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ORIGINAL PAPER

Metabolomic study of plasma of patients with abdominal aortic aneurysm

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Abstract Abdominal aortic aneurysm (AAA) is an important health problem, both because of AAA rupture and death and because of increased cardiovascular mortality. Identification of new biomarkers of AAA may suggest novel pathological mechanisms and targets for new medical treatments to slow AAA progression. Metabolic changes in AAA patients were mainly related to carbohydrate and lipid metabolism and many of these changes can be associated with a situation of insulin resistance (which can be related to metabolic syndrome) together with altered amino acid metabolism. For the first time, metabolites that can be associated with differential metabolism by the gut microflora of AAA patients have also been found. Moreover, aminomalonic acid in plasma has been shown to be the metabolite with the biggest difference between patients suffering from large aneurysm (>5 cm) and controls.

Keywords Metabonomics · Aortic abdominal aneurysm · Metabolic pathways

Introduction

Abdominal aortic aneurysm (AAA) is an important health problem which occurs in up to 9 % of adults older than 65 years of age, causing approximately 1–

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2 % of male deaths in Western countries [1]. In individuals with cardiovascular (CV) disease and/or multiple risk factors, those with diagnosed AAA are more likely to suffer cardiovascular hospitalization and deaths than non-AAA subjects, probably not only because of AAA rupture and death, but also because of more widespread polyvascular manifestation of atherothrombotic disease in the affected individuals [2]. In this regard, infrarenal aortic diameter is an indicator of AAA disease, but is also an independent marker of all-cause mortality, mainly related to CV disease [3].

AAA is characterized by localized structural deterioration of the wall of the aorta, leading to progressive dilation and eventual rupture [4]. Efforts to limit mortality from AAA rupture depend on early detection and elective AAA repair. However, most AAAs have discontinuous growth patterns and alternate periods of stability and non-growth with periods of acute expansion and occasionally rupture [5]. The indication for elective repair is based upon a maximum diameter of AAA above 5 to 5.5 cm [6]. However, management of small AAA remains controversial, which is why associating concentrations of circulating proteins with AAA has been of interest [7, 8]. Furthermore, identification of new biomarkers may suggest novel pathological mechanisms and targets for new medical treatments to slow AAA progression.

In the search for novel biomarkers of AAA, our recent proteomic and metabolomic studies using human AAA tissues obtained at surgery and kept in culture have identified candidate molecules released to the conditioned media [9, 10]. The compounds identified are related to different pathological mechanisms involved in AAA (e.g. oxidative stress). Some metabolites (e.g. hippuric acid) had not previously been associated with AAA; others (e.g. fatty acid amides) may arise as a promising line of research [9]. The utility of high-throughput "omics" in systems biology becomes clear when applied to human material from multifactorial diseases with complex biology, for example CV diseases [11]. These techniques will help to decipher the complexity and the biodiversity of human pathology that will enable us to ameliorate both prediction and treatment of patients on the way to the era of individualized medicine [12]. With this purpose in mind, in this study analysis of the plasma of AAA patients in a non-targeted global approach, by use of GC–MS and ^{1}H NMR, was undertaken.

Materials and methods

Solvents and reagents

The reference compounds for both GC–MS and ^{1}H NMR were purchased from Sigma (St Louis, MO, USA) and Aldrich (Steinhein, Germany). Compounds and reagents were all of analytical grade except where stated otherwise. *N*,*O*-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) plus 1 % trimethylchlorosilane (TMCS) were purchased from Pierce Chemical (Rockford, IL, USA). *O*-Methylhydroxylamine hydrochloride, pyridine (silylation grade), and heptane (analytical grade) were obtained from Sigma. Methanol, acetone, and acetonitrile (HPLC grade) were from Supelco (Bellefonte, PA, USA). Ultra pure water was produced by a Milli-Q reagent water system (Millipore, MA, USA). Deuterium oxide (D_2O; 99.9 % D) was purchased from Euriso-Top (Saint-Aubin, France).

Stock solutions of the reference compounds were prepared either in Milli-Q water or in methanol at the same concentration of 25 mmol L^{-1}. These solutions were kept at 4 °C or −20 °C. Before analysis the solutions were diluted 1:100 with methanol and derivatized in accordance with the GC–MS method.

Samples

The AAA patients and the controls were recruited in Fundación Jimenez Diaz and Galdakao Hospital, whose ethics committee approved the study. Blood was collected from two groups of patients with abdominal aortic aneurysm: small-AAA (AAA diameter = 3–5 cm) and large-AAA (AAA > 5 cm) and control subjects (AAA diameter<3 cm) matched by age and sex. Specifically, 22 patients and 11 controls were analyzed by GC–MS and NMR. All subjects were in a fasting state, and blood samples were taken in the morning (between 0800 and 1100). Fresh EDTA anticoagulated blood was centrifuged at 3000*g* for 15 min. Plasma was stored in aliquots at −80 °C until the day of analysis.

Plasma fingerprinting by GC–MS

Plasma was treated as described elsewhere [13, 14]. Briefly 50 μL plasma was methoxylated and silylated before GC–MS analysis with a Varian instrument 3900 Series GC. Helium was used as carrier gas at a constant flow rate of 1 mL min^{-1} through the column (FactorFour capillary column, VF-5 ms, 30 m×0.25 mm ID, DF=0.25 μm; Varian, USA). The mass spectrometer detector was a Saturn 2110 T with ion trap.

Metabolites in the profiles were identified from their retention times and by comparison with mass spectra either in the NIST library or in a library built in house by use of the pure standards analyzed under the same analytical conditions.

