Disclosure of a putative biosignature for respiratory chain disorders through a metabolomics approach

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Abstract The diagnosis of respiratory chain deficiencies (RCDs) is complicated and the need for a diagnostic biomarker or biosignature has been widely expressed. In this study, the metabolic profile of a selected group of 29 RCD patients, with a predominantly muscle disease phenotype, and 22 controls were investigated using targeted and untargeted analyses of three sub-sections of the human metabolome, including urinary organic acids and amino acids [measured by gas chromatography–mass spectrometry (GC–MS)], as well as acylcarnitines (measured by electrospray ionization tandem MS). Although MS technologies are highly sensitive and selective, they are restrictive by being applied only to sub-sections of the metabolome; an untargeted nuclear magnetic resonance (NMR) spectroscopy approach was therefore also included. After data reduction and pre-treatment, a biosignature comprising six organic acids (lactic, succinic, 2-hydroxyglutaric, 3-hydroxyisobutyric, 3-hydroxyisovaleric and 3-hydroxy-3-methylglutaric acids), six amino acids (alanine, glycine, glutamic acid, serine, tyrosine and α-aminoadipic acid) and creatine, was constructed from uni- and multivariate statistical analyses and verified by cross-validation. The results presented here provide the first proof-of-concept that the metabolomics approach is capable of defining a biosignature for RCDs. We postulate that the composite of organic acids ≈ amino acids > creatine > betaine > carnitines represents the basic biosignature for RCDs. Validated through a prospective study, this could offer an improved ability to assign individual patients to a group with defined RCD characteristics and improve case selection for biopsy procedures, especially in infants and children.

Keywords Metabolomics · Respiratory chain disorders · Urinary organic acids · Urinary amino acids · Data reduction · Biosignature

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1 Introduction

The identification of mitochondrial disorders (MDs) in patients is still a major clinical and diagnostic challenge in mitochondrial medicine, especially in view of the extensive genetic, phenotypic and clinical heterogeneity of these disorders (Koene and Smeitink 2011). Accordingly, a wide range of medical specialists, including paediatricians, cardiologists, gastroenterologists, neurologists and ophthalmologists, may first encounter these patients (Wong et al. 2010). As a consequence, various criteria have been developed from clinical, genetic and biochemical points of view to direct the diagnosis of the RCDs.

Skeletal muscle provides the key material for histological and biochemical analysis of mitochondrial function and RCD diagnosis. Given the invasive procedure of a

muscle biopsy under general or local anaesthesia in children or adults, respectively, distinct clinical and biochemical information is desirable as a directive for a biopsy. Elevated transaminases and creatine phosphokinase are generally accepted as non-specific enzymatic indicators of MDs (Wong et al. 2010). Recently, Suomalainen et al. (2011) proposed fibroblast growth factor (FGF-21) as a biomarker for muscle-manifesting mitochondrial respiratory chain deficiencies, which needs to be confirmed by a prospective study, including appropriate patient groups (Turnbull 2011). An analysis of urinary metabolites including lactate, alanine, other amino acids, Krebs cycle intermediates and other organic acids provides the least invasive indicators of RCDs, but still lacks specificity as well as selectivity, as pointed out by Koene and Smeitink (2011). By using metabolic profiling of data generated by mass spectrometry (MS), plasma creatine was recently proposed as a specific and sensitive indicator of RCDs (Shaham et al. 2010). In this regard, it has been suggested that "omics" approaches, such as metabolite profiling, might expand the global view of metabolism due to RCD pathology directly or indirectly (Suomalainen 2011), and so support the more efficient identification of improved biomarkers for RCDs. This concurs with the findings of a metabolomics investigation which disclosed the presence of 24 organic acid metabolites that were practically and statistically highly significant for a well-defined group of RCD patients (Reinecke et al. 2012).

A biomarker is defined as a feature that is objectively measured and evaluated as an indicator of normal biological processes, pathological conditions or pharmacological responses to a therapeutic intervention (Atkinson et al. 2001). A profile of combined biomarkers is called a biosignature. Measuring single markers seems insufficient in dealing with complex diseases, such as RCDs, as outlined above. It has been argued that for complex infectious diseases, such as tuberculosis, a combination of molecular profiles is likely to have more value than single biomarkers (Jacobsen et al. 2008), and that a global approach is the analytical route to reveal such markers. Metabolic profiling using a global approach thus proved valuable in the search for biomarkers of complex conditions employing an experimental model for an infectious condition (Wikoff et al. 2008) as well as for the inherited RCDs (Shaham et al. 2010). We recently proposed that a global approach might disclose a metabolite profile with the potential to define an extended and characteristic biosignature that can be used as a non-invasive screening instrument for RCDs (Reinecke et al. 2012).

In the study reported here we have further investigated the metabolite profile in RCDs by analysis of three sub-sections of the human metabolome, included in the evaluation by the Mitochondrial Medicine Society's Committee on Disease as different laboratory modalities that can contribute to the establishment of RCDs (Haas et al. 2008. The three sub-sections are the organic acids and amino acids [measured by gas chromatography–mass spectrometry (GC–MS)], and acylcarnitines [measured by electrospray ionization tandem mass spectrometry (TMS)]. MS technologies are highly sensitive and selective, but also restrictive by applying only to subsections of the metabolome. We therefore also included untargeted NMR spectroscopy in this investigation. Although less sensitive than MS analysis, NMR spectroscopy proved to be highly successful as a complementary technique in studies of inherited metabolic diseases (Engelke et al. 2004).

