WILEY
InterScience®
DISCOVER SOMETHING GREAT

Metabolic characterisation of plasma in juveniles with glycogen storage disease type 1a (GSD1a) by high-resolution ¹H NMR spectroscopy

Iola F. Duarte,[1]* Brian J. Goodfellow,[1] António Barros,[2] John G. Jones,[3] Cristina Barosa,[3] Luisa Diogo,[4] Paula Garcia[4] and Ana M. Gil[1]

[1]CICECO, Department of Chemistry, Campus Universitário de Santiago, University of Aveiro, 3810-193 Aveiro, Portugal
[2]QOPNAA, Department of Chemistry, Campus Universitário de Santiago, University of Aveiro, 3810-193 Aveiro, Portugal
[3]Center for Neuroscience and Cell Biology, Faculty of Science and Technology, University of Coimbra, 3001-401 Coimbra, Portugal
[4]Pediatrics Hospital of Coimbra, Avenida Bissaya Barreto, 3000-076 Coimbra, Portugal

ABSTRACT: This paper reports the first application of high-resolution ¹H NMR spectroscopy to the plasma of five juveniles with glycogen storage disease type 1a (GSD1a), permitting the characterisation of the plasma metabolic profile and the identification of alterations relative to a set of control samples. The relaxation-weighted spectra allowed changes in low molecular weight compounds to be detected more clearly, whereas diffusion-edited spectra were used to characterise the plasma lipoprotein profile. Low molecular weight metabolites with altered levels in most patients were lactate, ketone bodies, acetate, creatine/creatinine and glucose. One of the patients showed distinctively lower glucose levels and higher lactate and ketone body contents, suggesting poorer metabolic control of the disease compared with other patients. In addition, a metabolite tentatively identified as α-hydroxyisobutyrate was only detected in the spectra of GSD1a plasmas, representing, therefore, a possible novel GSD1a biomarker. Total lipoprotein contents were higher in the plasma from GSD1a patients. Furthermore, lower HDL and higher VLDL + LDL levels also characterised the plasma of these patients. Preliminary results on principal component analysis of ¹H NMR spectra allowed a clear separation between GSD1a and control plasmas. The specificity of the changes observed to GSD1a is discussed, together with the recognised potential of NMR and pattern recognition methods for aiding the diagnosis of GSD1a. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: NMR; GSD; glycogen storage disease; plasma; lipoproteins; biomarker

INTRODUCTION

The understanding and diagnosis of many diseases rely heavily on the metabolic profiling of biological fluids such as urine and plasma, as their composition is intimately related to the biochemical status of a living organism and carries information about the biological processes associated with pathological conditions. High-

*Correspondence to: I. F. Duarte, CICECO, Department of Chemistry, Campus Universitário de Santiago, University of Aveiro, 3810-193 Aveiro, Portugal. E-mail: ioladuarte@dq.ua.pt

Abbreviations used: BPPLED, bipolar pulse longitudinal eddy current delay; CPMG, Carr–Purcell–Meiboom–Gill; DOSY, diffusion ordered spectroscopy; EDTA, ethylenediaminetetraacetic acid; FID, free induction decay; GSD, glycogen storage disease; GSD1a, glycogen storage disease type 1a; GSD1b, glycogen storage disease type 1b; HDL, high-density lipoprotein; HSQC, heteronuclear single quantum correlation; LC–NMR, liquid chromatography–nuclear magnetic resonance; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acids; NOESY, nuclear Overhauser enhancement spectroscopy; PC1, principal component 1; PC2, principal component 2; PCA, principal component analysis; PUFA, polyunsaturated fatty acids; TOCSY, total correlation spectroscopy; VLDL, very low-density lipoprotein.

resolution ¹H NMR spectroscopy provides an overall view of the proton-containing metabolites at submillimolar concentrations in a body fluid, requiring minimal sample preparation and avoiding the use of extraction or derivatization steps to investigate specific groups of metabolites, as usually required by conventional metabolic screening techniques. To date, a significant number of studies have demonstrated the outstanding value of NMR spectroscopy for characterising the composition of biofluids and investigating their perturbed metabolic profiles in different diseases, as recently reviewed by Lindon and co-workers (1,2). Inborn errors of metabolism are a class of diseases where NMR spectroscopy has been shown to be particularly useful, as alterations in a wide range of metabolites can be detected simultaneously without making *a priori* assumptions about the metabolic processes involved, thus allowing the identification of possible biomarkers. Indeed, NMR has not only shown its diagnostic value for known errors of metabolism (3–8), but has also led to the discovery of new inborn errors (9–11).

Glycogen storage disease type 1a (GSD1a) is an autosomal recessive disease in which gene expression of glucose-6-phosphatase is absent. This deficiency impairs

the ability of the liver to produce free glucose from both glycogenolysis and gluconeogenesis, leading to severe hypoglycaemia and excessive accumulation of glycogen in the liver and kidneys, causing enlargement of both. Secondary clinical manifestations include lactic acidosis, hyperlipidaemia, hyperuricaemia and growth retardation (12). In addition, without effective treatment, long-term complications occur, namely hepatic adenomas, renal dysfunction, osteoporosis and gout (13–15). Currently, treatment of this disease concentrates on avoiding hypoglycaemia by continuously providing a dietary supply of glucose, either by frequent ingestion of cornstarch during day and night or nocturnal intragastric feeding (16–18). Good dietary management minimises the metabolic abnormalities of the disease and decreases the risk of long-term complications, hence the early diagnosis and initiation of treatment coupled with effective follow-up assessment of metabolic control are important objectives. This is particularly relevant for GSD1a patients since for reasons yet unknown, metabolic and glycaemic control often improves in adulthood to the extent that the frequency of carbo-hydrate feeding can be substantially curtailed (19). Possible mechanisms include the induction of extra-hepatic glucose-6-phosphatase activity in peripheral tissues such as skeletal muscle (20) or increased activity of lysosomal acid α-glucosidase, which generates glucose directly from glycogen (21). GSD1a is usually suspected on the basis of a set of clinical and biochemical features, the definitive diagnosis being confirmed by a liver biopsy and enzyme assay or, more recently, by mutation analysis (12). Therefore, the development of rapid and minimally invasive diagnostic methods continues to be a challenge.

In vivo localised ^{13}C MRS of human liver has been shown to be a fast and non-invasive tool for quantifying hepatic glycogen content in patients with different types of GSD (22,23) and *in vivo* [31]P magnetic resonance permitted the study of liver metabolism in GSD1a patients (24). Other relevant NMR studies of GSD include investigations of glucose carbon recycling by ^{13}C NMR of plasma using ^{13}C-labelled glucose (25,26). However, to our knowledge, there are no studies reporting the metabolic profiling of this disease by NMR.

The present work is the first application of high-resolution ^{1}H NMR spectroscopy to the plasma of children with GSD1a, aimed at characterising its metabolic profile and looking for consistent alterations relative to control plasma samples. In addition to standard 1D spectra, relaxation- and diffusion-edited experiments were recorded in order to improve the detection of changes in small metabolites and macromolecules, respectively. Principal component analysis (PCA) was applied to the spectra datasets as a means to validate the findings from visual inspection of the spectra and to identify the spectral features with larger variability between controls and patients.

EXPERIMENTAL

Patients

Five juveniles with GSD1a [3M, 2F, age 14 ± 6 years (minimum 9, maximum 25 years); weight $= 35 \pm 9$ kg (minimum 25, maximum 50 kg); labelled P1–P5] and five healthy controls [1M, 4F, age 20 ± 4 years (minimum 15, maximum 25 years); weight $= 53 \pm 13$ kg (minimum 40, maximum 71 kg); labelled C1–C5] were considered. The uncertainty ranges indicated correspond to the standard deviation intervals. Following informed consent, subjects were enrolled into a study protocol approved by the Pediatrics Hospital of Coimbra Ethics Committee. GSD1a patients were maintained on cornstarch feeding overnight whereas healthy controls had a meal at 20:00 the evening before the study and were fasted until the start of the protocol at 08:00 the next day. At 08:00, 11:00 and 14:00, all subjects ingested 1g/kg body weight of cornstarch dissolved in water to form a thin slurry. As part of a parallel study, all subjects also took paracetamol at 08:00 (10–15 mg/kg) and phenylbutyric acid (5 mg/kg) plus [U-^{13}C]glycerol (25–30 mg/kg) at 14:00. These reagents were all dissolved in the cornstarch meal and were not expected to affect the results of the present work. Indeed, ingested glycerol is quantitatively extracted from the splanchnic circulation by the liver and is not expected to perturb endogenous plasma glycerol levels and, in addition, paracetamol, phenylbutyrate and their metab-olites are rapidly cleared into the urine therefore not contributing to the ^{1}H NMR spectrum of plasma (data not shown). Blood (10 mL) was collected at 17:00, at which point the study was finished.