Plasma fingerprinting with ^{1}H NMR

^{1}H NMR spectra were recorded at 298 K on a Bruker Avance 400 spectrometer, equipped with a 5 mm BBI probe and *z*-gradient coil. A transverse relaxation-edited sequence (d1–90°–(τ/2–180 °–τ/2)n acquisition, called the Carr–Purcell–Meiboom–Gill (CPMG) sequence) was acquired with a total echo time of 508 ms (n=265, τ=1.9 ms) and a recycle delay of 2 s enabling the broad lines to be suppressed and the signals of the low-molecular-weight metabolites to be readily observed. 32 k-data points with a spectral width of 5,600 Hz and 1,024 scans were used for both experiments. Data were processed by use of Bruker TopSpin software 2.1. Before Fourier transformation, data were zero-filled by a factor of 1 and multiplied by an exponential function corresponding to line broadening of 0.3 Hz. Spectra were then phased and a baseline correction was applied. The chemical shifts of the signals were referenced to the lactate methyl doublet at 1.33 ppm. The signals of the metabolites were identified as described elsewhere [14].

Data alignment and treatment

First, raw signals were baseline corrected, then multialignment of the GC–MS chromatograms was performed with in-house software developed in Matlab 7.0, as described elsewhere [15], using correlation optimised warping (COW) as firstly described by Nielsen et al. [16], and later implemented for chromatographic data by Tomasi and van den Berg et al. [17, 18]. Briefly, the COW algorithm requires input of two variables—a section length and a value of the maximum deformation within a section. COW can be applied to any data but the values of the two variables depend on individual characteristics of the data set and must be customized. After multialignment, GC–MS profiles were normalized to the internal standard C18:0 methyl ester.

By analogy, baseline correction and multialignment of ^{1}H NMR spectra were performed after removing the spectral regions containing water (4.3–5.2 ppm) and EDTA signals (2.53–2.58, 2.68–2.70, 3.05–3.23, and 3.60–3.64 ppm).

To combine ^{1}H NMR and GC–MS data a ratio factor was calculated taking into account the highest signals from both. The factor was obtained by dividing the value of the higher signal by the value of the lower one. GC–MS data were multiplied by this factor and data sets from both techniques were merged into one matrix of comparable variables and exported as an MSExcel file for statistical analysis. Subsequently, the three sets of data were analyzed independently with SIMCA P+ 12.0 (Umetrics, Sweden) to obtain PCA, PLS-DA, and OPLS-DA results.

Results

Figures 1 and 2 show the comparative profiles for controls, and small and large AAA in ^{1}H NMR and GC–MS, respectively. After baseline correction and multialignment, pattern-recognition techniques were applied. The final set contained 33 samples for ^{1}H NMR and GC–MS profiles.

PCA data analysis was performed individually for each technique and the combined data in order to spike strong outliers and to obtain a general overview of the data. In a plot of tree groups no significant separation was observed (data not shown) but the group of large AAA showed a tendency to separate from small AAA and control groups.

To achieve optimum disease-related differentiation and to identify candidate biomarkers of the pathology, partial least-squares discriminant analysis (PLS-DA) was performed. Univariance (UV) scaling was used because it proved to work the best for this kind of data. Score plots of PLS-DA of all observations revealed significant differences between groups (Fig. 3).

To identify metabolic changes each possible pair of the groups were compared by performing PLS-DA and orthogonal partial least-squares discriminant analysis (OPLS-DA). OPLS-DA was applied to sharpen the separation between groups and to obtain the significance of the correlation between the variable and the scores on the predictive vector.

When clustering according to the pathology was observed in PLS-DA and OPLS-DA the loading plot considering variables with higher weight in the classification was investigated. Variables in the data matrix are a numerical analogue of either a chemical shift (δ (ppm)) in ^{1}H NMR or a retention time (min) in GC–MS. Therefore, identifying discriminant variables enabled them to be assigned to 45 specific metabolites in the profiles, listed in Table 1, together with the technique that was used for its determination.

Discriminant variables were picked up in PLS-DA analysis as those that had the heaviest weight in the loading plot when two compared groups were separated. Discriminators were confirmed by OPLS-DA as those that had a high correlation with the predictive component in a score plot. Thirty-three metabolites were exclusive to one of the techniques (NMR or GC–MS) whereas 12 metabolites were identified with both techniques (myoinositol, pyruvate, lactate, 3-hydroxybutyrate, citrate, alanine, serine, threonine, creatinine, oxalate, and glycerol). In each case of co-identification, common trends were observed, and the analytical reliability of the signal was evaluated. For further quantitative evaluation one of the signals (GC–MS or NMR) was chosen. Thirty-nine of the metabolites were significant in the discriminant analysis both for clustered (NMR+GC–MS) and non-clustered data, whereas six metabolites disappeared from the clustered loading plot (2-aminobutyrate,