2 Materials and methods

2.1 Reagents

Reagents and standards for the extraction of the organic acids were purchased from Merck Chemical Co. (Darmstadt, Germany) andethylacetate, diethylether and sodium sulphate and 3-phenylbutyric acid from Sigma-Aldrich (St. Louis, MO, USA). All the reagents for the amino acid analysis, including the standards (200 μM each), GC column (10 m × 0.25 mm ZB-AAA) and liner were provided in the EZ:faast^{TM} amino acid analysis sample testing kit by Phenomenex, Inc., (Torrance, CA, USA). For the carnitine analysis, acetonitrile, formic acid, and methanol were purchased from Merck Chemical Co., Butanolic HCl (3 N) was purchased from Sigma-Aldrich Co. The following standards were obtained from Dr. HJ ten Brink, Free University Medical Center (VUMC), Amsterdam, The Netherlands: L-carnitine·HCl, acetyl-L-carnitine·HCl, propionyl-L-carnitine·HCl, isovaleryl-L-carnitine·HCl, octanoyl-L-carnitine·HCl, hexadecanoyl-L-carnitine·HCl, [methyl-d_3]-L-carnitine·HCl, [d_3]-acetyl-L-carnitine·HCl, [3,3,3-d_3]-propionyl-L-carnitine·HCl, [d_9]-isovaleryl-L-carnitine·HCl, [8,8,8-d_3]-octanoyl-L-carnitine·HCl, and [16,16,16-d_3]-hexadecanoyl-L-carnitine·HCl.

2.2 Subjects and the selection of samples for the metabolomics analysis

Ethical approval for the study was obtained from the relevant Ethics Committees of the University of Pretoria (No. 91/98 and amendments) and North-West University (No. 02M02). Informed consent was obtained from the parents of patients and controls for the use of the urine samples and biopsy material (where applicable) of their children for research purposes.

The original RCD experimental group consisted of 101 clinically selected patients, including the cohort of South African patients described by Smuts et al. (2010). Urine samples were obtained at the Paediatric Neurology Unit of the Steve Biko Academic Hospital, Pretoria, South Africa,

at the time when the muscle biopsy was performed; the patients did not receive any specific treatment or supplements often given to patients with MDs. The use of anti-convulsants, such as valproate, is known to cause metabolic derangements and mitochondrial toxicity (Sztajnkrycer 2002), which might lead to biased mitochondrial-related markers; its use was not stopped in the patients, however, because of health risks and attendant ethical consequences. The controls were selected from among children referred to the clinic, but for whom no prevailing disorder was detected. Aliquots of all samples were stored at −80 °C prior to metabolomics analyses. This cohort provided the basis for the selection of samples from patients and controls for these analyses.

Metabolomic investigations are most successfully conducted with control and patient groups which are clearly distinguished from one another, because sample selection is one of the most important aspects of any metabolomics analysis. As mutational analyses of mtDNA and nDNA were not part of the routine procedures used to diagnose the present RCD patient group, other available clinical as well as biochemical parameters were selected to ensure the clear distinction between controls and patients, while retaining the intrinsic heterogeneity of the selected RCD group. Three inclusion criteria were thus formulated to define the patient and control groups: (1) clinical criteria characteristic of RCDs [including the intrinsic property of having a predominantly myopathic phenotype as described in Smuts et al. (2010)]; (2) a proven deficiency in one or more complexes of the RC as measured by biochemical enzyme analyses; and (3) elevated excretion of the total urinary organic acids in the patient group, to the extent of there being no overlap of these values for the controls and patients. Although elevated urinary excretion of organic acids is not a recognized principle for diagnosis of RCDs, it was included to ensure separation between the groups used in unsupervised multivariate analyses for the comparative metabolomics (Reinecke et al. 2012). Samples from 51 cases (29 patients and 22 controls, designated as Group 1) satisfied these criteria and were available for the MS analyses of the organic acids, amino acids and acylcarnitines; sufficient urine from only 34 of these cases (20 patients and 14 controls, designated as Group 2), however, was available for the NMR analyses. The characteristics of Groups 1 and 2 are shown in Tables 1 and 2.

2.3 Biopsy material and enzyme analyses from the patient group

Enzyme analyses were performed on muscle biopsies from the *vastuslateralis* muscle of all patients complying with the Mitochondrial Disease Criteria as defined by Wolf and Smeitink 2002. The analyses were conducted according to the procedures fully described previously; we also recognized the two criteria used to identify an enzyme deficiency in this patient group (Reinecke et al. 2012). As summarized in Table 1, the 29 patients selected thus had a muscle deficiency of either complex I (CI; five cases), complex III (CIII; four cases) or several different deficiencies of more than one RC enzyme (CM; 20 cases).

