).

Therefore, in the present study we sought to address the following questions: (i) can we identify changes in global metabolite profiles in plasma and urine samples of T2DM after 6 weeks of *in vivo* treatment with the PPAR γ agonist rosiglitazone vs. placebo that could serve as 'treatment fingerprint'? (ii) If so, can we identify a similar PPAR γ 'treatment fingerprint' in (more easily studied and recruited) healthy volunteers (HVs)? (iii) Can we identify potentially disease-related changes ('disease fingerprint') by comparing the baseline global metabolite profiles of T2DM and HVs?

Materials and methods

Patients

Eight male and eight female T2DM patients uncontrolled by diet alone, aged between 40 and 75 years, with a body mass index $>25 \text{ kg m}^{-2}$, increased fasting plasma glucose concentrations $>7.0 \text{ mmol L}^{-1}$ and C-peptide $>0.17 \text{ nmol L}^{-1}$ were included in the study. Patients were excluded if they had a significant medical history or current symptoms of clinically relevant conditions, or had used any nonsteroidal anti-inflammatory drug, TZD or insulin preparation within 2 weeks of the expected start of the study.

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 study. The subjects were studied in an 8-week period consisting of five visits and a follow-up visit (Figure 1). Within 1 week after medical screening, all subjects started with a 2-week single blind placebo run-in ('wash-out') period. At the end of the run-in period (baseline) they were randomly assigned to a 6-week treatment with oral 4 mg RSG or placebo twice daily. Subsequently, all subjects visited the study centre after 2, 4 and 6 weeks of treatment.

Fasting urine and blood plasma samples were collected in the run-in period (visits 1 and 2) and treatment period (visits 3 and 5). At baseline, one predose blood sample was collected followed by two blood samples

Figure 1

Overview of the study populations, placebo run-in and active treatment period, and double-blind placebo-controlled randomized design of the study. Urine and blood plasma samples for ^1H NMR spectroscopic analysis were collected on visits 1, 2, 3 and 5

at 3.5 and 10 h postdose for RSG pharmacokinetic assessments. In addition, measurements of biochemical efficacy markers (including fasting measurements for parameters of glucoregulation, lipid profile and inflammation markers) were performed at baseline and at all subsequent visits.

The protocol for this study was approved by the Medical Ethical Committee of Leiden University Medical Centre and performed according to the principles of the International Conference on Harmonization – Good Clinical Practice (ICH-GCP), the Helsinki Declaration and Dutch law, and all subjects gave written informed consent. This study was part of a larger study in which several biochemical plasma markers were measured and comprehensive transcriptomic analyses were performed. The results of these analyses will be published separately.

Analytical methods for plasma markers

Fasting concentrations of biochemical efficacy parameters [glucose, glycosylated haemoglobin, insulin, C-peptide, fructosamine, total cholesterol, high-density lipoprotein- and low-density lipoprotein-cholesterol, triglycerides, free fatty acids, tumour necrosis factor- α , interleukin (IL)-6, IL-1 β , high-sensitivity C-reactive protein and white blood cell count] were determined as previously described [22].

NMR metabolite profiling in plasma

Fasting venous blood samples were collected in standard 5-ml sodium heparin tubes. Immediately after sampling, the samples were stored on ice water and centrifuged (4°C, 10 min at 2000 g) within 30 min. Each of the resultant plasma samples was divided into two aliquots and stored at –20°C until analysis. Blood plasma from all subjects collected at visits 1, 2, 3 and 5 were thawed immediately prior to use following storage at –20°C. Samples were then centrifuged to remove proteins which had come out of solution after freezing and ali-

quots (500 μl) were transferred to standard 5-mm o.d. NMR tubes. Deuterium oxide (100 μl) was added to each sample in order to provide a field frequency lock. Spectra were acquired using the standard Bruker ‘Carr-Purcell-Meiboom-Gill’ (CPMG) spin-echo pulse sequence with water presaturation and delays as described previously [9].

The free induction decays (FIDs) were multiplied by an exponential weighing function corresponding to a line broadening of 0.5 Hz prior to Fourier transform (FT) and the acquired NMR spectra were manually phased, baseline-corrected and chemical shift referenced to the methyl lactate doublet at δ 1.33.