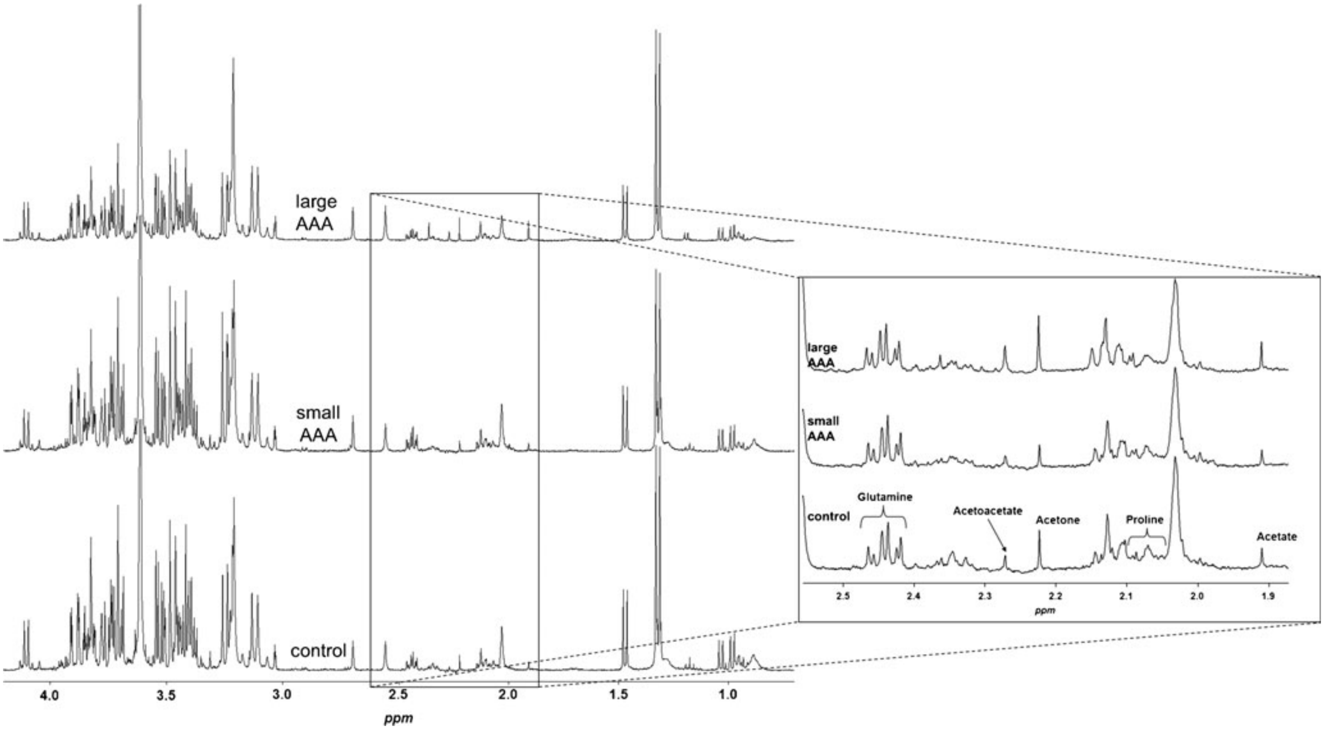

Fig. 1 Comparative ^{1}H NMR profiles obtained from plasma samples from controls and from patients with small and large aneurysms. For conditions, see text

octanoate, and stearate from GC–MS; glucose, acetate, and threonine from NMR).

The values for the variation (ratio) between the respective pathological situations and the controls were estimated. To calculate the ratio for the metabolites from GC–MS, the abundances (areas of peaks) of the metabolites were averaged by group; for NMR the intensities of all the drifts were averaged sample by sample, and those assigned to a single metabolite were averaged by group. For all metabolites in both techniques, the differences between the pathological and control groups divided by the average control (percentage) were calculated (Table 1). These values are also depicted in Figs. 4, 5, and 6, in the representation of specific metabolic pathways in which the metabolites are involved (glycolysis, Fig. 4; amino acid catabolism, Fig. 5; lipid metabolism, Fig. 6).

Mannitol was also found significant, but was discarded from the study because it was administered to four of the AAA patients before surgery.

Among the 45 metabolites, different patterns of variation relating to controls can be observed. For eighteen a "common" trend was observed: they were higher in both small-AAA and large-AAA or lower in both small-AAA and large-AAA than in controls. Among the metabolites with common trends, glucose, phosphate (Fig. 4), creatine (Fig. 5), and formate, caprylate, and oleate (Fig. 6) were higher in the small-AAA group than in controls, and even higher in the large-AAA group, whereas the trend for serine, tryptophan (Fig. 5), laurate, methanol, and ethanol (Fig. 6), was the opposite: they were smaller in small-AAA than in controls, and even smaller in large-AAA. On the other hand, for myoinositol, uric acid (Fig. 4), proline, creatinine, 2-aminobutyrate, and urea (Fig. 5), the differences between small-AAA and controls were larger than those between large-AAA and controls. Similar behaviour, but with the opposite trend, was observed for threonine (Fig. 5): it was higher in both small and large-AAA than in controls, but the difference compared with controls was smaller for large-AAA.

For twenty-seven metabolites variations between controls, and small and large-AAA were observed that did not share a common trend: fructose, glycerate (Fig. 6), succinate, glutamine, and oxalate (Fig. 5), were higher in small-AAA than in controls but smaller when compared with the large-AAA group. For twenty-two metabolites average values in small-AAA were lower than in controls, but when large-AAA was present, lactate, aminomalonate (Fig. 4), pyruvate (Figs. 4 and 5), 3-hydroxybutyrate, acetoacetate, acetone, acetate, citrate, glycerol, caproate, stearate, vaccinate, linoleate, palmitate, (Fig. 6), alanine, asparagine, histidine, valine, leucine, isoleucine, tyrosine, and 2-hydroxybutyrate, (Fig. 5) were also higher than in controls.

For small-AAA the variations ranged from −38 % (myoinositol) to +35 % (succinate), whereas changes were larger for the large-AAA group. The largest difference in the large-AAA group compared with controls (+803 %) was observed for aminomalonate; the ethanol ratio was the lowest in the AAA group (−41 %).