2.4 Acquisition of metabolite data

2.4.1 Untargeted metabolic analysis using nuclear magnetic resonance spectroscopy

Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy was included in this investigation for its high selectivity, provision of unambiguous information about a metabolite and the direct analysis of samples that did not require any prior sub-fractionation for metabolite selection. This work was conducted at the Laboratory for Genetic, Endocrine and Metabolic Diseases, Department of Laboratory Medicine, Radboud University Nijmegen Medical Centre according to standard procedures used there (Engelke et al. 2007). Urine samples from 34 cases of Group 2 were used in the ^{1}H-NMR study. These urine samples were analysed using one-dimensional (1D) ^{1}H-NMR spectroscopy. One millilitre of urine was centrifuged at 3,000 rpm for 10 min and 700 µL supernatant was transferred into a clean test tube. To this, 70 µL of 20.2 mM standard trimethylsilyl-2,2,3,3-tetradeuteropropionic acid sodium salt (TSP) in ^1H_2O was added, the pH adjusted to 2.5 ± 0.05 with concentrated HCl, and 650 µL was transferred to a 5-mm NMR tube. Each sample was analysed in a 500 MHz Bruker DRX spectrometer at 256 scans with a pulse of 7 µs and a delay of 4 s. The resulting free induction decay (FID) was converted into frequency domain by Fourier transformation, thereby yielding a ^{1}H-NMR spectrum. The instrument was equipped with a sample changer and each urine sample's ^{1}H-NMR spectrum was analysed individually. The dominant metabolites typically present in urine were detected in all samples. Six notable metabolites were identified based upon their chemical shift resonances at pH 2.5, namely, alanine [1.51 ppm (doublet)], betaine [3.26 ppm (singlet)], creatinine [3.13 ppm (singlet)], creatine [3.05 ppm (singlet)], lactic acid [1.41 ppm (doublet)] and succinic acid [2.66 ppm (singlet)]. Each of the above peaks was manually selected and the area under the peak was calculated using a software program (BrukerAmix). Each selected metabolite was quantified relative to creatinine, using the integral and the number of protons with respect to each peak. Interference from medication made the selection, and thus the quantification, of certain metabolites (particularly alanine) impossible for some urine samples. The creatine and betaine values obtained for

Table 1 Summary of the three inclusion criteria and selected metabolomics data of patients used in this study

Patients Number	Criterion 1 Clinical profile[1]	Criterion 1 MDC score[2]	Criterion 1 Lactate (mmol/l)	Criterion 1 Pyruvate (mmol/l)	Criterion 1 L/P	Criterion 2 RC enzyme defect: Percentage of the lowest control value	Criterion 3 Total OA (mmol/mol Cr)	Data 1 Total AA (mmol/mol Cr)	Data 2 Acetyl-Car (mmol/mol Cr)	Data 3 Carnitine (mmol/mol Cr)	Data 4 DC372/370	Data 5 OC344/342	Data 6 Creatine (mmol/mol Cr)	Data 7 Betaine (mmol/mol Cr)
P10_1	M, CNS, DD, DYS	6	2.50	0.10	25.00	CI: 95	1,159	1,485	2.10	3.39	0.12	0.86	0.84	0.05
P27_1	M, CNS, Eye, ENT, DD, DYS	7	6.00	0.47	12.80	CI: 94	2,695	4,765	15.27	35.80	0.10	0.15	Nd	Nd
P30_1	CNS	6	3.53	0.11	32.18	CI: 76	619	1,874	8.45	13.30	0.24	1.81	Nd	Nd
P47_5	M, Eye, R, DD, PNS	7	1.60	0.07	22.85	CI: 66	3,049	9,719	17.96	44.24	0.42	0.08	3.3	0.38
P73.1_A	M, CNS, Eye, L, DD, PNS	8	0.80	0.08	10.00	CI: 72	1,514	901	25.48	22.88	0.41	2.83	0.46	0.04
P36_1	M, CNS, DD	3	1.60	0.13	12.31	CIII: 90	1,397	1,034	12.85	7.43	0.10	0.11	0.77	0.04
P59_1	M, CNS, Eye, L, DR	7	2.40	0.12	20.70	CIII: 71	1,137	3,284	14.71	35.02	0.10	0.08	0.66	0.19
P60_1	M, AID	4	1.07	0.13	8.20	CIII: 95	1,223	1,399	4.57	3.82	0.41	0.24	Nd	Nd
P84_1_A	M, CNS, Eye, G, R, DD	8	Nd	Nd	Nd	CIII: 97	1,297	1,119	5.06	6.56	0.14	0.07	0.56	0.03
P01_2	M, Eye, PNS, DD	7	2.80	0.20	14.00	CI, CII + III: 0, 0	873	2,877	4.12	2.29	0.41	0.67	Nd	Nd
P07B_1	M, Eye, DD	3	1.90	0.14	13.57	CII, CII + III: 80, 64	2,041	2,068	6.18	12.35	0.21	0.25	3.23	0.06
P11_1	M, CNS, Eye, R, DD	8	2.50	0.35	7.10	CI, CIII: 79, 78	845	1,058	2.12	1.32	0.58	2.76	0.26	0.01
P14_1_1	M, CNS, Eye, DD, DR	6	1.10	0.10	11.00	CCI, CII + III: 77, 89	1,985	2,492	4.94	12.86	0.39	12.51	2.03	0.1
P21_1	M, Eye, PNS, End	8	2.80	0.32	8.80	CIII, CII + III: 98, 92	3,218	4,781	10.07	15.36	0.17	0.09	0.52	1.44
P39_3	M, R, DD	4	1.30	0.20	6.50	CI, CII, CIII, CII + III: 91, 93, 81, 83	1,841	6,544	29.42	74.93	0.40	0.13	Nd	Nd
P43_1	M, Eye, DD	7	2.00	Nd	Nd	CII, CIII, CII + III, CIV: 98, 94, 70, 91	1,208	3,961	9.71	27.13	0.28	0.35	2.56	0.07
P55_1	M, End, ENT, G, DD	10	Nd	Nd	Nd	CI, CIII: 86, 71	849	1,784	5.33	7.55	0.25	0.13	2.24	0.13
P62_1	M, CNS, End, S, DD	8	3.30	0.17	19.41	CIII, CIV: 87, 80	864	904	1.80	0.54	0.07	0.19	0.92	0.04
P67_1	M, CNS, L, DD	6	1.01	0.08	12.60	CIII, CIV: 65, 92	941	850	3.94	7.83	0.29	0.55	0.88	0.12
P69.1_A	M, End, DD, DYS	5	1.10	0.10	11.00	CCII + III, CIV: 90, 97	779	1,901	9.44	2.07	0.06	1.21	Nd	Nd
P70.1_A	BE, CNS & PNS	4	1.90	0.16	11.90	CCI, CIII: 84, 82	586	1,862	6.44	12.06	0.08	0.17	0.85	0.06
P71_1_A	M, G, Car, DD	4	2.20	0.12	18.30	CIII, CIV: 99, 71	1,077	1,745	5.56	4.78	0.38	0.18	0.93	0.03
P75.1_A	CNS, Eye, S, DD, DR, BE	6	3.00	0.14	22.40	CI, CIII, CIV: 90, 97, 37	820	695	9.85	6.54	0.20	0.26	0.13	0.01
P76.1_A	M, CNS, Eye, ENT, S, DD	5	Nd	Nd	Nd	CI, CIII: 37, 33	814	1,622	6.47	1.82	0.31	0.19	Nd	Nd