NMR metabolite profiling in urine

Fasting urine samples (1 ml) were freeze-dried and re-constituted in 0.1 M deuterated sodium phosphate buffer (pH 6.0), in order to reduce the magnitude of the inherent signal from partially deuterium-labeled water and control sample pH to a narrow range. Samples were then transferred to 5 mm o.d. NMR tubes and spectra acquired in automation using the standard 1D ^1H NMR pulse sequence with water suppression (zgpr). Typically 256 transients were acquired into 64 k data points using a spectral width of 8 kHz. The FIDs were multiplied by an exponential weighing function corresponding to a line broadening of 0.5 Hz prior to FT. The acquired NMR spectra were manually phased, baseline corrected and referenced to the methyl lactate resonance at δ 1.33.

^1H NMR spectral acquisition and data preprocessing

All spectra were acquired on a Bruker AVANCE 600 spectrometer, operating at 600.13 MHz ^1H resonance frequency. All spectra were measured at 300 K internal probe temperature. After peak picking of the NMR data using standard Bruker NMR software (WINNMR version 3.1 for Windows NT), peak lists were imported into the TNO in-house pattern recognition software, WinLin, and shift correction was made manually.

Table 1

Summary of the number of high-quality urine and plasma samples per visit and treatment group available for ^1H NMR spectrometry measurements

Treatment	Group	Run-in (V1)		Run-in (V2)		Post 2 week (V3)		Post 6 week (V5)	
		Urine	Plasma	Urine	Plasma	Urine	Plasma	Urine	Plasma
Placebo	DF	4	6	4	5	4	1	3	3
Placebo	HF	4	4	4	4	4	NA	4	4
Placebo	DM	4	5	4	5	4	2	2	2
Placebo	HM	4	4	4	4	4	NA	4	4
RSG	DF	4	2	4	4	4	1	3	3
RSG	HF	4	4	4	3	4	NA	4	4
RSG	DM	4	4	4	4	4	1	3	3
RSG	HM	4	4	4	4	4	NA	4	4

Plasma samples collected on visit 3 (2-weeks post) were measured only if no sample was available at visit 5 (last observation carried forward). DF, Diabetic female; HF, healthy female; DM, diabetic male; HM, healthy male; RSG, rosiglitazone; Vx, visit x; NA, not analysed.

Statistical analysis

Included data Triplicate measurements of the urine samples collected at visits 1, 2, 3 and 5 (including the data from drop-outs) and the plasma samples collected at visits 1, 2 and 5 were used in the multivariate analysis. For two plasma samples (diabetic female visit 1 and healthy female visit 1) and four urine samples (diabetic female visit 3, male diabetic visit 2, male diabetic visit 3 and male diabetic visit 5) only duplicate measurements were performed and for two plasma samples (diabetic female visit 2 and healthy male visit 2) only a single measurement was available.

Plasma samples collected on visit 3 (2-weeks post) were analysed only if no sample was available at visit 5 (last observation carried forward). The total number of high-quality samples (per group and visit) available for measurements is summarized in Table 1.

Multivariate analysis All multivariate analyses – Principal Component Analysis (PCA) and Principal Component Discriminant Analysis (PC-DA) – were carried out using TNO in-house software, Winlin. After preprocessing, data were normalized using the ‘sum of the squares’ method so that vector lengths were equal in order to account at least partially for differences in the concentration of samples. Prior to multivariate analysis, all data were autoscaled (otherwise known as scaling to unit-variance). This scaling method applies equal weight to all variables regardless of their absolute magnitude in

the raw data, since the relative importance of variables cannot be preassumed.

Results

Subjects

Seven T2DM patients were withdrawn: one subject developed a clinically significant elevated triglyceride concentration (12.9 mmol l^{-1} ; RSG treatment group), five subjects had repetitive measurements of glucose $>15\text{ mmol l}^{-1}$ (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 (e.g. withdrawal prior to visit 4; after 2 weeks of active treatment). One of these replacement patients developed repetitive measurements of glucose $>15\text{ mmol l}^{-1}$ and was withdrawn after visit 4. Consequently, a total of 11 T2DM patients and 16 HVs completed the entire study period.