Samples

Blood was collected by venipuncture and placed in vials containing EDTA as an anticoagulant, except in one control subject (C5), where a heparinised vial was used. The plasma was separated immediately by centrifugation ($3000 \times g$, 10 min), frozen and stored at -20 °C until NMR analysis. Typically, 200 µL of each sample were mixed with 400 µL of aqueous saline (0.9% NaCl) and centrifuged (8000 rpm, 5 min). Owing to the small volume available, one of the samples (C5) was prepared with a higher dilution factor (100 µL to 500 µL saline). A volume of 500 µL was then transferred to an NMR tube and 50 µL of D_2O were added.

NMR measurements

High-resolution NMR spectra of blood plasma were recorded at 298 K on a Bruker Avance DRX-500 spectrometer, operating at 500.13 MHz for proton and equipped with an actively shielded gradient unit with a

maximum gradient strength output of 53.5 G/cm. Standard 1D ^{1}H spectra were acquired using a 1D NOESY pulse sequence (RD–90°–t_1–90°–t_m–90°–acquire) with a relaxation delay (RD) of 13s, a mixing time (t_m) of 100 ms and a fixed t_1 delay of 3 μs. Water suppression was achieved by presaturation of the water signal during the relaxation delay and mixing time. Each spectrum consisted of 128 free induction decays (FIDs) collected into 32K complex data points with a spectral width of 8012.82 Hz and an acquisition time of 2.0 s. Three to six replicate spectra were recorded for each sample, for multivariate analysis. 1D relaxation-edited ^{1}H spectra were acquired (with no replicas) using the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence (RD–90°–{τ–180°–τ}$_n$–acquire) (27) with simple presaturation of the water peak, a total spin–spin relaxation time ($2n\tau$) of 200 ms and a relaxation delay of 13 s. Typically, 256 FIDs were collected into 32K complex data points. 1D diffusion-edited spectra were recorded (with no replicas) using the bipolar pulse longitudinal eddy current delay (BPPLED) pulse sequence (28). In order to attenuate the signals from low molecular weight compounds without affecting the lipoprotein signals, square gradients with a duration of 2 ms and a strength of 48.15 G/cm were used, together with a diffusion time of 200 ms; 128 FIDs were collected into 32K complex data points, using a 13s relaxation delay. Prior to Fourier transformation, the FIDs were zero-filled to 64K points and multiplied by an exponential line-broadening function of 0.3 Hz. The 1D spectra were manually phased, the baseline corrected and the chemical shifts referenced internally to the α-glucose signal at δ 5.23. Additionally, 2D TOCSY and HSQC spectra were recorded for selected samples to aid spectral assignment.

Principal component analysis (PCA)

For each set of spectra to which PCA was applied (standard 1D, relaxation-edited and diffusion-edited), a data matrix was built from δ 0 to 6, excluding the water region (δ 4.5–5.0) and the segments containing EDTA-related signals (δ 2.53–2.58 and 3.06–3.18). All the remaining data intensity values were used for the PCA. Each spectrum was normalized by adjusting the total area to unity. The calculations were performed using a program co-developed in the University of Aveiro and the Institut National Agronomique Paris-Grignon (29).

RESULTS

Figure 1(a) shows a standard 1D ^{1}H spectrum of blood plasma collected from a GSD patient. As typically observed, this spectrum is dominated by the broad resonances of lipoproteins and other plasma proteins such as albumin, which mask the signals from low molecular

weight metabolites. Some degree of spectral editing can be achieved by employing a CPMG pulse sequence in order to attenuate or even eliminate resonances from macromolecules (or bound small molecules) with shorter T_2 relaxation times (30,31). Indeed, the CPMG spectrum [Figure 1(b)] gives a much clearer representation of small metabolites, especially in the low-frequency (δ 0–3) and high-frequency (δ 6–10) regions. Based on the literature (1,30,31) and on the 2D spectra recorded, several low molecular weight compounds such as amino acids (e.g. valine, leucine, isoleucine, alanine, proline, histidine, tyrosine), organic acids (e.g. lactate, acetate, citrate, formate), acetone, creatine, creatinine and glucose have been identified, as indicated in the figure caption. On the other hand, the macromolecular profile can be visualized without the sharp signals by utilizing the difference in translational diffusion coefficients between macromolecules and metabolites, an approach which is particularly useful for allowing the direct study of plasma lipoproteins (32–37). Figure 1(c) shows the diffusion-edited spectrum obtained for the same plasma sample. Compared with the standard 1D spectrum [Figure 1(a)], the peaks from fast-diffusing, small metabolites have been strongly attenuated, whereas the broad resonances corresponding to slow-diffusing macromolecules are not affected. A significant number of signals arise from the various types of protons in the fatty acyl chains of lipoproteins, the most prominent corresponding to the terminal methyl protons (δ 0.87) and the long-chain methylene protons (δ 1.27) [peaks 18 and 19, respectively, in Fig. 1(c)]. Other parts of the lipoprotein molecules are observed through the signals arising from the head groups (N$^+$Me$_3$) of choline-containing phospholipids (peak 26). Signals corresponding to the *N*-acetyl groups of glycoproteins and the lysyl groups of albumin are also detected [peaks 8 and 25 in Fig. 1(c)].