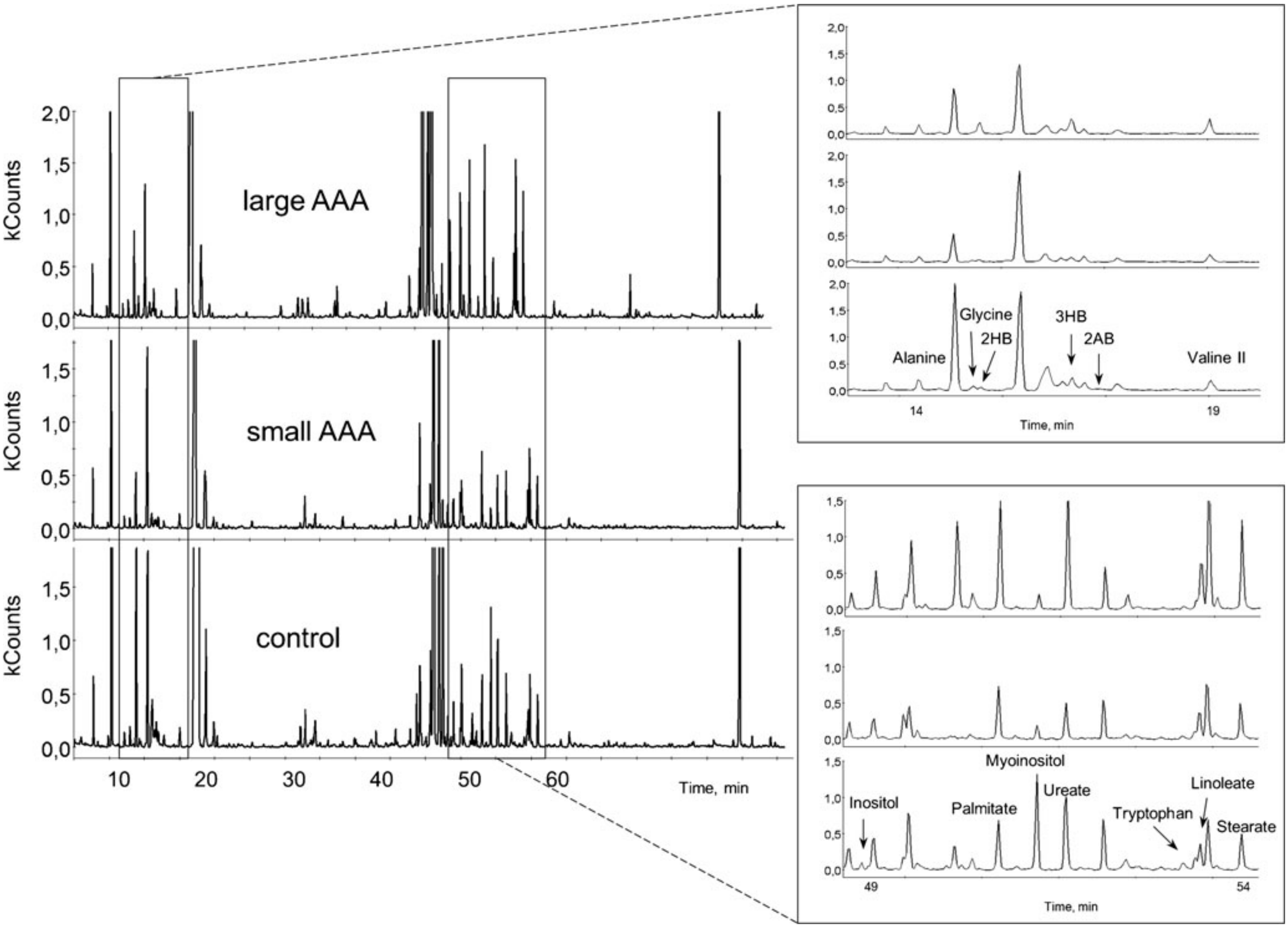

Fig. 2 Comparative GC–MS profiles obtained from plasma samples from controls and from patients with small and large aneurysms. For conditions, see text

Discussion

PLS-DA of the aligned signals from NMR and GC–MS showed that samples were successfully classified (Fig. 3). The classification achieved by use of NMR signals was slightly improved by adding the results from GC–MS, i.e. more information about significant metabolites was obtained by means of this strategy.