Baseline characteristics and effects on plasma markers

Detailed demographics and baseline characteristics of both study populations and effects on plasma markers have been published previously [22]. Briefly, we observed significant decreases in fasting measurements of plasma glucose, fructosamine, insulin, C-peptide, IL-6 and white blood cell count after 6 weeks of treatment with RSG vs. placebo, whereas free fatty acid

Table 2

Summary of demographics of both study populations as well as the baseline characteristics for the glycaemic control parameters as measured after the 2-week placebo run-in period

Parameter	T2DM patients		Healthy volunteers	
	Male	Female	Male	Female
Gender (n)	9	9	8	8
Age (years)	56.8 (10.53)	54.0 (8.56)	22.1 (4.79)	24.4 (7.25)
Body mass index (kg m ⁻²)	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 (years)	5 (2.1)	2 (1.6)	NA	NA
Prior treatment (n)				
No medication	0	0	8	8
OAD monotherapy	7	5	0	0
OAD combination	2	4	0	0
Statin	3	3	0	0
Antihypertensive	3	2	0	0
Glucose (mmol l ⁻¹)	11.8 (3.09)	11.2 (4.96)	4.7 (0.31)	4.6 (0.63)
HbA _{1c} percentage (%)	7.3 (0.84)	6.9 (1.74)	4.6 (0.32)	4.7 (0.27)
Insulin (mU l ⁻¹)	11.7 (3.00)	12.6 (6.67)	10.4 (8.03)	9.8 (4.89)
C-peptide (nmol l ⁻¹)	1.1 (0.25)	1.0 (0.37)	0.6 (0.33)	0.8 (0.38)

The data in parentheses represent the SD. OAD, oral antidiabetic drug.

levels, lipid and lipoprotein parameters remained virtually unchanged. A demographics/baseline characteristics summary is provided in Table 2.

Overview of blood plasma and urine NMR spectra

Initial visual inspection of the data showed typical higher concentrations of glucose in the urine and higher concentrations of glucose and lipids in the plasma samples from T2DM patients compared with those from the HVs (Figure 2). Further investigation of the metabolic consequences of the disease was performed using multivariate analysis techniques.

T2DM 'disease fingerprint'

An initial analysis of the data using PCA (not shown here) revealed no clearly distinguishable differences between any of the male vs. female visit 1 or 2 or between T2DM vs. HV classifications. The only observable difference in the plot of PC1 vs. PC2 was a separation between the samples containing either high or low glucose concentrations. Even after removing the glucose signals, the resulting PCA did not reveal a separation between the samples from the different categories. Hence, an unsupervised PCA approach alone was not sufficient to separate the samples into different groups. In order to focus the statistical analysis on the available class information it was decided to use a supervised PC-DA method, which uses the T2DM/HV and male/female classification as category information. As we were mainly interested in observing the global T2DM vs.

HV separation without the gender interaction, and because of the exploratory nature of this study, the PC-DA results were not further validated at this stage.

PC-DA in plasma was initially carried out using data from the run-in period (visits 1 and 2) and included glucose resonances. Whilst this showed very clear separation between the groups (Figure 3a), this was highly dependent on glucose concentration. In order to identify nonglucose metabolites related to the disease state, PC-DA was repeated after exclusion of glucose resonances from the data (Figure 3b). The components contributing to the first discriminant axis (Figure 3b) can be considered as a putative plasma 'disease fingerprint'. Interpretations of the peaks with distinctive 1D NMR signatures from the corresponding factor spectrum (not shown) are given in Table 3. It reveals metabolic changes indicated by a number of different plasma metabolites, which appear mainly related to increased plasma lipids and lactate, coinciding with decreased levels of several amino acids in the T2DM patients vs. HV group. In addition, gender differences could also be determined in the ¹H NMR plasma data (Figure 3b).

Analogous to the plasma samples, supervised PC-DA was carried out on data from urine samples collected during the run-in period after removal of glucose resonances from the data. A clear distinction between urine samples collected from T2DM and HVs can be made in the first discriminant axis and, although not illustrated here, the third discriminant axis shows a clear separation between the female and male diabetic samples, resulting

Figure 2

Typical 600-MHz ^1H NMR spectra (δ 0.0–4.5) of urine (left) and blood plasma (right) from a (randomly chosen) healthy male subject (a) and a diabetic male subject (b) both at visit 1 of the study. The spectra illustrated are scaled to the same signal-to-noise ratio in order to highlight the differences in typical glucose and lipid resonance intensities

in four well-separated clusters in the data (Figure 4). An overview of all urinary metabolites with distinctive 1D NMR signatures in the 'disease fingerprint' factor spectrum (not shown) is given in Table 4. Briefly, T2DM patients showed a relative increase in urinary levels of alanine, citrate, phenylalanine, tyrosine, hippurate and phospho(enol)-pyruvate.