Variations in plasma low molecular weight metabolites

In order to look for qualitative or quantitative differences in the low molecular weight metabolites present in the plasma of GSD1a patients relative to control subjects, the CPMG spectra of both groups were compared. For some compounds with reduced spectral overlap, a semi-quantitative analysis was carried out by integrating selected signals and dividing their integrals by the total spectral area to account for differences in sample dilution (Fig. 2). This approach is based on the assumption that for each compound there is no significant variation in the proton T_2 times between different samples, which seems to be reasonable, since all samples were analysed under the same conditions. Therefore, given the high degree of spectral overlap in the standard 1D spectrum, integration of the CPMG spectra is a possible alternative to obtain semi-quantitative information, although it cannot be used
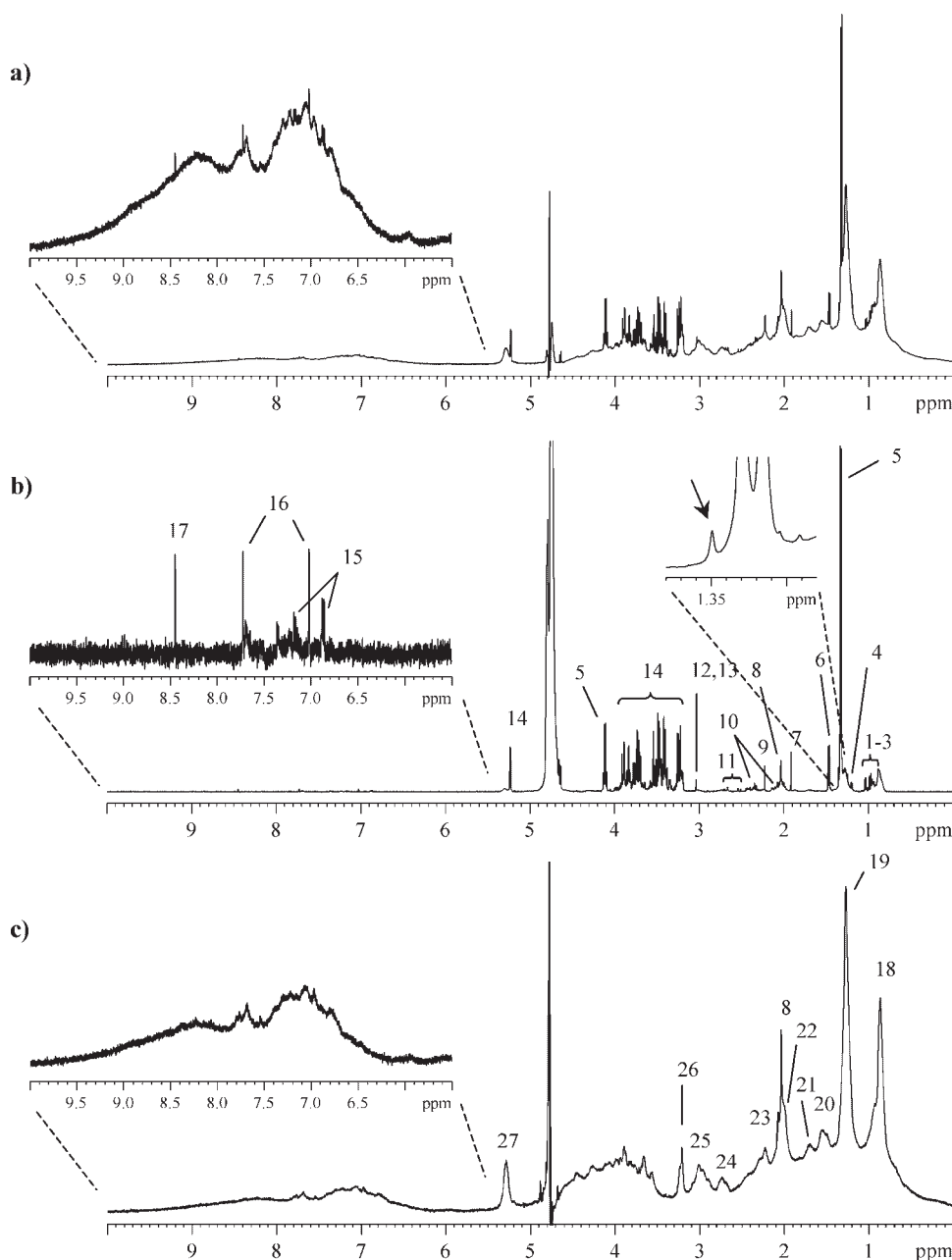
Figure 1. 500 MHz ^{1}H NMR spectra of plasma from a patient with GSD1a: (a) standard 1D spectrum; (b) 1D relaxation-edited (CPMG) spectrum; (c) 1D diffusion-edited spectrum. Assignment: 1, valine; 2, leucine; 3, isoleucine; 4, β-hydroxybutyrate; 5, lactate; 6, alanine; 7, acetate; 8, *N*-acetyl groups of glycoproteins; 9, acetone; 10, proline; 11, citrate; 12, creatine; 13, creatinine; 14, glucose; 15, tyrosine; 16, histidine; 17, formate; 18, lipid CH_3; 19, lipid (CH_2)$_n$; 20, lipid CH_2CH$_2$CO; 21, lipid CH_2CH$_2$CH=CH; 22, lipid CH_2CH=CH; 23, lipid CH_2CO; 24, lipid CH=CHCH_2CH=CH; 25, CH_2 lysyl (albumin); 26, N(CH_3)$_3$ choline in phospholipids; 27, lipid CH=CH. The high-field inset in (b) shows the singlet tentatively assigned to α-hydroxyisobutyrate

for absolute metabolite quantification owing to selective signal loss for compounds with different T_2 relaxation times.

The average lactate level is higher in patients than in controls, although there is a significant intra-group variation, especially within patients [Figure 2(a)]. The plasma of patient P3 contains a markedly higher lactate

concentration, whereas in patient P5 the lactate content is close to the average value found for the control group. While the aggregate amount of ketone bodies is generally higher in GSD patients, individual ketone bodies tend to be less specific markers for GSD. For example, levels of β-hydroxybutyrate and acetone for the GSD group are similar to controls, with the exception of P3, who has high
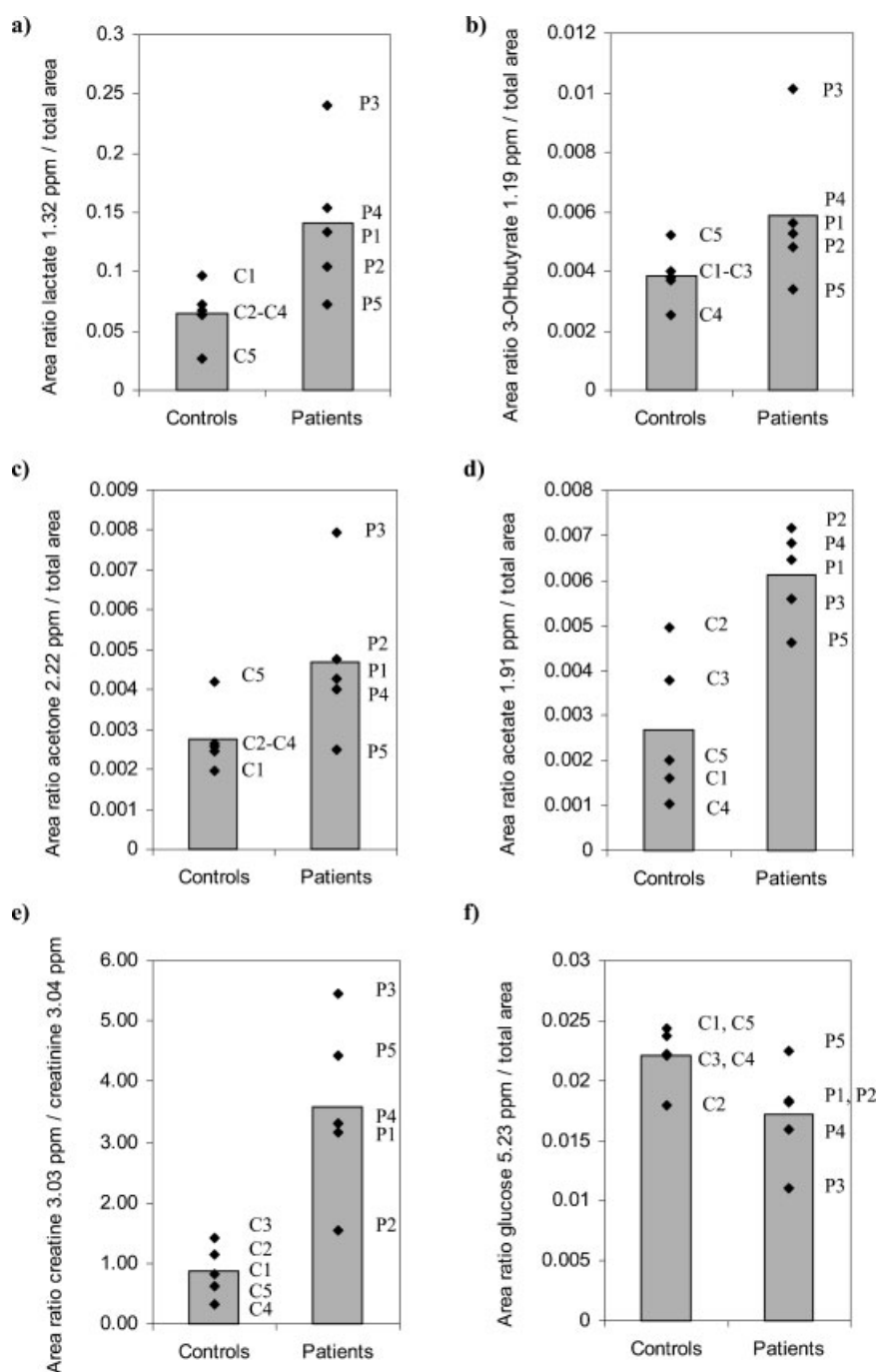
Figure 2. Representation of signal areas of some metabolites measured in the CPMG spectra of controls (C1–C5) and GSD1a patients (P1–P5), where the vertical bars represent average values: (a) lactate (δ 1.32); (b) β-hydroxybutyrate (δ 1.19); (c) acetone (δ 2.22); (d) acetate (δ 1.91); (e) creatine (δ 3.03)/creatinine (δ 3.04); (f) glucose (δ 5.23)

concentrations of both metabolites [Fig. 2(b) and (c)]. Further, acetoacetate is present in all GSD patients with the exception of P5, but is not found in any of the controls.

The level of acetate also shows great variation within each subject group, but has a clearly higher average concentration in GSD patients compared with control subjects [Fig. 2(d)]. In addition, the CPMG spectra of GSD patients show a signal at δ 1.35, not detected in

control plasmas, which is possibly a singlet assignable to α-hydroxyisobutyrate [inset in Fig. 1(b)]. This compound has been detected previously by NMR in some spectra of urine samples (1), but not, to our knowledge, in human blood plasma.