Table 1 continued

Patients Number	Criterion 1 Clinical profile[1]	Criterion 1 MDC score[2]	Criterion 1 Lactate (mmol/l)	Criterion 1 Pyruvate (mmol/l)	Criterion 1 L/P	Criterion 2 RC enzyme defect: Percentage of the lowest control value	Criterion 3 Total OA (mmol/ mol Cr)	Data 1 Total AA (mmol/ mol Cr)	Data 2 Acetyl-Car (mmol/ mol Cr)	Data 3 Carnitine (mmol/ mol Cr)	Data 4 DC372/ 370	Data 5 OC344/ 342	Data 6 Creatine (mmol/ mol Cr)	Data 7 Betaine (mmol/ mol Cr)
P78.1_A	M, CNS, L, S, DD	5	1.30	0.08	16.25	CI, CIII, CIV: 28, 38, 54	746	1,054	6.39	1.25	1.25	11.29	Nd	Nd
P82.1_A	M, CNS, Eye, G, ENT, DD	8	3.60	0.15	24.00	CI, CIII, CIV: 72,70, 65	2,297	1,739	31.15	8.41	0.14	0.33	Nd	Nd
P83.1_A	M, CNS, End, G, DD, DYS	6	2.30	0.14	16.40	CIII, CIV: 65, 93	1,762	4,222	3.29	5.94	10.22	0.67	0.14	0.12
P86_1_A	M, CNS, G, End, BE, DD, DR	8	3.70	0.20	18.50	CI, CIII, CII + III, CIV: 11, 65, 76, 39	1,416	980	12.30	9.05	0.12	0.16	0.21	0.01
P87_1_A	M, CNS, Eye, Skin, DD, DR	8	1.90	0.17	11.20	CII + III: 86	580	3,186	10.01	30.93	0.11	0.18	0.02	0.15

[1] Clinical profile includes *M* muscle involvement, *CNS* central nervous system involvement, *Eye* vision involvement, *DD* developmental delay, *DR* developmental regression, *Dys* dysmorphism (minor and major), *BE* behaviour and emotional abnormalities, *ENT* sensori-neural deafness, *PNS* peripheral neuropathy, *G* gastro-intestinal tract involvement, *R* renal involvement, *Car* cardiac involvement, *End* endocrine abnormalities, *AID* auto-immune disorder, *L* liver involvement, *S* skeletal involvement

[2] MDC score: Mitochondrial Disease Score (Wolf and Smeitink 2002). *AA* amino acids, *AcCar* acylcarnitines, *CI-IV* complexes I-IV, *CAR* carnitines, *Crea* creatine, *Cr* creatinine, *L/P* lactate:pyruvate ratio, *nd* not done, *OA* organic acids, *RC* respiratory chain, *SD* standard deviation, *nd:* not determined

14 controls and 20 patients are included in Table 2, where applicable.

2.4.2 *Analyses of organic acids using gas chromatography–mass spectrometry*

The organic acids were isolated from the urine, derivatized and separated by gas chromatography according to a procedure described previously (Reinecke et al. 2012). The volume of urine used for organic acid analysis was based on urinary creatinine values, transferred to salinized glass tubes (Kimax) and the internal standard (3-phenylbutyric acid) was added to a final concentration of 180 mmol/mol creatinine. The samples were acidified with 5 N HCl to a pH less than 2, followed by the addition of 6 ml of ethyl acetate to each sample which was then shaken on a rotary wheel for 20 min. After centrifugation of each mixture for 2 min at 1,300×*g*, the upper ethyl acetate phase was transferred to a clean glass tube; 3 ml of diethylether was added to the water phase, shaken for a further 10 min and centrifuged at 1,300×*g* for 10 min. The upper phase was removed and added to the ethylacetate. A small amount of sodium sulphate (BDH) was added to the ethylacetate/diethylether mixture to remove any residual water. After a subsequent centrifugation step, the organic phase was transferred to a clean glass tube. The organic solvents were evaporated to dryness under nitrogen at 37 °C.