Treatment effects

Using PC-DA, the spectra derived from the plasma samples in the T2DM group allowed only subtle separation of the different treatment visits, and was difficult

to interpret reliably due to gender-dependent effects of the treatment. Further investigation into the effect of RSG on plasma metabolites was therefore carried out by studying male and female patients separately. In contrast, gender-dependent treatment effects were not observed for urine metabolites. Therefore, investigation into the effect of RSG on urine metabolites was carried out using pooled male and female patient samples.

Urine samples Figure 5 illustrates the supervised analysis of ^1H NMR urine spectra from all T2DM patients, separated according to treatment regime. The first dis-

Figure 3

Results of Supervised Principal Component Discriminant Analysis (PC-DA) of plasma samples from run-in visits (visits 1 and 2). A good distinction between diabetic patients and healthy volunteers as well as separation by gender are accomplished when the glucose resonances are included in the analysis (a). The separation of the different groups is less pronounced but still clearly visible when the glucose resonances are excluded from the analysis (b)

criminant axis (D-1) appears to show treatment-related markers, whereas the second discriminant axis (D-2) appears to show a treatment-independent effect on metabolic markers (Figure 5). Figure 6 provides an overview (factor spectrum) of the metabolites that appear to correlate with either RSG or placebo treatment in the urine samples of the T2DM patient group. This figure shows a relative reduction of hippurate and a further increase of aromatic amino acids in the T2DM patient group. We could not identify significant treatment-related changes in metabolite profiles of the urine samples in the HV group.

Plasma samples In the female T2DM group, a number of metabolites appeared to increase after treatment with RSG, for example the branched chain amino acids (0.94–1.05 p.p.m.), alanine (1.46 p.p.m.), glutamine/glutamate (2.15 and 2.46 p.p.m.) and citrate (2.55 and 2.67 p.p.m.), whereas lactate (1.33 and 4.11 p.p.m.), acetate (1.91 p.p.m.), tyrosine (6.91 and 7.18 p.p.m.) and phenylalanine (7.36 and 7.45 p.p.m.) all appeared to decrease compared with placebo. There was no effect of RSG vs. placebo on plasma lipids.

In the case of the male T2DM patients, metabolites such as the branched chain amino acids, alanine,

Table 3

Relative metabolite concentrations in plasma samples of the T2DM patients and HVs collected during the run-in period (visits 1 and 2)

Metabolite	Chemical shift (multiplicity)	T2DM vs. HVs
Leucine	0.95 (t), 0.97 (d), 1.74 (m)	↓
Isoleucine	1.00 (d), 3.67	↓
Valine	1.05 (d), 3.61	↓
3-D-hydroxybutyrate	1.20	↓
Methylene groups from mobile fatty acids in lipoproteins	1.29 (m)	↑
Lactate	1.33 (d)	↑
Alanine	1.48 (d)	↓
Lipid (C[H2]-CH ₂ -CO)	1.59 (m), 5.33	↑
Glutamine	2.14 (m), 2.45 (m)	↓
Lipid (CH ₂ -CO)	2.25 (m)	↑
Citrate	2.55 and 2.67 (dd)	↓
Lipid (C=C-CH ₂ -C=C)	2.76 (m)	↑
Tyrosine	3.06, 3.94, 6.92 (d), 7.20 (d),	↓
Formate	8.48 (s)	↓

The up and down arrows indicate a respective increased or decreased concentration of each metabolite in the T2DM vs. HV group. Where multiplicity is indicated, s=singlet; d=doublet; t=triplet; dd=doublet of doublets; m=multiplet. T2DM, Type 2 diabetes mellitus; HV, healthy volunteer.

Figure 4

Results of Supervised Principal Component Discriminant Analysis (PC-DA) of urine samples from visits 1 and 2 (excluding glucose resonances). A clear separation between diabetic patients and healthy volunteers can be made in the first discriminant axis (D-1) and, although not illustrated here, the third discriminant axis shows a clear separation between the female and male diabetic samples, resulting in four well-separated clusters in the data

glutamine/glutamate and threonine (4.28 p.p.m.) appeared to increase with RSG treatment. As for the female T2DM patients, RSG appeared to induce little or no NMR-detectable changes in the

lipid profile compared with placebo. We could not identify significant treatment-related changes in metabolite profiles of plasma samples from the HV group.