Another difference between controls and patients, detected in the CPMG spectra, concerns the relative intensities of creatine (δ 3.03 and 3.92) and creatinine
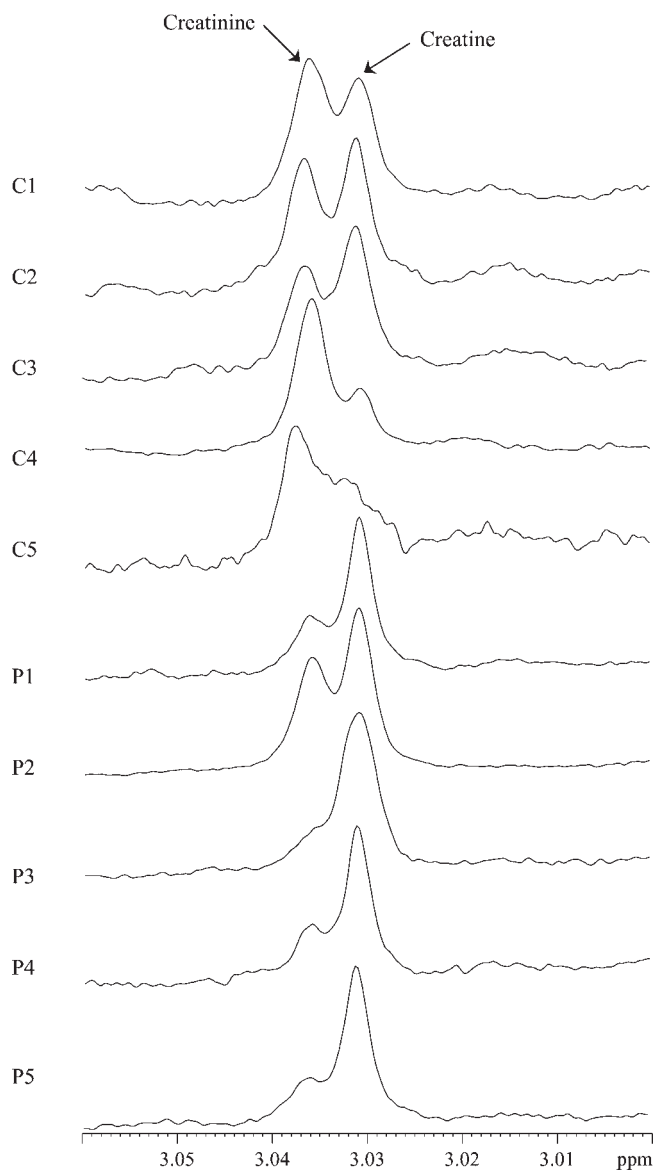
Figure 3. Expansion of the CPMG spectra of controls (C1–C5) and GSD1a patients (P1–P5) showing the signals of creatine (δ 3.03) and creatinine (δ 3.04)

(δ 3.04 and 4.05) signals (Fig. 3). These compounds show varying proportions in the five control subjects, while creatine predominates over creatinine in all GSD cases. By deconvolution of the two signals at δ 3.03 and 3.04, arising from creatine and creatinine methyl groups, respectively, the creatine/creatinine area ratio is found to have an average value of 0.9 for controls and 3.6 for patients [Fig. 2(e)]. However, the large dispersion within patients should be noted once more. Whereas the creatine/creatinine ratio of patient P2 is about 1.5, being close to some of the control values, in patient P3 the signal area of creatine is found to be more than five times higher than that of creatinine.

In terms of glucose levels, spectral integration gives higher average values in controls than in patients,

although there is considerable overlap between the ranges found for the two groups [Fig. 2(f)]. The exception is patient P3, who has much lower glucose than all other samples.

Variations in plasma lipoproteins

In order to compare the levels of total lipoproteins between subjects, spectral integration was performed on diffusion-edited spectra, which are not affected by overlap with signals from small metabolites and, therefore, permit more accurate semi-quantitative analysis than standard 1D spectra. Obviously, the areas in diffusion-edited spectra are not representative of the proton stoichiometry, and hence cannot be used for absolute quantification. Still, assuming that lipoproteins have similar diffusion behaviour in all samples under the experimental conditions used and, therefore, that their NMR signals show comparable visibility in all spectra, the overall changes in peak area relative to the spectral noise level provide an indication of how concentration levels differ between the different samples. Figure 4(a) shows the area of the signal arising from $(CH_2)_n$ protons of the various lipoprotein fractions (δ 1.24–1.27) divided by the total spectral area to account for differences in sample dilution. It is found that all GSD patients have higher plasma lipoprotein levels than control subjects, this difference being more noticeable for patient P3.

In addition to this difference in total quantity, the diffusion-edited spectra show slight differences in the qualitative profile, as illustrated in Fig. 5 for the signals arising from the methyl (δ 0.84–0.87) and methylene (δ 1.24–1.27) protons of fatty acyl groups of the lipid components in lipoproteins. It is well described in the literature that each of these resonances comprises a superimposition of signals arising from different types of lipoproteins in plasma (34,38). The three main lipoprotein types, traditionally classified according to their density and hence their size, are high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL). They all consist of a lipid core of triglycerides and cholesteryl esters inside a layer of phospholipid, free cholesterol and apolipoproteins, differing in the lipid and protein proportion. Owing to their similar chemical composition, these fractions show heavily overlapping signals, although there are small differences in chemical shifts according to the size of the lipoprotein particles. As shown in the spectra of physically separated fractions (38,39), larger size particles, namely VLDL complexes, give rise to methyl and methylene group signals shifted to higher frequencies. A first observation when comparing the spectra shown in Fig. 5 is the similarity of profiles within patients in contrast with the qualitative variations characterising control spectra. Second, it is found that the lipoprotein signals in all patients' spectra have their maxima slightly
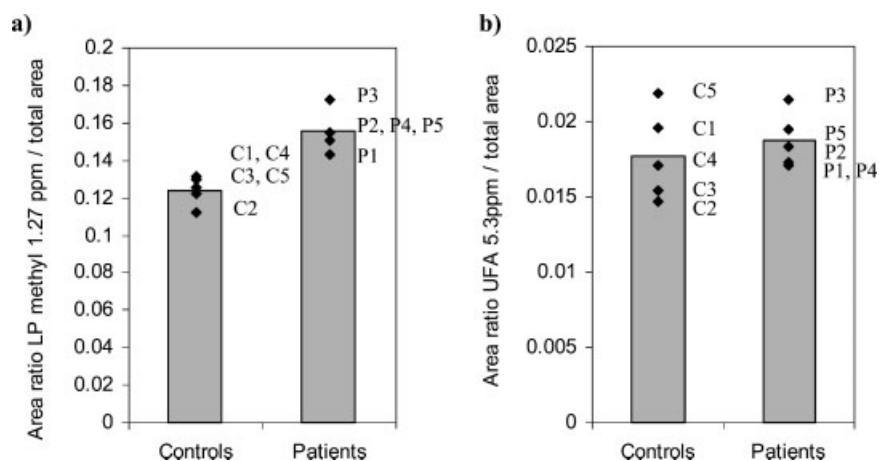
Figure 4. Representation of signal areas measured in the diffusion-edited spectra of controls (C 1–C5) and GSD1a patients (P1–P5), where the vertical bars represent average values: (a) lipoprotein (LP) methylene signal at δ 1.24–1.27; (b) olefinic protons in fatty acyl groups signal at δ ca 5.3

shifted to higher frequency relative to the spectra of most control plasmas. In the case of the methylene resonance, the spectra of all control plasmas except C4 show a more prominent shoulder at δ 1.23 relative to the main peak at about δ 1.27 which, according to previous NMR studies of plasma lipoproteins (34,39), arise mainly from HDL and LDL + VLDL, respectively. In the case of the methyl peak, the contribution of the shoulder at δ 0.83, corresponding mainly to HDL, is also higher for controls (except C4). Therefore, it may be suggested that the proportion of HDL in control plasma is higher than that in the plasma of GSD1a patients, although this hypothesis needs further confirmation by quantification of the individual lipoprotein fractions. Ultracentrifugation followed by specific enzymatic assays is the standard reference method for lipoprotein analysis, but it requires a large amount of plasma, which was not available in the present study. Alternative methods based on NMR spectroscopy, namely liquid chromatography coupled to NMR (LC–NMR) and 2D-DOSY NMR, are currently being explored in our laboratory, aimed at quantifying the lipoprotein main fractions in a new set of plasmas from GSD1a patients, and this work will be reported in a subsequent paper.