O-bis(trimethylsilyl)trifluoroacetamide (BSTFA):trimethylchlorosilane (TMCS):piridine (5:1:1, and volume added based on the creatinine values) was used for derivatization. The volume of urine used gave a creatinine concentration equivalent to 21 µmol/ml derivatization reagent. The samples were derivatized at 85 °C for 45 min in a sand bath. The derivatized mixture was transferred to a 1.5 ml vial for GC–MS analysis. The Agilent GC–MS system used in this study consisted of a model 7890A gas chromatograph, a model 5975C mass selective detector, an HP 5970C MS and Agilent Chemstation (Revision E.02.00), and the GC–MS analysis was done as previously described (Reinecke et al. 2012). Peak identification and feature annotation was done by using AMDIS software (Version 2.66) linked to NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library (Version 2.0F, built Oct 8, 2008). The semi-quantitative identification of the organic acids was conducted according to Chen et al. (2009). All organic acids identified above the detection limit of the equipment used were expressed as mmol per mol creatinine.

2.4.3 *Analyses of amino acids using gas chromatography–mass spectrometry*

GC–MS analysis of the amino acids was conducted on an Agilent Technologies (Chemetrix, Midrand, South Africa)

Table 2 Summary of the urinary parameters for the respective controls (22/12) and patients (29/22)

	Organic acids (mmol/mol Cr)	Amino acids (mmol/mol Cr)	Acylcarnitines (mmol/mol Cr)	Carnitines (mmol/mol Cr)	DC372/370	OC344/342	Creatine (mmol/mol Cr)	Betaine (mmol/mol Cr)
Patients								
Minimum	579	694	1.80	0.5	0.06	0.07	0.02	0.01
Mean	1,366	2,479	9.83	24.2	0.62	1.33	1.08	0.15
Maximum	3,217	9,719	31.15	74.9	10.22	12.51	3.3	1.44
SD	724	2,006	7.73	22.4	1.86	3.02	1.01	0.31
Controls								
Minimum	164	246	0.59	0.26	0.05	0.08	0.02	0.01
Mean	348	494	3.96	6.5	0.19	0.26	0.12	0.02
Maximum	565	882	20.54	10.6	0.41	0.72	0.5	0.03
SD	123	164	4.05	5.8	0.09	0.17	0.15	0.01
P value	>0.0001	>0.0001	>0.001	>0.001	0.232	0.070	>0.0001	0.047

SD standard deviation, *DC372/370* decanoyl-carnitine:decenoyl-carnitine, *OC344/342* octanoyl-carnitine:octenoyl-carnitine

6890 series GC system with an Agilent Technologies 5973 Mass Selective Detector and a 7683 series dual tower and autosampler, all controlled by the MSD ChemStation E.02.00 (Palo Alto, CA, USA). The amino acid standards and urine were prepared as prescribed by the suppliers of the EZ:faast^{TM} amino acid analysis sample testing kit. One hundred microlitres of internal standard (norvaline at 200 μM) and amino acid standards (10, 25, 50, and 100 μL of each standard at 200 μM) or 100 μL urine were combined in a glass vial and further procedures were conducted according to the method supplied with the testing kit. Two microlitres of the extracts prepared according to the prescribed method was injected into the GC–MS for analysis and also analysed according to the prescribed method. The standard range analysis was used to calibrate the identification and quantification of the amino acids, using the MSD ChemStation E.02.00 software with a linear regression curve fit.

2.5 Analyses of carnitines using tandem mass spectrometry

The electrospray ionization TMS method was used to quantify urinary acylcarnitines. Ten μL of urine was added to a 1.5-ml centrifuge tube before 400 μL of the deuterated acylcarnitines (internal standard solution) with the following concentrations was added: [methyl-d_3]-L-carnitine·HCl (30.45 μM), [d_3]acetyl-L-carnitine·HCl (20.83 μM), [3,3,3-d_3]propionyl-L-carnitine·HCl (19.69 μM), [d_9]isovaleryl-L-carnitine·HCl (17.73 μM), [8,8,8-d_3]octanoyl-L-carnitine·HCl (15.43 μM) and [16,16,16-d_3]hexadecanoyl-L-carnitine·HCl·(11.47 μM). After the samples were evaporated to dryness under a gentle stream of nitrogen (55 °C), the remaining procedures were followed as described by Mels et al. (2011). Acylcarnitines were quantified by comparing the signal intensities of carnitine and acylcarnitines against those of the corresponding deuterated analogues. The concentrations of carnitine and acylcarnitines analysed were expressed as mmol per mol creatinine.