Metabolite	Chemical shift (multiplicity)	T2DMvs. HV
Alanine	1.48 (d)	↑
Glutamate	2.10 (m), 2.39 (m), 2.50 (m)	↓
Glutamine	2.12 (m)	↓
Citrate	2.57 and 2.72 (dd)	↑
Phenylalanine	3.12 (m), 3.27 (m), 4.00 (m), 7.30 (m), 7.38 (m), 7.42 (m)	↑
Tyrosine	3.20 (dd), 3.32 (dd), 6.90 (d), 7.20 (d)	↑
Hippurate	3.97 (d), 7.56 (t), 7.65 (t), 7.84 (d)	↑
N-methyl nicotinamide	4.49 (s), 8.91 (d), 8.99 (d), 9.30 (s)	↓
Phospho(enol)pyruvate	5.19 (t), 5.36 (t)	↑
Uridine	5.81 (d), 5.87 (d), 7.78 (d)	↓

The up and down arrows indicate a respective increased or decreased concentration of each metabolite in the T2DM vs. HV group. Where multiplicity is indicated, s=singlet; d=doublet; t=triplet; dd=doublet of doublets; m=multiplet. T2DM, Type 2 diabetes mellitus; HV, healthy volunteer.

Table 4

Relative metabolite differences of the T2DM patients and HVs in urine samples collected during the run-in period (visits 1 and 2)

Figure 5

Results of Supervised Principal Component Discriminant Analysis (PC-DA) from urine collected from Type 2 diabetes mellitus patients (pooled for male and female samples) classified according to treatment regime, excluding glucose resonances. The first discriminant axis (D-1) appears to show the effect on treatment-related markers, whereas the second discriminant axis (D-2) appears to show a treatment-independent effect on metabolic markers

Discussion

The main objective of the present study was to evaluate whether ^1H NMR-based spectroscopy could identify metabolite profiles that could serve as molecular biomarkers for the pharmacological effects of TZDs. To this end, we conducted a small intensive clinical study that investigated the pharmacological effects of RSG (as prototype TZD) on endogenous metabolite profiles in blood plasma and urine samples of T2DM patients and HVs. In parallel, we investigated and confirmed the efficacy of

RSG using typical parameters of gluco-regulation and inflammation as published previously [22].

We identified several endogenous metabolites in urine and plasma of T2DM patients that responded to RSG treatment. In urine these changes related to a (gender-independent) relative reduction of hippurate and a further increase of aromatic amino acids (Figure 6). The (gender-dependent) changes observed in plasma samples included an increase in branched chain amino acids, alanine, glutamine/glutamate and citrate, coindicating

Figure 6

Factor spectrum corresponding to the first discriminant axis (D-1) in Figure 5. In this plot the variables contributing to the first discriminant are expressed as correlation coefficients. A high positive peak (correlation) belongs to a corresponding peak in the NMR spectrum of a metabolite that has a relatively high concentration in the Type 2 diabetes mellitus (T2DM) placebo-treated group, and a relative low concentration in the T2DM rosiglitazone (RSG)-treated group. Similarly, a high negative peak belongs to a corresponding peak in the NMR spectrum of a metabolite that has a relatively high concentration in the T2DM RSG-treated group and a relatively low concentration in the T2DM placebo-treated group. As such, the figure shows the urinary metabolites that appear to alter with the RSG-treatment regime relative to placebo. Subsequent interpretation of the peaks with distinctive ^1H NMR signatures showed that RSG treatment appears to induce a decrease in hippurate and a further increase in aromatic amino acids

with a decrease in lactate, acetate, tyrosine and phenylalanine in the female T2DM group, whereas changes in the male T2DM group included an increase in branched chain amino acids, alanine, glutamine/glutamate and threonine. In addition, we identified several putative disease- and gender-specific metabolites in urine and plasma samples of T2DM patients and HVs (Figures 2, 3 and 4; Tables 3 and 4).