Regarding the total amount of unsaturated fatty acids, there is no significant difference between controls and GSD1a patients, as found by integration of the broad signal at about δ 5.3 arising from olefinic protons (CH= CH) in fatty acyl chains [Fig. 4(b)]. However, a small shift is noted for this signal between the spectra of controls (δ 5.28) and patients (δ 5.30), as illustrated in Fig. 6(a) for two representative cases, which is accompanied by a higher intensity of the signal at δ 2.74 [lipid CH=CH–(CH$_2$–CH=CH)$_n$] in the spectra of GSD1a plasmas compared with controls [Fig. 6(b)]. According to the assignment of unsaturated fatty acids in lipids of tissues

and body fluids (40), these differences suggest a higher proportion of polyunsaturated fatty acids (PUFA) and a lower proportion of monounsaturated fatty acids (MUFA) in the plasma of GSD1a patients.

PCA of ^{1}H NMR spectra of plasma

Although the number of samples included in this study was limited owing to the low incidence of the disease [1/100 000 births (14)] and consequent small number of patients at any one time, PCA was applied to the dataset in order to check the findings from the visual inspection of the spectra and to determine the most important sources of variability between controls and patients. The scores scatter plot resulting from applying PCA to the standard 1D spectra (water and EDTA signals excluded) is shown in Fig. 7(a). The good reproducibility of the standard 1D spectra is visible in the results, as the scores corresponding to replicate spectra of the same sample overlap. There is a clear separation between control and GSD1a samples along PC1, patient P3 showing the most negative scores, and, therefore, the largest separation from the control group. The PC1 loadings vector [Fig. 7(b)] shows the spectral features responsible for this separation. Glucose signals are positive in PC1 loadings, confirming the higher average values in controls and the lower value in patient P3, as observed by spectral integration. On the other hand, strong negative loadings are found for the signals of lactate (δ 1.32 and 4.11), indicating elevated levels of this metabolite in patients, especially in those with the most negative PC1 scores. However, when the segments including the lactate signals are removed from the matrix used for PCA, a similar PC1 vs PC2 scores plot is obtained (not shown), indicating that other compounds also contribute strongly to the observed separation. In
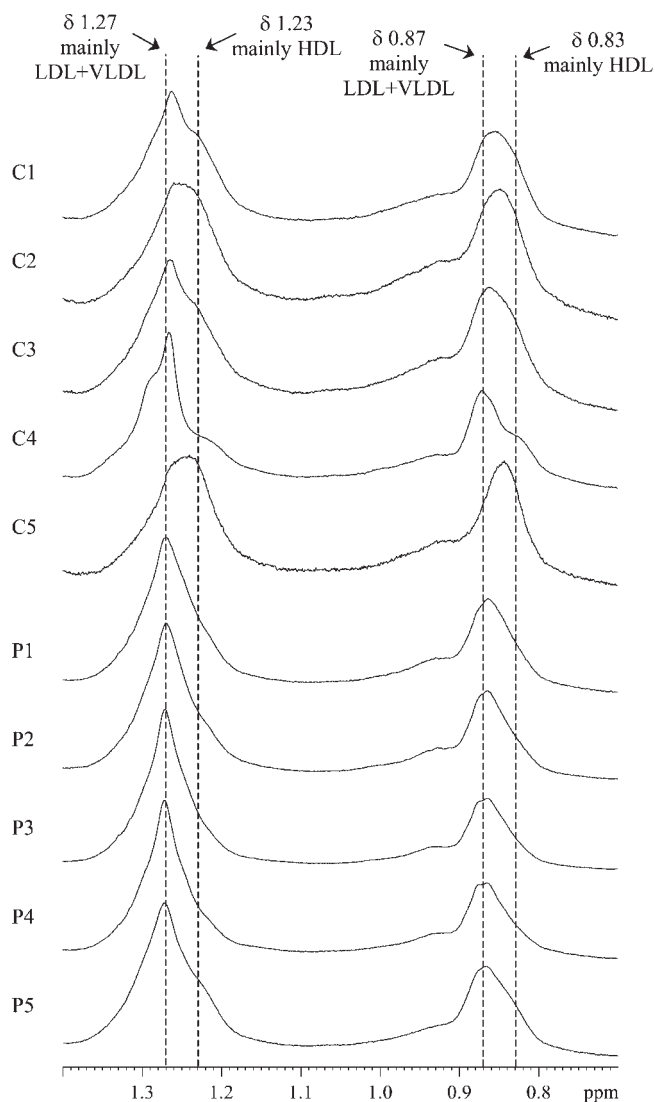
Figure 5. Expansion of the diffusion-edited spectra of controls (C1–C5) and GSD1a patients (P1–P5) showing signals from the methyl (δ 0.84–0.87) and methylene (δ 1.24–1.27) protons of fatty acyl groups of lipoproteins. Each spectrum is scaled to maximum peak intensity

Figure 6. Expansion of the diffusion-edited spectra of control C2 and patient P3 showing signals from (a) CH=CH (δ*ca* 5.3) and (b) CH=CH–C*H₂*–CH=CH (δ 2.65–2.80) of fatty acyl groups

particular, the contributions of acetate (δ 1.91) and acetone (δ 2.22) are enhanced, showing negative PC1 loadings and, therefore, higher levels in patients. These results are in general agreement with the results of spectral integration and were confirmed by PCA applied to the relaxation-edited spectra (not shown). These same PCA results highlighted the difference in the levels of creatine and creatinine, as already detected by visual comparison of the spectra.

Furthermore, the loadings in Fig. 7(b) suggest the contribution of broad lipoprotein resonances to the observed distribution of samples scores. In order to investigate this further and evaluate the variability in the macromolecule profile, PCA was also applied to the diffusion-edited spectra. Again, a good separation between control and GSD1a samples is seen along PC1
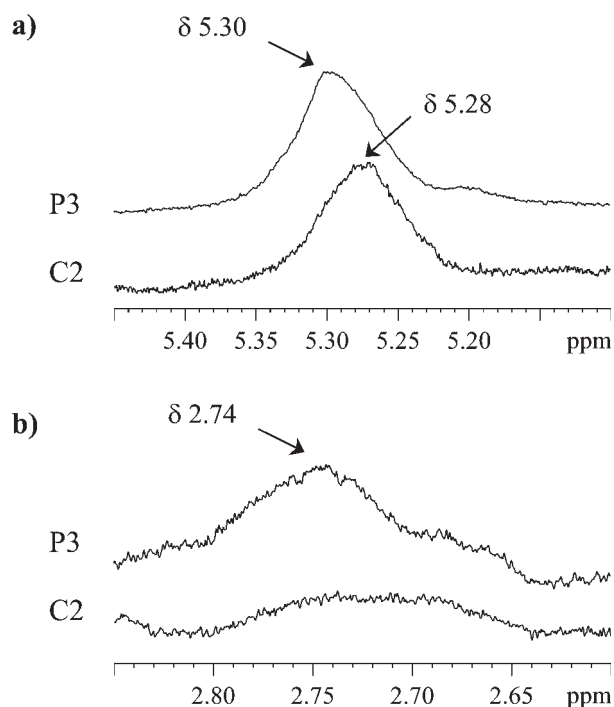
in the scores scatter plot of PC1 vs PC2 [Fig. 8(a)]. The PC1 loadings [Fig. 8(b)] show positive values at δ 0.83 and negative values at δ 0.87 and 1.27. As argued in the previous section based on the literature, the former chemical shift is characteristic of HDL, whereas the 0.87 and 1.27 ppm signals arise mainly from LDL + VLDL. Therefore, in agreement with the visual comparison of the spectra, the PCA results suggest that GSD1a patients show a lower proportion of HDL and a higher proportion of LDL + VLDL relative to control subjects. In addition, the signal at δ 3.22, assigned to the methyl head groups of choline-containing phospholipids, also shows clearly positive loadings, together with the signal at δ 0.83, which supports the assignment of this resonance to HDL, since phosphatidylcholine is known to be the most predominant lipid in the HDL fraction (34). Finally, the PC1 loadings in Fig. 8(b) show a split of the signal arising from lipid olefinic protons, with positive values for δ 5.28 and negative values for δ 5.30, which confirms the shift of this signal to higher frequencies in the GSD plasma spectra relative to control spectra, as already observed by visual comparison of the spectra.