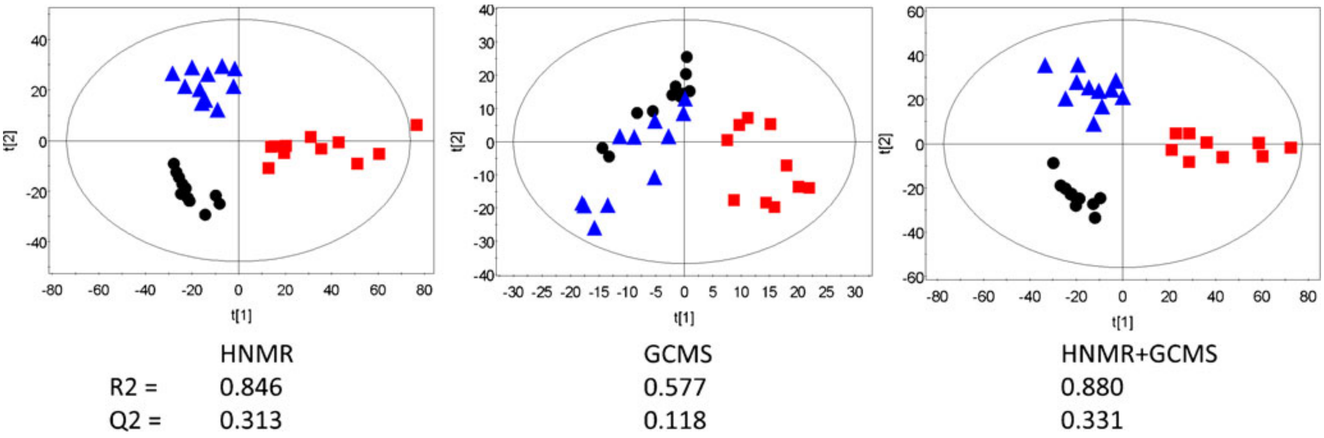

Fig. 3 PLS-DA—1/2 component—discrimination between large aneurysms (AAA size > 5 cm, *red squares*), small aneurysms (AAA size = 3–5 cm, *black dots*), and controls (*blue triangles*). From the left: NMR, GC–MS, NMR + GC–MS

Table 1 Metabolites found to be significant by GC–MS and NMR in the models from AAA patients and control subjects

Abbreviation	Compound	Formula	Technique	Identification[a]	Ratio, small AAA/Ctrl (%)	Ratio, large AAA/Ctrl (%)	Fig.
GLC	Glucose	$C_6H_{12}O_6$	^{1}H NMR	L/ST	4	36	4
FRC	Fructose	$C_6H_{12}O_6$	GC–MS	L/ST	1	−20	4
MYO	Myoinositol	$C_6H_{12}O_6$	GC–MS	L/ST	−48	−20	4
GLRT	Glycerate	$C_3H_5O_4$	GC–MS	L/ST	3	−2	4
PYR	Pyruvate	$C_3H_3O_3$	GC–MS	L/ST	−7	64	4, 5, 6
LAC	Lactate	$C_3H_5O_3$	^{1}H NMR	L	−33	8	4
CIT	Citrate	$C_6H_5O_7$	^{1}H NMR	L	−29	71	5
SUC	Succinate	$C_4H_2O_4$	GC–MS	L/ST	21	−25	5
2HB	2-Hydroxybutyrate	$C_4H_7O_3$	GC–MS	L/ST	−14	132	5
2AB	2-Aminobutyrate	$C_4H_8NO_2$	GC–MS	L/ST	−37	−5	5
THR	Threonine	$C_4H_9NO_3$	^{1}H NMR	L/ST	9	3	5
ALA	Alanine	$C_3H_7NO_2$	GC–MS	L/ST	−1	13	5
ASN	Asparagine	$C_4H_8N_2O_3$	GC–MS	L/ST	−27	145	5
SER	Serine	$C_3H_7NO_3$	^{1}H NMR	L/ST	−1	−30	5
PRO	Proline	$C_5H_9NO_2$	^{1}H NMR	L/ST	−6	−5	5
GLN	Glutamine	$C_5H_{10}N_2O_3$	^{1}H NMR	L/ST	5	−10	5
HIS	Histidine	$C_6H_9N_3O_2$	^{1}H NMR	L/ST	−9	6	5
VAL	Valine	$C_5H_{11}NO_2$	^{1}H NMR	L/ST	−6	1	5
LEU	Leucine	$C_6H_{13}NO_2$	^{1}H NMR	L/ST	−21	22	5
ILE	Isoleucine	$C_6H_{13}NO_2$	^{1}H NMR	L/ST	−22	23	5
TYR	Tyrosine	$C_9H_{11}NO_3$	^{1}H NMR	L/ST	−7	10	5
TRP	Tryptophan	$C_{11}H_{12}N_2O_2$	GC–MS	L/ST	−13	−21	5
CRE	Creatine	$C_4H_9N_3O_2$	^{1}H NMR	L	0	92	5
CRN	Creatinine	$C_4H_7N_3O$	GC–MS	L/ST	−16	−2	5
OXA	Oxalate	C_2O_4	GC–MS	L/ST	26	−1	5
UREA	Urea	CH_4N_2O	GC–MS	L/ST	−12	−2	5
AC	Acetate	$C_2H_3O_2$	^{1}H NMR	L	−16	17	5, 6
ACAC	Acetoacetate	$C_4H_5O_3$	^{1}H NMR	L	−34	96	5, 6
FOR	Formate	CHO_2	^{1}H NMR	L	14	74	5, 6
3HB	3-Hydroxybutyrate	$C_4H_7O_3$	GC–MS	L/ST	−40	41	6
ACE	Acetone	C_3H_6O	^{1}H NMR	L/ST	−32	39	6
CAP	Caproate	$C_6H_{11}O$	GC–MS	L	−29	68	6
CAY	Caprylate	$C_8H_{15}O_2$	GC–MS	L/ST	12	344	6
LAU	Laurate	$C_{12}H_{23}O_2$	GC–MS	L/ST	−2	−40	6
PAL	Palmitate	$C_{16}H_{31}O_2$	GC–MS	L/ST	−15	21	6
STE	Stearate	$C_{18}H_{35}O_2$	GC–MS	L/ST	−22	7	6
VAC	Vaccenate	$C_{18}H_{33}O_2$	GC–MS	L/ST	−15	16	6
OLE	Oleate	$C_{18}H_{33}O_2$	GC–MS	L/ST	8	13	6
LIN	Linoleate	$C_{18}H_{31}O_2$	GC–MS	L/ST	−35	19	6
GOL	Glycerol	$C_3H_8O_3$	^{1}H NMR	L/ST	−12	127	6
EOH	Ethanol	C_2H_6O	^{1}H NMR	L/ST	−12	−41	6
MOH	Methanol	CH_4O	^{1}H NMR	L/ST	−30	−51	6
PO4	Phosphate	PO_4	GC–MS	L	42	68	4
URIC	Uric acid	$C_5H_4N_4O_3$	GC–MS	L/ST	−31	−13	4
AMA	Aminomalonate	$C_3H_5NO_4$	GC–MS	L/ST	−15	830	4