Baseline comparisons revealed several putative disease-related metabolic differences between T2DM patients and HVs, as indicated by different concentrations of a large number of metabolites in plasma and urine samples. In plasma, these differences were related mainly to increased concentrations of several lipid fractions and lactate, whereas there were decreased levels of several amino acids in the T2DMvs. HV group (Table 3). In urine, the same comparison

identified differences mostly indicated by a relative increase in amino acid concentrations (Table 4). Our findings appear to differ from previous investigations, which showed increased plasma concentrations of the branched chain amino acids and alanine in insulin-resistant states such as obesity [23, 24]. On the other hand, a more recent study in Zucker diabetic fatty rats showed that the progression from the insulin-resistant stage to frank Type 2 diabetes was associated with a pronounced decrease in plasma concentrations of gluconeogenic amino acid [25], which is compatible with the results of the present study. However, as both groups were not age matched, some of our findings may be attributed to differences in age [26]. We chose to study unmatched groups since the primary objective of the study was to identify treatment-responsive metabolite changes in T2DM patients that would also

respond in easily recruited healthy volunteers, who are usually between 18 and 30 years of age.

Our results with regard to other (urinary) metabolites partly corroborate previous findings by Messana *et al.*, who also reported citrate and hippurate to be elevated in urine of T2DM patients vs. healthy controls using NMR spectroscopy [17]. These authors also reported dimethylamine (DMA), trimethylamine-N-oxide (TMAO), betaine and acetate to be elevated in T2DM patients. Increased urinary concentrations of these metabolites have previously been linked to the hyperosmotic effect of glucose and nephropathy in T2DM, including renal papillary dysfunction and tubular distortion [27–29]. These findings could not be reproduced in the present study. However, only a limited number of patients in the present study exhibited glucosuria and all patients had normal renal function without microalbuminuria, which might explain the discrepancy with previous findings.

The metabolic differences in metabolite concentrations, coinciding with previously described elevated levels of inflammation markers observed in this study [22], could be related to a hypercatabolic state which is a common feature of chronic diseases such as T2DM, heart failure, coronary artery disease and acute liver cirrhosis [30, 31]. According to this ‘hypercatabolic state hypothesis’, these conditions are characterized by an increase of circulating catabolic molecules, such as proinflammatory cytokines and hormones, coinciding with the impairment of anabolic hormones, such as insulin [32]. Insulin resistance and catabolic/anabolic hormonal imbalance can significantly affect the metabolism of the whole body, including the biochemistry of muscle and adipose tissue. In the musculature, both the lack of anabolic stimulation and insulin resistance cause protein degradation and amino acid release. These amino acids are used in the liver to produce glucose by gluconeogenesis [32].

Our results appear to be compatible with this hypothesis. We speculate that the excess (gluconeogenic) amino acids in plasma originating from increased protein degradation in T2DM patients, in addition to being used as substrate for gluconeogenesis, are cleared from the circulation by the kidneys and thus appear in higher concentrations in the urine compared with HVs.

The effects of RSG on metabolite profiles in plasma of T2DM patients were mainly related to increases in amino acid concentrations without any changes in plasma lipid concentrations. The latter observation is consistent with the results of the biochemical markers assessed in this study, which showed no significant effect on any of the lipid/lipoprotein parameters [22]. In contrast, increases in amino acid concentrations in urine

or plasma following treatment with RSG vs. placebo have not been previously reported. It is conceivable that these changes are related to the early improvement in hepatic insulin sensitivity reported to occur after 3 weeks of TZD treatment in T2DM patients [33]. An increase in hepatic insulin sensitivity after short-term TZD treatment will diminish the demand for amino acids as substrate for hepatic gluconeogenesis. Therefore, the increase in urine amino acid concentrations following RSG treatment in the T2DM group could be related to renal clearance of the growing pool of amino acids in plasma, which have become less important as fuel for hepatic gluconeogenesis. Furthermore, absence of amendable hepatic insulin resistance would also explain why virtually no changes in metabolite concentrations were observed after RGS treatment in the HV group.

Figure 5 shows that the maximum shift to the left of the cluster designating T2DM patients treated with RSG on the second discriminant axis is similar for visit 3 (after 2 weeks’ active treatment) and visit 5 (after 6 weeks’ active treatment). This could imply that the maximum treatment effect observed on metabolite concentrations in the urine samples of the T2DM RSG group was achieved after 2 weeks of treatment, and did not increase further after 6 weeks of treatment. If indeed changes in concentrations of amino acids (and perhaps other metabolites shown in Figure 6) in urine of T2DM patients reflect the occurrence and degree of early improvements in hepatic insulin sensitivity, they could serve as important and easy to assess early responding biomarkers for the action of TZDs in T2DM patients.

Although these results are promising, caution is needed considering the relatively small sample size of the study compared with the large number of analytes measured, and the subsequent complex multivariate statistical analyses. Hence, the results of this study need to be confirmed in one or more independent studies to increase confidence in this approach.