2.6 Statistical analysis

Variables with no variation (e.g. the internal standards) were removed from the original data sets for the organic acids, amino acids and carnitines and each of these data sets was initially analysed separately to identify their role as potential biomarkers. In addition, a data filter, based on the approach of Bijlsma et al. (2006), was applied to each variable to eliminate those that contained more than 40 % zero values ("60 % rule") for the control and patient groups. Standard univariate analyses, including *t* tests and the Mann–Whitney *U* test, were applied to all the remaining variables after application of the 60 % rule to assess the statistical significance of those variables that would eventually be considered as components of a putative biosignature. The subsequent data pre-treatment, in the first instance, consisted of zero replacement, where the zero values represented the detection limit of the analytical equipment. The zeros were replaced by a random sample of values from a Beta (0.1;1) distribution bounded between zero and the detection limit. Thereafter, a shifted logarithmic transformation with a shift parameter set at one was performed, ensuring that the scales of the various metabolite concentrations were more comparable, after which the transformed data were centred prior to further statistical analyses.

The effect size of each individual variable was measured to ascertain the importance of the single variables (Ellis and Steyn 2003). An effect size of $d > 0.5$ can be

considered as being of medium practical importance, whereas an effect size of $d > 0.8$ can be considered as highly practically significant. Descriptive statistics, such as minimum and maximum values, means and standard deviations, were included as applicable.

Multivariate analyses used for the identification of important variables were principal component analysis (PCA), as an unsupervised pattern recognition method (Johnson and Wichern 1998), and a partial least squares discriminant analysis (PLS-DA) as a supervised method (Barker and Rayens 2003). Variables listed by the PCA with a modelling power greater than 0.5 were regarded as potential biomarkers (Brereton 2003); and for variables important in projection (VIPs) from PLS-DA, the 'greater than one rule' was used as the criterion for variable selection (Chong and Jun 2005). The primary criterion for selection of important metabolites was that identified by PLS-DA, based on a VIP > 1.0 for each variable in the three data sets. The specificity and sensitivity estimates of the outcomes of the PLS-DA approach were evaluated by cross-validation as described below. Fit statistics of the PCA and PLS-DA models were reported as the percentage variance explained for the metabolites (R^2X), the percentage variance for the group membership of the patients and controls (R^2Y), and the predictive R^2Y values (Q^2).

A putative biosignature was derived from a consolidated data set, consisting of the important metabolites identified by the PLS-DA of the three MS-based analyses and the two important variables from the ^{1}H-NMR analysis. Because the scales of these four data sets were quite different, we compared various approaches of scaling for normalization, from which we selected the scale function provided in the R-statistical program, expressed as $Z = \log[X/\sqrt{\{1/(n-1)\ \Sigma X^2\}}]$. The scaled variables were then further transformed by using a shifted logarithmic transformation ($Y = \log[Z + 1]$). The transformed data were subsequently centred prior to PLS-DA analysis, and the important variables identified and validated as described below.

A generic description of the cross-validation, which was constructed on the outcomes of the applicable PLS-DA models, includes the following aspects. A data set was constructed which included only the important metabolites that were identified. Next, a PLS-DA model was built for this data set and an appropriate cut-off point was determined by calculating the Youdin index (Fluss et al. 2005). Then, we let P_{CON} and P_{PAT} be the observed occurrence probabilities of a control and a patient, respectively, and let α be the fraction of cases to be removed in the cross-validation. Next, 10,000 unique stratified samples of size $n_{CV} = [\alpha{\cdot}n]$, with n = total number of controls and patients, were selected from the data, stratified according to the observed occurrence probabilities, that is, $n_{CV} = n_C + n_P$, where n_C and n_P are the sample sizes from the controls and patients, respectively. For each of the 10,000 samples, the n_{CV} cases were withheld, a PLS-DA model was built using the remaining cases and the group membership of the withheld cases was predicted. For this, the sensitivity and specificity as well as the percentage of misclassified cases were recorded. Lastly, the standard deviation and the average of the recorded information were calculated over the 10,000 samples and reported as cross-validated estimates of sensitivity, specificity and percentage of misclassifications, as well as the respective values for α and the cut-point.

3 Results and discussion

3.1 Profile of the control and patient groups

Table 1 summarizes the inclusion criteria and selected metabolomics data of the 29 RCD patients investigated. With regard to criterion 1 (5 aspects), assessmentswere based on a detailed history and clinical examination of all patients (Smuts et al. 2010), indicating that an intrinsic property of the selected patients was their predominant myopathic phenotype. Original baseline investigations included lactate (L), pyruvate (P), creatine kinase (CK), and ammonia (NH_3) determinations. Lactic acidosis was present in five (17 %) and a raised pyruvate and lactate:pyruvate ratio (>18) in nine (31 %) cases of the selected group. All patients had a deficiency in one or more complexes of the RC (criterion 2), established by enzyme assays of biopsy material; and no patients with a deficiency in the pyruvate dehydrogenase complex (PDH) were included in the group. The total excretion of organic acids (criterion 3) of the patients was statistically significantly increased relative to the controls [mean value of the 22 controls was 348.9 (SD = 123.5) and 1336.6 (SD = 724.5) mmol per mol creatine for the 29 patients with $P < 0.0001$ for the t as well as the Mann–Whitney U tests]. No patients (minimum = 579 mmol/mol creatinine) or controls (maximum = 565 mmol/mol creatinine) were included in the group with an overlap in the total organic acid content, as shown in Table 2. With regard to the 7 sets of metabolomics data shown, statistically significant differences between the patients and controls were also found for the total amino acid excretion ($P < 0.0001$), total acylcarnitines ($P < 0.001$), free carnitine ($P < 0.001$), creatine ($P < 0.0001$) and betaine ($P < 0.047$). Although the mean values for the ratios of octanoyl-carnitine:octenoyl-carnitine and decanoyl-carnitine:decenoyl-carnitine were, respectively three times and five times higher than the controls, these differences were not statistically significant (the P values from the robust Mann Whiney U test were 0.424 and 0.246 for these two ratios, respectively), which coincides with the view that high values for the ratio of certain acylcarnitine esters may in certain cases be useful in