DISCUSSION

This paper reports the direct analysis of plasma from GSD1a patients by ¹H NMR spectroscopy, using relaxation- and diffusion-edited experiments to detect
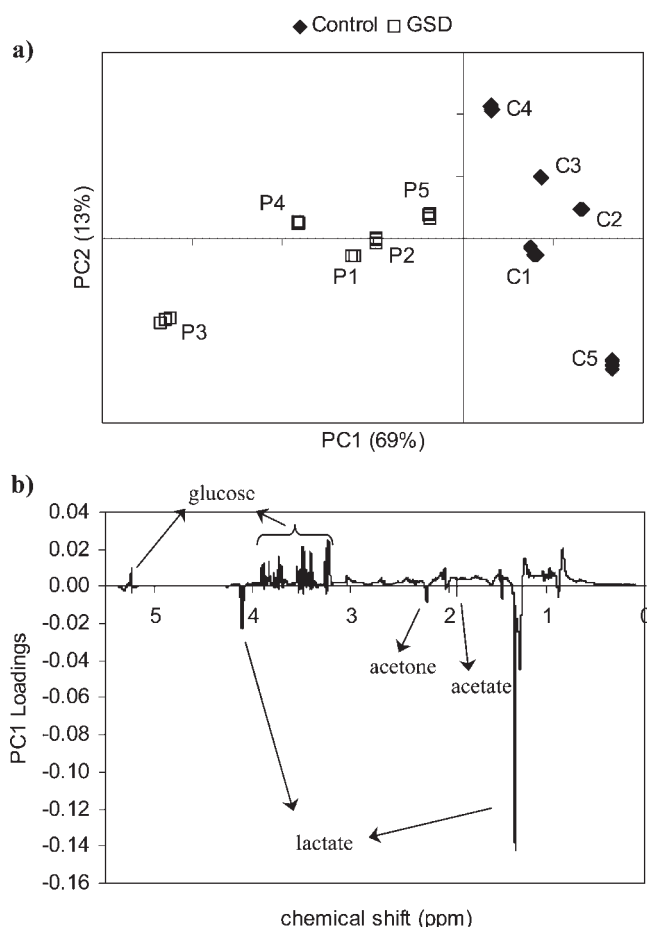
Figure 7. (a) Scores scatter plot of PC1 vs PC2 resulting from PCA of standard 1D spectra of plasma from controls (C1–C5) and GSD1a patients (P1–P5), the different spots for each subject correspond to replicate spectra; (b) PC1 loadings vector

Figure 8. (a) Scores scatter plot of PC1 vs PC2 resulting from PCA of 1D diffusion-edited spectra of plasma from controls (C1–C5) and GSD1a patients (P1–P5); (b) PC1 loadings vector

changes in low molecular weight compounds and in macromolecules, respectively.

The low molecular weight metabolites whose levels in plasma were most different between GSD1a patients and healthy controls were lactate, ketone bodies, acetate, α-hydroxyisobutyrate, creatine/creatinine and glucose. Elevated plasma lactate levels are a common feature of GSD1a, reflecting the inability of systemic lactate to be recycled into glucose via gluconeogenesis coupled with high rates of glycolytic lactate production due to frequent carbohydrate feeding. In the GSD1a patients enrolled in this study, plasma lactate levels were generally high [see Fig. 2(a)], particularly in the case of P3, where lactate levels were about three times higher than in controls. This patient, but not the others, also had significant levels of lactate in urine (data not shown). In agreement, this is also the patient with lower glucose levels than any other samples. Persistent hyperlacticacidaemia has been reported in several GSD1a cases despite dietary treatment and represents a risk factor for long-term complications such as renal dysfunction (41,42).
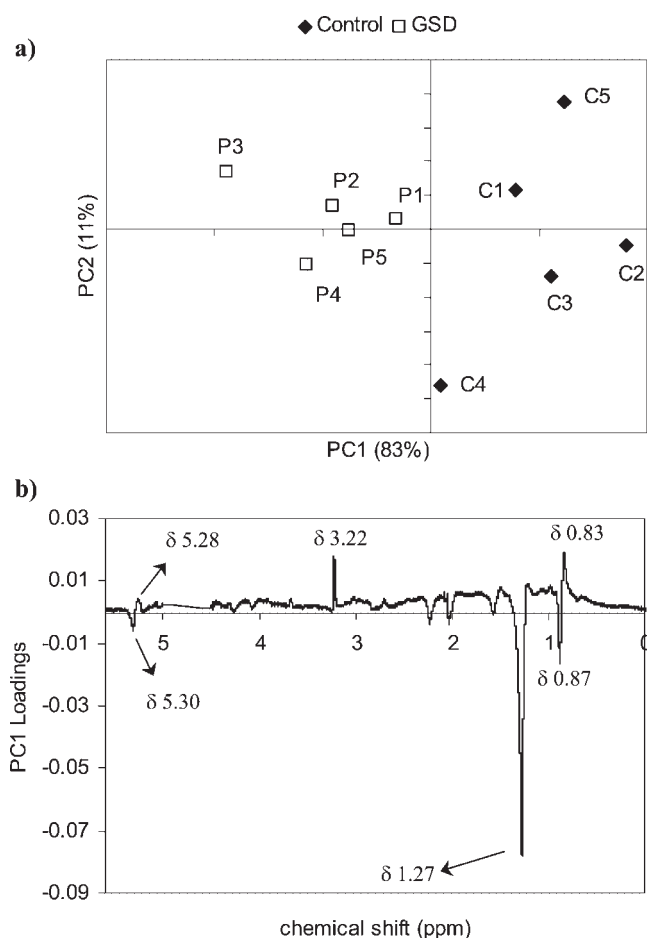
Two ketone bodies, β-hydroxybutyrate and acetone, had markedly elevated levels in patient P3, but normal levels in all other patients. On the other hand, acetoacetate could only be detected in all patients but one (P5) and not in the control plasmas. Ketone bodies are generated from acetyl-CoA under conditions of hypoglycaemia and serve as alternative oxidisable fuels for the brain and central nervous system when there is insufficient glucose available. The literature contains conflicting data about ketone body concentrations in GSD1 patients. Decreased ketone body levels have been reported (43), although a different study did not confirm this finding (44). Furthermore, in an acute GSD1b model in rats, the plasma concentrations of β-hydroxybutyrate were identical with those in control rats (45). In the case of the subjects included in this study, it may be suggested that ketogenesis is increased in at least one of the patients, P3.

Elevated levels of acetate were also found in GSD1a patients, although the range of acetate concentrations within controls was also considerable. Other diseases such as severe liver disease and severe acidosis have both

been associated with increased plasma acetate concentrations (46). Plasma acetate levels are also sensitive to dietary state (47), since acetate is generated endogenously by microbial fermentation of non-digestible carbohydrates in the lower intestine.

An unexpected metabolite, tentatively identified as *α*-hydroxyisobutyrate, has been detected in the spectra of GSD1a plasmas and might represent a novel biomarker of this disease. Unequivocal identification of this metabolite, for instance using LC–NMR, as well as the determination of its concentration are important tasks to be accomplished in the future. Another subtle difference between control and patient spectra was found for the creatine/creatinine ratio, which was higher in the plasma of most GSD1a patients. This is due to relatively lower creatinine levels in patients (with the exception of P2) compared with the control group. This rules out any possible correlation with the onset of renal dysfunction, known to be accompanied by relatively high levels of blood creatinine. Furthermore, it is probable that some relationship exists between the average higher body weights of the patients and the observed differences in creatine/creatinine, a fact which hinders any GSD-specific interpretation.

^{1}H NMR spectroscopy, in particular 1D diffusion-edited NMR, also allowed a preliminary study of the plasma lipoprotein profile of GSD1a patients. A key observation was the higher total lipoprotein contents relative to control subjects. Hyperlipidaemia is a well-known metabolic derangement of GSD1 and is not well controlled by dietary therapy (48–52). Possible causes include sharply increased rates of *de novo* lipogenesis (51,52) and decreased rates of fat oxidation (48,50), both of which are favoured by the high and frequent intake of carbohydrate. These studies have reported increased triglyceride and cholesterol concentrations, especially in LDL and VLDL particles, which were found to be increased not only in number but also in size. On the other hand, HDL cholesterol and apolipoprotein A-1 concentrations are reported to be decreased. These statements are in general agreement with the evidence found in this work suggesting a lower proportion of HDL and a higher proportion of LDL + VLDL in GSD1a patients relative to control subjects. However, to confirm these findings, the NMR results have to be explored further, a task which is under way in our laboratory for a new set of GSD1a plasma samples. In recent years, a significant effort has been put into quantifying the different lipoprotein fractions in human blood plasma, as disorders in lipoprotein metabolism are critical to the development of many diseases such as atherosclerosis, coronary heart disease, liver dysfunction and cancer. NMR-based methods show great potential in this respect and different approaches have been reported. These include curve fitting of the overlapped NMR bands based on known positions and linewidths for individual lipoproteins measured from purified fractions (38), multivariate

analysis of NMR spectra generally involving large numbers of samples (37,53), 2D-DOSY NMR (34,37) and LC–NMR (39).