[a] L, library; ST, standard

Plasma glucose was higher in large-AAA patients, which is indicative of disturbances in insulin signalling. Moreover, other changes also support this hypothesis: in large-AAA patients, plasma fructose was found to be lower than for controls whereas pyruvate and lactate were higher (Fig. 4). These changes suggest that glycolysis was more important for energy supply in these patients than in controls. Together with these disturbances in carbohydrate metabolism, free phosphate and ketones (acetoacetate, 3-hydroxybutyrate, and acetone) were also higher in AAA patients (Fig. 6). These variations are common to diabetic ketoacidosis, and these findings add evidence to the association between CV events and insulin resistance and/or metabolic syndrome [19–21]. Even more, all the fatty acids found in this investigation (except laurate) were significantly higher in the AAA groups than in controls, together with higher levels of glycerol (Fig. 6), suggesting that higher lipolysis is occurring, as would be expected with higher glucose, higher pyruvate, and higher ketones. From the information provided by metabolites related to carbohydrate and lipid metabolism, the biggest changes can be seen in large-AAA, and many of these changes can be associated with insulin resistance (which can be related to metabolic syndrome).

Furthermore, oxidative stress or detoxification demands can dramatically increase the rate of hepatic glutathione synthesis. Under such metabolic stress glutathione can be de-novo synthesized from cystathionine, releasing 2-hydroxybutyrate as a by-product [22]. In other studies we found increased 2-hydroxybutyrate [13, 14], and this compound has been proposed as an early biomarker of insulin resistance [23]. In addition, 2-hydroxybutyrate and 2-aminobutyrate are metabolites of 2-oxobutyrate, a product of catabolism of threonine (Fig. 5). They are generated by lactate dehydrogenase (LDH) or by transaminases, respectively. Because it has previously been reported that LDH activity is reduced in AAA [24], it seems more probable that the higher values of 2-hydroxybutyrate were related to the synthesis of glutathione, enhanced because of to greater inflammation.

In the list of significant metabolites analysed, some that could be related not only to changes in host metabolism, but also to different metabolism by the gut microflora (formate, acetate, acetone, ethanol, methanol, caprylate, caproate, and laurate) (Fig. 6) have been reported. As far as we are aware, gut microflora are not involved in either the incidence or progression of aneurysms, but different metabolomic approaches have correlated gut microflora to diabetes [25] and obesity [26].

It is difficult to draw conclusions from results for the amino acids found to be significant, because most of the changes observed are relatively small; in the small-AAA group the variations range from 16 % lower than controls

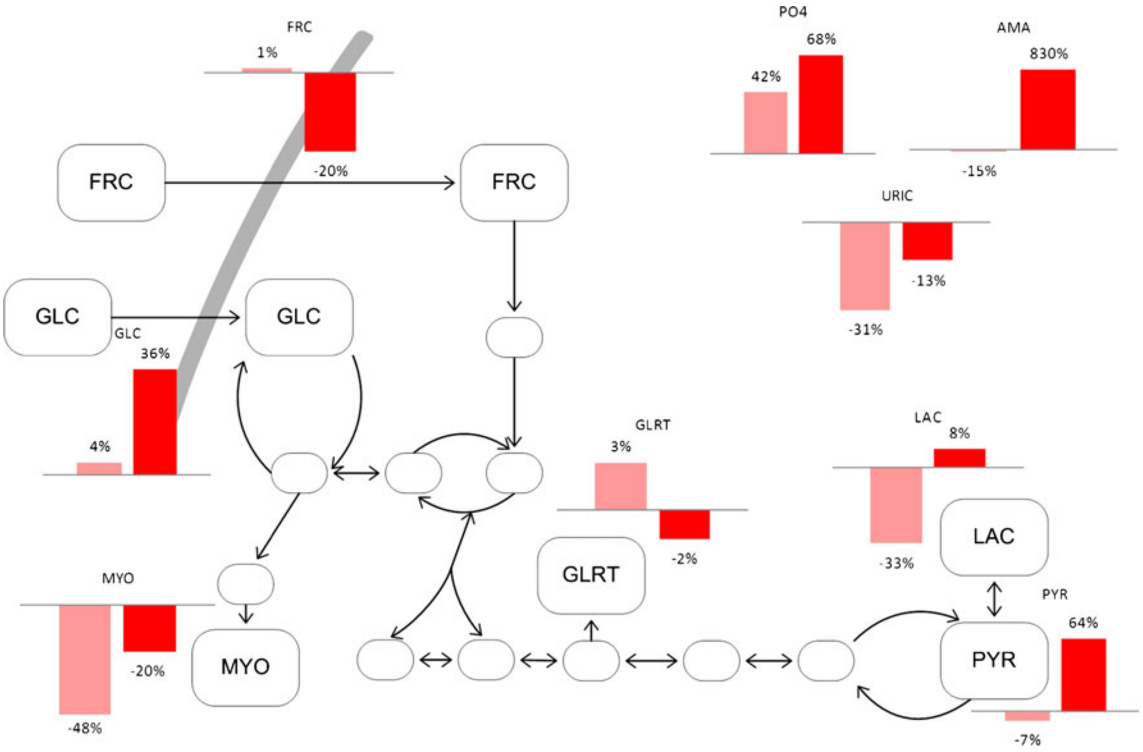

Fig. 4 Glycolysis-related metabolites and other metabolites found to be significant, and their differences compared with the control group. First column (*light red*), small AAA vs. control (100 %); second column (*deep red*), large AAA vs. control (100 %). *AMA*, aminomalonate; *FRC*, fructose; *FOR*, formate; *GLC*, glucose; *GLRT*, glycerate; *LAC*, lactate; *MYO*, *myo*-inositol; *PO4*, phosphate; *PYR*, pyruvate; *URIC*, uric acid