In conclusion, the current study demonstrates that metabolic profiling of biofluids using NMR spectroscopy can shed more light on mechanisms of drug action and can yield novel nonglucose, early responding molecular biomarkers for the effects of RSG in T2DM patients (the ‘treatment metabolome’). Since the RSG treatment effects could not be replicated in HVs, it appears that T2DM patients cannot be effectively substituted with more easily recruited and studied HVs in ‘proof of concept’ metabolomics studies investigating novel TZDs.

The current study has also provided further support for the usefulness of ^1H NMR spectroscopy-based

metabolomics for the discovery of putative disease biomarkers in plasma and urine samples (the 'disease metabolome'). The metabolites identified in urine may be of especial clinical relevance, since measuring these markers may provide the means for easy and non-invasive screening or staging of T2DM.

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References

- 1 Bailey WJ, Ulrich R. Molecular profiling approaches for identifying novel biomarkers. *Expert Opin Drug Saf* 2004; 3: 137–51.
- 2 Lindon JC, Holmes E, Nicholson JK. So what's the deal with metabonomics? *Anal Chem* 2003; 75: 384A–391A.
- 3 van der Greef J, Verheij ER, Vogels J, van der Heijden R, Davidov E, Naylor S. The role of metabolomics in systems biology, a new vision for drug discovery and development. In: *Metabolic Profiling: its Role in Biomarker Discovery and Gene Function Analysis*, eds Harrigan GG, Goodacre R. Boston: Kluwer Academic Publishing 2003: 170–98.
- 4 Garrod S, Bollard ME, Nicholls AW, Connor SC, Connelly J, Nicholson JK, Holmes E. Integrated metabonomic analysis of the multiorgan effects of hydrazine toxicity in the rat. *Chem Res Toxicol* 2005; 18: 115–22.
- 5 Lindon JC, Nicholson JK, Holmes E, Everett JR. Metabonomics: metabolic processes studied by NMR spectroscopy of biofluids. *Concepts Magn Reson* 2000; 12: 289–320.
- 6 Bock JL. Analysis of serum by high-field proton nuclear magnetic resonance. *Clin Chem* 1982; 28: 1873–7.
- 7 Wevers RA, Engelke U, Heerschap A. High-resolution ^1H -NMR spectroscopy of blood plasma for metabolic studies. *Clin Chem* 1994; 40: 1245–50.
- 8 Nicholson JK, Foxall PJ, Spraul M, Farrant RD, Lindon JC. 750 MHz ^1H and ^1H – ^{13}C NMR spectroscopy of human blood plasma. *Anal Chem* 1995; 67: 793–811.
- 9 Daykin CA, Foxall PJ, Connor SC, Lindon JC, Nicholson JK. The comparison of plasma deproteinization methods for the detection of low-molecular-weight metabolites by (^1H) nuclear magnetic resonance spectroscopy. *Anal Biochem* 2002; 304: 220–30.
- 10 Deprez S, Sweatman BC, Connor SC, Haselden JN, Waterfield CJ. Optimisation of collection, storage and preparation of rat plasma for ^1H NMR spectroscopic analysis in toxicology studies to determine inherent variation in biochemical profiles. *J Pharm Biomed Anal* 2002; 30: 1297–310.
- 11 Brindle JT, Antti H, Holmes E, Tranter G, Nicholson JK, Bethell HW, Clarke S, Schofield PM, McKilligan E, Mosedale DE, Grainger DJ. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ^1H -NMR-based metabonomics. *Nat Med* 2002; 8: 1439–44.
- 12 Nicholson JK, Sadler PJ, Bales JR, Juul SM, MacLeod AF, Sonksen PH. Monitoring metabolic disease by proton NMR of urine. *Lancet* 1984; 2: 751–2.
- 13 Nicholson JK, O'Flynn MP, Sadler PJ, MacLeod AF, Juul SM, Sonksen PH. Proton-nuclear-magnetic-resonance studies of serum, plasma and urine from fasting normal and diabetic subjects. *Biochem J* 1984; 217: 365–75.
- 14 Tate AR, Damment SJ, Lindon JC. Investigation of the metabolite variation in control rat urine using (^1H) NMR spectroscopy. *Anal Biochem* 2001; 291: 17–26.
- 15 Williams RE, Lenz EM, Evans JA, Wilson ID, Granger JH, Plumb RS, Stumpf CL. A combined (^1H) NMR and HPLC-MS-based metabonomic study of urine from obese (fa/fa) Zucker and normal Wistar-derived rats. *J Pharm Biomed Anal* 2005; 38: 465–71.
- 16 Daykin CA, Van Duynhoven JP, Groenewegen A, Dachtler M, Van Amelsvoort JM, Mulder TP. Nuclear magnetic resonance spectroscopic based studies of the metabolism of black tea polyphenols in humans. *J Agric Food Chem* 2005; 53: 1428–34.
- 17 Messana I, Forni F, Ferrari F, Rossi C, Giardina B, Zuppi C. Proton nuclear magnetic resonance spectral profiles of urine in type II diabetic patients. *Clin Chem* 1998; 44: 1529–34.
- 18 Spiegelman BM. PPAR- γ : adipogenic regulator and thiazolidinedione receptor. *Diabetes* 1998; 47: 507–14.
- 19 Kliewer SA, Lehmann JM, Willson TM. Orphan nuclear receptors: shifting endocrinology into reverse. *Science* 1999; 284: 757–60.
- 20 Willson TM, Brown PJ, Sternbach DD, Henke BR. The PPARs: from orphan receptors to drug discovery. *J Med Chem* 2000; 43: 527–50.
- 21 van der Greef J, McBurney RN. Rescuing drug discovery. *In vivo systems pathology and systems pharmacology*. *Nat Drug Discovery* 2005; 4: 1–7.
- 22 van Doorn M, Kemme M, Ouwens M, van Hoogdalem EJ, Jones R, Romijn H, de Kam M, Schoemaker R, Burggraaf K, Cohen A. Evaluation of proinflammatory cytokines and inflammation markers as biomarkers for the action of thiazolidinediones in type 2 diabetes mellitus patients and healthy volunteers. *Br J Clin Pharmacol* 2005; 62: 391–402.
- 23 Felig P, Marliss E, Cahill GF Jr. Plasma amino acid levels and insulin secretion in obesity. *N Engl J Med* 1969; 281: 811–6.
- 24 Felig P, Wahren J, Hendler R, Brundin T. Splanchnic glucose and amino acid metabolism in obesity. *J Clin Invest* 1974; 53: 582–90.
- 25 Wijekoon EP, Skinner C, Brosnan ME, Brosnan JT. Amino acid metabolism in the Zucker diabetic fatty rat: effects of insulin resistance and of type 2 diabetes. *Can J Physiol Pharmacol* 2004; 82: 506–14.