Another finding suggested by the present study is the different composition in fatty acids, namely in the proportion of PUFA, which seems to be higher in GSD1a patients, although the total amount of unsaturated fatty acids (poly- and monounsaturated) seems to be similar for controls and patients. Since this may be related to the oxidizability of lipoproteins, it is important to investigate this point further through a fuller characterisation of the fatty acids present.

A clear separation between GSD1a and control plasmas was achieved by applying PCA to either standard 1D, relaxation-edited or diffusion-edited spectra, showing that the distinctive metabolic features of the disease arise both from plasma small metabolites and lipoproteins. Lactate and glucose were the low molecular weight metabolites with the highest variability between controls and patients. Other compounds contributing to the separation of the two groups, especially highlighted by PCA of the relaxation-edited spectra, were acetate, acetone and creatine/creatinine. Furthermore, PCA of standard 1D spectra suggested the contribution of lipoprotein signals for the observed distribution of samples. Indeed, this could be confirmed by PCA of diffusion-edited spectra, which resulted in a clear separation between healthy controls and GSD1a patients due to the higher relative LDL + VLDL content and lower HDL content in the latter. Therefore, in spite of the limited number of samples available for this study, these results show the promising diagnostic value of the application of pattern recognition techniques to the ^{1}H NMR spectra of plasma from children suspected to have an inborn error of metabolism with the clinical symptoms of GSD1a.

In conclusion, this work demonstrates the significant value of ^{1}H NMR spectroscopy as a rapid and non-invasive method to (a) find GSD biomarkers, knowledge of which may aid in designing new disease diagnosis and tracking methods, (b) contribute with new biochemical information about the disease and (c) form the basis of pattern recognition models to aid rapid and objective GSD diagnosis. The specificity of the changes observed to GSD1a is not fully known, at this stage. However, the detection of a new indicator peak (possibly arising from *α*-hydroxyisobutyrate) and particular changes in lipoprotein composition, together with the simultaneous changes in lactate, glucose acetate and ketone bodies, should start to form a picture of increasing specificity for GSD diagnosis. The work described here is currently being pursued along several parallel lines, namely the detailed characterisation of the nature of lipoproteins and new biomarkers and their relation to the disease and the building of NMR-based models allowing rapid diagnosis of the disease. The latter require a larger number of samples compared with those shown here and will

therefore require sample collection during long periods of time, owing to the low incidence of the disease.

Acknowledgements

REFERENCES

1. Lindon JC, Nicholson JK, Everett JR. NMR spectroscopy of biofluids. *Annu. Rep. NMR Spectrosc*. 1999; **38**: 1–88.
2. Lindon JC, Holmes E, Bollard ME, Stanley EG, Nicholson JK. Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. *Biomarkers* 2004; **9**: 1–31.
3. Holmes E, Foxall PJD, Nicholson JK, Neild GH, Brown SM, Beddell CR, Sweatman BC, Rahr E, Lindon JC, Spraul M, Neidig P. Automatic data reduction and pattern recognition methods for analysis of ^{1}H nuclear magnetic resonance spectra of human urine from normal and pathological states. *Anal. Biochem*. 1994; **220**: 284–296.
4. Holmes E, Foxall PJD, Spraul M, Farrant RD, Nicholson JK, Lindon JC. 750 MHz ^{1}H NMR spectroscopy characterisation of the complex metabolic pattern of urine from patients with inborn errors of metabolism: 2-hydroxyglutaric aciduria and maple syrup urine disease. *J. Pharm. Biomed. Anal*. 1997; **15**: 1647–1659.
5. Wevers RA, Engelke UFH, Moolenaar SH, Brautigam C, de Jong JGN, Duran R, de Abreu RA, van Gennip AH. ^{1}H-NMR spectroscopy of body fluids: inborn errors of purine and pyrimidine metabolism. *Clin. Chem*. 1999; **45**: 539–548.
6. Moolenaar SH, Engelke UFH, Abeling NGGM, Mandel H, Duran M, Wevers RA. Prolidase deficiency diagnosed by ^{1}H NMR spectroscopy of urine. *J. Inherit. Metab. Dis*. 2001; **24**: 843–850.
7. Sewell AC, Murphy HC, Iles RA. Proton nuclear magnetic resonance spectroscopy detection of sialic acid storage disease. *Clin. Chem*. 2002; **48**: 357–359.
8. Engelke UFH, Liebrand-van Sambeek MLF, de Jong JGN, Leroy JG, Morava E, Smeitink JAM, Wevers RA. *N*-Acetylated metabolites in urine: proton nuclear magnetic resonance spectroscopic study on patients with inborn errors of metabolism. *Clin. Chem*. 2004; **50**: 58–66.
9. Moolenaar SH, Poggi-Bach J, Engelke UFH, Corstiaensen JMB, Heerschap A, de Jong JGN, Binzak BA, Vockley J, Wevers RA. Defect in dimethylglycine dehydrogenase, a new inborn error of metabolism: NMR spectroscopy study. *Clin. Chem*. 1999; **45**: 459–464.
10. Moolenaar SH, van der Knaap MS, Engelke UFH, Pouwels PJW, Janssen-Zijlstra FSM, Verhoeven NM, Jakobs C, Wevers RA. *In vivo* and *in vitro* NMR spectroscopy reveal a putative novel inborn error involving polyol metabolism. *NMR Biomed*. 2001; **14**: 167–176.
11. Moolenaar SH, Gohlich-Ratmann G, Engelke UFH, Spraul M, Humpfer E, Dvortsak P, Voit T, Hoffmann GF, Brautigam C, van Kuilenburg AB, van Gennip A, Vreken P, Wevers RA. β-Ureidopropionase deficiency: a novel inborn error of metabolism discovered using NMR spectroscopy on urine. *Magn. Reson. Med*. 2001; **46**: 1014–1017.
12. Wolfsdorf JI, Weinstein DA. Glycogen storage diseases. *Rev. Endocr. Metab. Disord*. 2003; **4**: 95–102.
13. Chen YT. Type-I glycogen storage disease – kidney involvement, pathogenesis and its treatment. *Pediatr. Nephrol*. 1991; **5**: 71–76.
14. Talente GM, Coleman RA, Alter C, Baker L, Brown BI, Cannon RA, Chen YT, Crigler JF, Ferreira P, Haworth JC, Herman GE, Issenman RM, Keating JP, Linde R, Roe TF, Senior B, Wolfsdorf JI. Glycogen storage disease in adults. *Ann. Intern. Med*. 1994; **120**: 218–226.
15. Lin CC, Tsai JD, Lin SP, Lee HC. Renal sonographic findings of type I glycogen storage disease in infancy and early childhood. *Pediatr. Radiol*. 2005; **35**: 786–791.
16. Weinstein DA, Wolfsdorf JI. Effect of continuous glucose therapy with uncooked cornstarch on the long-term clinical course of type 1a glycogen storage disease. *Eur. J. Pediatr*. 2002; **161**: S35–S39.
17. Daublin G, Schwahn B, Wendel U. Type I glycogen storage disease: favourable outcome on a strict management regimen avoiding increased lactate production during childhood and adolescence. *Eur. J. Pediatr*. 2002; **161**: S40–S45.
18. Bodamer AO, Feillet F, Lane RE, Lee PJ, Dixon MA, Halliday D, Leoard JV. Utilization of cornstarch in glycogen storage disease type Ia. *Eur. J. Gastroenterol. Hepatol*. 2002; **14**: 1251–1256.
19. Powell RC, Wentworth SM, Brandt IK. Endogenous glucose-production in type-I glycogen-storage disease. *Metabolism* 1981; **30**: 443–450.
20. Shieh JJ, Pan CJ, Mansfield BC, Chou JY. A glucose-6-phosphate hydrolase, widely expressed outside the liver, can explain age-dependent resolution of hypoglycemia in glycogen storage disease type Ia. *J. Biol. Chem*. 2003; **278**: 47098–47103.
21. Hers HG, Van Hoof F, De Barsy T. Glycogen Storage Disease. In *The Metabolic Basis of Inherited Disease*, Scriver CR, Beaudet AL, Sly WS, Valle D (eds). McGraw Hill: New York, 1989; 425–452.
22. Labrune P, Jehenson P, Syrota A, Odievre M. *In vivo* ^{13}C-NMR evaluation of glycogen-content in a patient with glycogen storage disease. *J. Inherit. Metab. Dis*. 1992; **15**: 723–726.
23. Roser W, Beckmann N, Wiesmann U, Seelig J. Absolute quantification of the hepatic glycogen content in a patient with glycogen storage disease by ^{13}C magnetic resonance spectroscopy. *Magn. Reson. Imaging* 1996; **14**: 1217–1220.
24. Oberhaensli RD, Rajagopalan B, Taylor DJ, Radda GK, Collins JE, Leonard JV. Study of liver metabolism in glucose-6-phosphatase deficiency (glycogen storage disease type IA) by P-31 magnetic resonance spectroscopy. *Pediatr. Res*. 1988; **23**: 375–380.
25. Kalderon B, Korman SH, Gutman A, Lapidot A. Estimation of glucose carbon recycling in children with glycogen storage disease: a ^{13}C NMR study using (U-^{13}C)glucose. *Proc. Natl. Acad. Sci. USA* 1989; **86**: 4690–4694.
26. Lapidot A. Inherited disorders of carbohydrate metabolism in children studied by ^{13}C-labelled precursors, NMR and GC–MS. *J. Inherit. Metab. Dis*. 1990; **13**: 466–475.
27. Meiboom S, Gill D. Modified spin-echo method for measuring nuclear relaxation times. *Rev. Sci. Instrum*. 1958; **29**: 688–691.
28. Wu DH, Chen AD, Johnson CS Jr. An improved diffusion-ordered spectroscopy experiment incorporating bipolar-gradient pulses. *J. Magn. Reson. A* 1995; **115**: 260–264.
29. Barros AS. Contribution à la sélection et la comparaison de variables caractéristiques. PhD Thesis, Institut National Agronomique Paris-Grignon, Paris, 1999.
30. Nicholson JK, Foxall PJD, Spraul M, Farrant RD, Lindon JC. 750 MHz ^{1}H and ^{1}H–^{13}C NMR spectroscopy of human blood plasma. *Anal. Chem*. 1995; **67**: 793–811.
31. Tang H, Wang Y, Nicholson JK, Lindon JC. Use of relaxation-edited one-dimensional and two-dimensional nuclear magnetic resonance spectroscopy to improve detection of small metabolites in blood plasma. *Anal. Biochem*. 2004; **325**: 260–272.
32. Liu M, Nicholson JK, Lindon JC. High-resolution diffusion and relaxation edited one- and two-dimensional ^{1}H NMR spectroscopy of biological fluids. *Anal. Chem*. 1996; **68**: 3370–3376.
33. Liu M, Nicholson JK, Parkinson JA, Lindon JC. Measurement of biomolecular diffusion coefficients in blood plasma using two-dimensional ^{1}H–^{1}H diffusion-edited total-correlation NMR spectroscopy. *Anal. Chem*. 1997; **69**: 1504–1509.
34. Liu M, Tang H, Nicholson JK, Lindon JC. Use of ^{1}H NMR-determined diffusion coefficients to characterize lipoprotein fractions in human blood plasma. *Magn. Reson. Chem*. 2002; **40**: S83–S88.
35. de Graaf RA, Behar KL. Quantitative ^{1}H NMR spectroscopy of blood plasma metabolites. *Anal. Chem*. 2003; **75**: 2100–2104.
36. Yang Y, Bai G, Zhang X, Ye C, Liu M. ^{1}H NMR spectroscopic evidence of interaction between ibuprofen and lipoproteins in human blood plasma. *Anal. Biochem*. 2004; **324**: 292–297.
37. Dyrby M, Petersen M, Whittaker AK, Lambert L, Norgaard L, Bro R, Engelsen SB. Analysis of lipoproteins using 2D diffusion-edited