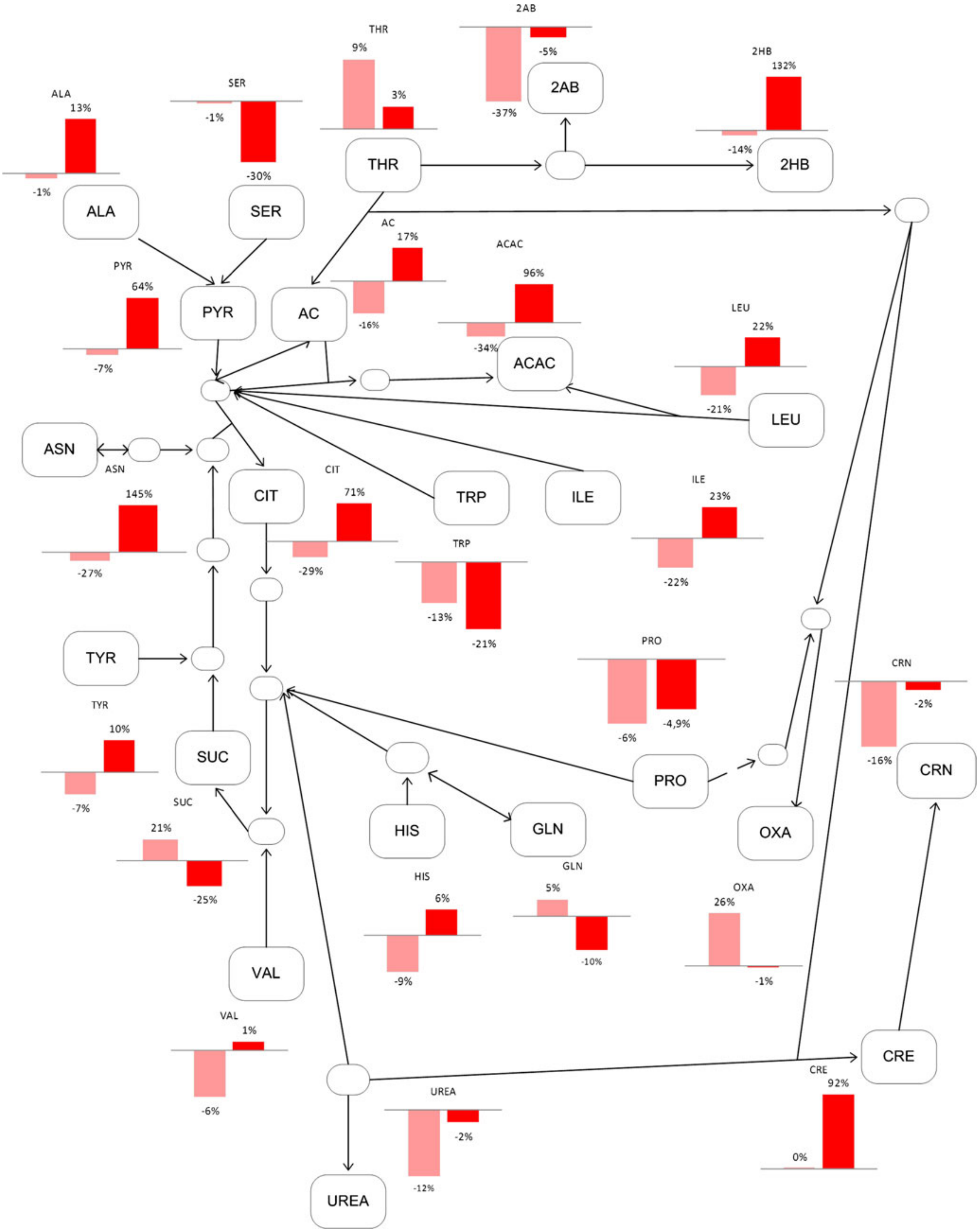

Fig. 5 Amino acid-related metabolites found to be significant, and their differences compared with the control group. First column (*light red*), small AAA vs. control (100 %); second column (*deep red*), large AAA vs. control (100 %). *2AB*, 2-aminobutyrate; *2HB*, 2-hydroxybutyrate; *ALA*, alanine; *ASN*, asparagine; *CIT*, citrate; *CRE*, creatine; *CRN*, creatinine; *GLN*, glutamine; *HIS*, histidine; *ILE*, isoleucine; *LEU*, leucine, *OXA*, oxalate; *PRO*, proline; *PYR*, pyruvate; *SER*, serine; *SUC*, succinate; *THR*, threonine; *TRP*, tryptophan; *TYR*, tyrosine; *UREA*, urea; *VAL*, valine