supporting specific diagnosis (Haas et al. 2008). It has also been suggested that the severity of symptoms observed in mitochondrial fatty acid β-oxidation defects may correlate with the concentrations of accumulating acyl carnitines, with possible application in newborn screening programmes (Giak-Sim et al. 2002). We therefore, finally, made a cluster and conducted other univariate analyses to investigate a possible correlation between some distinct clinical and other phenotypes of the patient group and the metabolite profiles described above. Comparison between phenotypes and metabolite profiles did not reveal any meaningful relationships. A way of improving genotype, phenotype and metabolite interrelationships might be to classify phenotypes in greater depth by also including transcriptional information as reported for genetic networks in liver metabolism (Ferrara et al. 2008).

A distinct difference between the biochemical profile of the patients and the controls, as shown in Table 2, is an important point of departure for metabolomics investigations. This was substantiated by a PCA conducted on the 29 patients and 22 controls for all the original 291 variables measured in the organic acid, amino acid and acylcarnitine (including free carnitine) analysis (Fig. 1). The outcome of the PCA, shown as a two-dimensional (PC1 and PC2) score plot for all the cases, indicates that the patient group was distinguished from the controls. This reveals that the metabolic profiles of the two groups were distinctly different due to the perturbation induced by the respective CI, CIII or CM deficiencies. Moreover, the heterogeneity, which is characteristic of RCDs, was retained in the patient group, as shown by the spread of these cases in the PCA. These observations already had the potential to identify biomarkers that could distinguish RCDs, but required data reduction for this identification.

3.2 Identification of important metabolites

The work-flow followed to identify important metabolites is shown schematically in Fig. 2. NMR-based metabolic profiling enables the simultaneous examination of a complex mixture of metabolites in a biological sample and requires only a limited knowledge of sample composition prior to analysis. NMR metabolomics may thus be regarded as an untargeted mode of analysis. By contrast, MS-based analyses are mostly semi-targeted as they distinguish a specific sub-section of the metabolome, extracted from a biofluid by an appropriate analytical procedure. Thus, as shown in Fig. 2, we included both these analytical approaches in our metabolomics investigation to optimize the detection of possible biomarkers for RCDs.

As indicated, urine samples from only 20 patients and 14 controls, which included information on the urinary organic acids, amino acids and acylcarnitines, were available for the ^{1}H-NMR analysis. Although these cases were fewer than the 51 cases of the total group, the clinical profile of the 34 cases strongly resembled that of the group of 51 patients. Alanine, lactic acid, succinic acid, creatine and betaine were found to be the important variables that distinguished the patient and control groups. As the first three of these are included in the MS-based analysis, only the values obtained for creatine and betaine were considered for the final consolidation of all important variables identified by the different analytical approaches.

Identification of important metabolites from GC–MS and TMS data required preprocessing to generate a data matrix of variables and cases of an operational size, to be followed by multivariate analyses to identify only the relevant analytical information. The total number of features in the original data set of the 51 cases, generated by an untargeted analysis in each of the three metabolite groups, yielded 291 substances that could be annotated as metabolites, namely, 189 organic acids, 51 amino acids, 50 acylcarnitines and free carnitine. Using the data filter, these compounds were reduced to 120 metabolites: 39 organic acids, 36 amino acids, 44 acylcarnitines and free carnitine. With regard to the long-chain acylcarnitines, it should be noted that they are strongly protein-bound in the plasma and thus escape excretion into the urine, as do the free fatty acids. Their presence in the urine in appreciable amounts may thus be due to renal malfunctioning or damage resulting in proteinuria and the relatively elevated amino acid excretion found in the RCD patients.

The subsequent data pretreatment first included zero replacement and logarithmic scaling. Mean values, as well as the standard deviation of all variables for the controls and patients, were determined on the unscaled data, followed by t-test and Mann–Whitney analyses. The two traditional methods of multivariate analysis chosen (PCA and PLS-DA) proved to be valuable for selection of variables and were subsequently applied to all three data sets, followed by effect size analyses. All variables with a VIP > 1.0 and/or a power value >0.5 and an effect size >0.8 were designated as important metabolites due to the RCDs in the patient group. A total of 26 metabolites was identified by this selection method, and included 11 organic acids (adipic, fumaric, homovanillic, lactic, suberic, succinic, vanilmandelic, 2-hydroxyglutaric, 3-hydroxyisobutyric, 3-hydroxyisovaleric, and 3-hydroxy-3-methylglutaric acids), 13 amino acids (alanine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, lysine, proline, serine, threonine, tyrosine, α-amino adipic acid and β-alanine), acetyl carnitine and free carnitine. Thus, from the ^{1}H-NMR-based and the MS-based analyses a total of $2 + 26 = 28$ metabolites were identified as important indicators of RCDs, from which a final list of biomarkers was selected and