NMR spectroscopy and multi-way chemometrics. *Anal. Chim. Acta* 2005; **531**: 209–216.

38. Ala-Korpela M, Korhonen A, Keisala J, Horkko S, Korpi P, Ingman LP, Jokisaari J, Savolainen MJ, Kesaniemi YA. ^{1}H NMR-based absolute quantification of human lipoproteins and their lipid contents directly from plasma. *J. Lipid Res.* 1994; **35**: 2292–2304.

39. Daykin CA, Corcoran O, Hansen SH, Bjornsdottir I, Cornett C, Connor S, Lindon JC, Nicholson JK. Application of directly coupled HPLC NMR to separation and characterization of lipoproteins from human serum. *Anal. Chem.* 2001; **73**: 1084–1090.

40. Willker W, Leibfritz D. Assignment of mono- and polyunsaturated fatty acids in lipids of tissues and body fluids. *Magn. Reson. Chem.* 1998; **36**: S79–S84.

41. Wolfsdorf JI, Laffel LMB, Crigler JF Jr. Metabolic control and renal dysfunction in type I glycogen storage disease. *J. Inherit. Metab. Dis.* 1997; **20**: 559–568.

42. Hagen T, Korson MS, Wolfsdorf JI. Urinary lactate excretion to monitor the efficacy of treatment of type I glycogen storage disease. *Mol. Genet. Metab.* 2000; **70**: 189–195.

43. Binkiewicz A, Senior B. Decreased ketogenesis in von Gierke's disease (glycogenosis type I). *J. Pediatr.* 1973; **83**: 973–978.

44. Havel RJ, Balasse EO, Williams HE, Kane JP, Segel N. Splanchnic metabolism in von Gierke's disease (glycogenosis type I). *Trans. Assoc. Am. Physicians* 1969; **82**: 305–323.

45. Bandsma RHJ, Wiegman CH, Herling AW, Burger HJ, ter Harmsel A, Meijer AJ, Romijn JA, Reijngoud DJ, Kuipers F. Acute inhibition of glucose-6-phosphatase translocator activity leads to increased *de novo* lipogenesis and development of hepatic steatosis without affecting VLDL production in rats. *Diabetes* 2001; **50**: 2591–2597.

46. Tollinger CD, Vreman HJ, Weiner MW. Measurement of acetate in human blood by gas chromatography: effects of sample preparation, feeding and various diseases. *Clin. Chem.* 1979; **25**: 1787–1790.

47. Fleming SE, Arce DS. Volatile fatty acids: their production, absorption, utilization and roles in human health. *Clin. Gastroenterol.* 1986; **15**: 787–814.

48. Levy E, Thibault LA, Roy CC, Bendayan M, Lepage G, Letarte J. Circulating lipids and lipoproteins in glycogen storage disease type I with nocturnal intrastric feeding. *J. Lipid Res.* 1988; **29**: 215–226.

49. Keddad K, Razavian SM, Baussan C, Chalas J, Abella A, Levenson J, Simon A, Moatti N, Legrand A. Blood lipids and rheological modifications in glycogen storage disease. *Clin. Biochem.* 1996; **29**: 73–78.

50. Wierzbicki AS, Watts GF, Lynas J, Winder AF, Wray R. Very low-density lipoprotein apolipoprotein B-100 turnover in glycogen storage disease type Ia (von Gierke disease). *J. Inherit. Metab. Dis.* 2001; **24**: 527–534.

51. Bandsma RHJ, Smit GPA, Kuipers F. Disturbed lipid metabolism in glycogen storage disease type 1. *Eur. J. Pediatr.* 2002; **161**: S65–S69.

52. Bandsma RHJ, Rake JP, Visser G, Neese RA, Hellerstein MK, van Duyvenvoorde W, Princen HMG, Stellaard F, Smit GPA, Kuipers F. Increased liopgenesis and resistance of lipoproteins to oxidative modification in two patients with glycogen storage disease type 1a. *J. Pediatr.* 2002; **140**: 256–260.

53. Bathen TF, Krane J, Engan T, Bjerve KS, Axelson D. Quantfication of plasma lipids and apolipoproteins by use of proton NMR spectroscopy, multivariate and neural network analysis. *NMR Biomed.* 2000; **13**: 271–288.