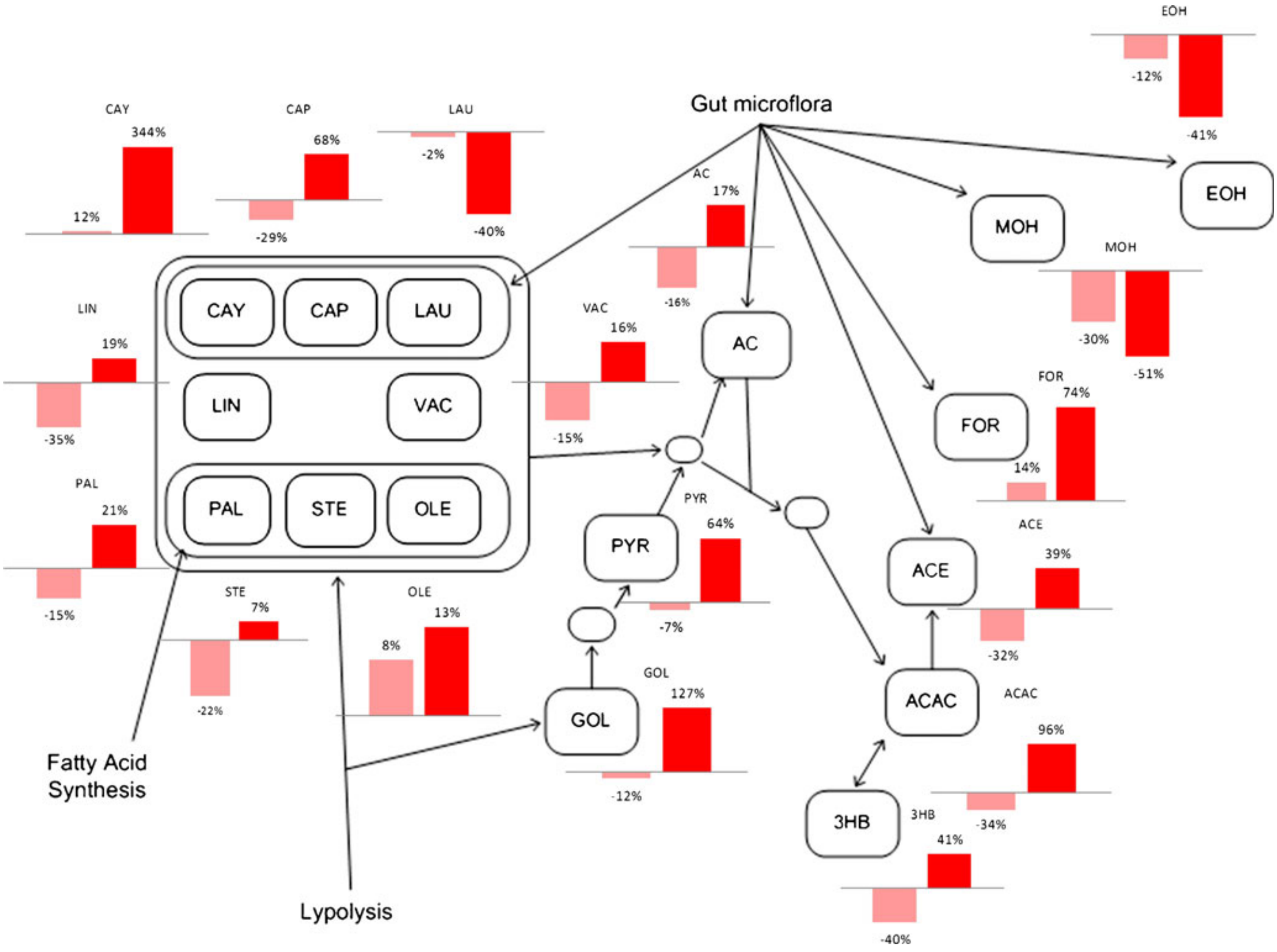

Fig. 6 Lipid-related metabolites found to be significant, and their differences compared with the control group. First column (*light red*), small AAA vs. control (100 %); second column (*deep red*), large AAA vs. control (100 %). *3HB*, 3-hydroxybutyrate; *AC*, acetate; *ACAC*, acetoacetate; *ACE*, acetone; *CAY*, caprylate; *CAP*, caproate; *GOL*, glycerol; *LAU*, laurate; *LIN*, linoleate; *OLE*, oleate; *PAL*, palmitate; *STE*, stearate; *VAC*, vaccinate

(asparagine) to 13 % higher than controls (threonine) (Fig. 6). Nevertheless, larger changes were found for large-aneurysm patients, from 32 % lower (serine) to 150 % higher than controls (asparagine), and it is becoming more evident that amino acid metabolism is altered in AAA patients.

It is worthy of note that the largest difference between aneurysm patients and controls was for aminomalonate, which was more than 800 % higher when a large aneurysm was present. Knowledge about this metabolite is scarce, and its relationship with aneurysm is unknown. In contrast, its presence in atherosclerotic plaque was described as early as 1984 [27]. Moreover, biodistribution studies of antibodies against aminomalonate injected in normal and hyperlipidemic rabbits revealed significantly greater staining for aminomalonate in atheromatous aortas than in normal aortas [28]. Its origin has, in addition, been attributed to radical-mediated oxidation of glycine [29], and it has recently been found to be reduced in the plasma of patients who have suffered from acute coronary syndrome [30].

This metabolomic study has revealed the relationship between inflammation, metabolic syndrome, and AAA. Furthermore, aminomalonate has emerged as a potential important biomarker in AAA, potentially associated with oxidative stress and proteolysis, two major pathological mechanisms in AAA. Further studies will be needed to confirm the potential association of aminomalonate with AAA.

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