- 26 Moulias R, Meaume S, Raynaud-Simon A. Sarcopenia, hypermetabolism, and aging. *Z Gerontol Geriatr* 1999; 32: 425–32.
- 27 Gartland KP, Bonner FW, Nicholson JK. Investigations into the biochemical effects of region-specific nephrotoxins. *Mol Pharmacol* 1989; 35: 242–50.
- 28 Lever M, Sizeland PC, Bason LM, Hayman CM, Robson RA, Chambers ST. Abnormal glycine betaine content of the blood and urine of diabetic and renal patients. *Clin Chim Acta* 1994; 230: 69–79.
- 29 Lundina TA, Knubovets TL, Sedov KR, Markova SA, Sibeldin LA. Variability of kidney tubular interstitial distortions in glomerulonephritis as measured by ¹H-NMR urinalysis. *Clin Chim Acta* 1993; 214: 165–73.
- 30 Pasini E, Aquilani R, Gheorghiadu M, Dioguardi FS. Malnutrition, muscle wasting and cachexia in chronic heart failure: the nutritional approach. *Ital Heart J* 2003; 4: 232–5.
- 31 Swan JW, Anker SD, Walton C, Godsland IF, Clark AL, Leyva F, Stevenson JC, Coats AJ. Insulin resistance in chronic heart failure: relation to severity and etiology of heart failure. *J Am Coll Cardiol* 1997; 30: 527–32.
- 32 Pasini E, Aquilani R, Dioguardi FS. Amino acids: chemistry and metabolism in normal and hypercatabolic states. *Am J Cardiol* 2004; 93: 3A–5A.
- 33 Tonelli J, Li W, Kishore P, Pajvani UB, Kwon E, Weaver C, Scherer PE, Hawkins M. Mechanisms of early insulin-sensitizing effects of thiazolidinediones in type 2 diabetes. *Diabetes* 2004; 53: 1621–9.