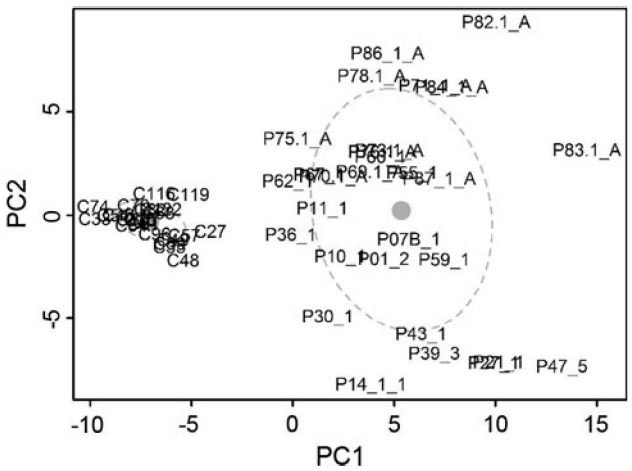

Fig. 1 Two-dimensional principal component analysis of the controls (indicated by a *C* and the case number) and patients (indicated by a *P* and the case number). This analysis was based on all 291 variables present before data reduction. The *circles* were drawn to indicate a 50 % probability level and the averages of the group scores are indicated by the solid dots. Owing to the density of the data references of the controls, the corresponding dot and most of the circle are obscured (see grouping in left-hand side of figure). Principal component 1 explained 35.7 % of the total variance whereas principal component 2 explained 10.58 % of the variance

validated according to the cross-validation procedure described in the statistical methods section.

The outcome of the cross-validation for the two experimental groups (51 or 34 cases, respectively) is shown in Table 3. The cut-off points for the metabolite groups were determined for each group separately; the differences relate to the numerical characteristics of the data sets for these variables. The sensitivity refers to the percentage of patients in the experimental group who were correctly classified as such by using the important metabolites identified from the organic acids (11), amino acids (13), carnitine (2) and creatine plus betaine, respectively. The specificity relates to the ability of the selected metabolites to identify the controls. The percentage misclassification includes the results obtained for the patients and controls taken together. The value of 100 obtained for the selectivity and the specificity for the organic acids clearly relates to the selection of the control and patient groups on the basis of a complete separation of the total urinary organic acids excreted by the groups (criterion 3). From the misclassification outcome it is clear that the ranking of importance of the metabolite groups is organic acids $\approx$ amino acids $>$ creatine and betaine $>$ carnitines, with the respective percentage of misclassifications being 0, 3.08, 16.58 and 26.64 %, respectively. A comparable ranking was obtained for the outcome of the sensitivity and specificity measures. The final conclusion from these cross-validations is that all 26 important metabolites from the three MS-based analyses should be included in a consolidated matrix, with the two metabolites identified by ^{1}H-NMR analysis. From this matrix a biosignature for the group of RCD patients could then be constructed.

3.3 Identification of a biosignature for the RCD patient group

First, the consolidated data set of 28 metabolites was formed, followed by data pretreatment as described above. Subsequently, a PLS-DA model was constructed for this data set to identify the metabolites that could qualify for a biosignature for the group of RCD patients. Sixteen metabolites with a VIP > 1.0 were identified as possible components of a biosignature, of which 13 are eventually summarized in Table 4, following exclusion of three of the original 16 as indicated below.

The RC is essentially involved in cellular reduction/oxidation (redox) status and energy (ATP) production; deficiencies in any component of this supramolecular complex inevitably affect a wide array of metabolic and other processes (Reinecke et al. 2009; Elstner and Turnbull 2011). Eight of the organic acids could accordingly directly be related to a consequence of RCDs and were included in the biosignature. Vanilmandelic acid (VMA) and homovanillic acid (HVA) were excluded from the biosignaturebecause of their properties as indicators of neurological stress conditions (Frankenhaeuser et al. 1986; Rauste-von Wright and Frankenhaeuser 1989), rather than being specifically related to RCDs. Betaine, the final component, was not included into the biosignature, because of a *P* value ($P = 0.047$) on the borderline between significant and not significant. Thus, 13 components can be related to RCDs, and are shown in Table 4 as part of the putative biosignature for RCD.

An important consequence of RCDs is a relative increase in levels of NADH (NADH/NAD^+ ratio) and $FADH_2$, as well as decreased ATP production, which may result from a defect at any site within the RC and lead to the well-established elevations in lactic acid and the lactate/pyruvate ratio. Increased succinic acid, 3-hydroxyisobutyric acid, 3-hydroxyisovaleric acid, 3-hydroxy-3-methylglutaric acid and 2-hydroxyglutaric acid were all reported in a metabolomics investigation on global changes in organic acid metabolism (Reinecke et al. 2012).

Amino acids and solutes such as bicarbonate, phosphate and glucose are transported across the apical membrane of the proximal renal tubular cells. This transport is driven by the sodium gradient, which is established by the basolateral ATP-dependent sodium pump. A disruption of the ATP supply in the kidney is therefore likely to occur in a multisystem disease, such as RCD (Martín-Hernández et al. 2005), or might even occur owing to a single enzyme

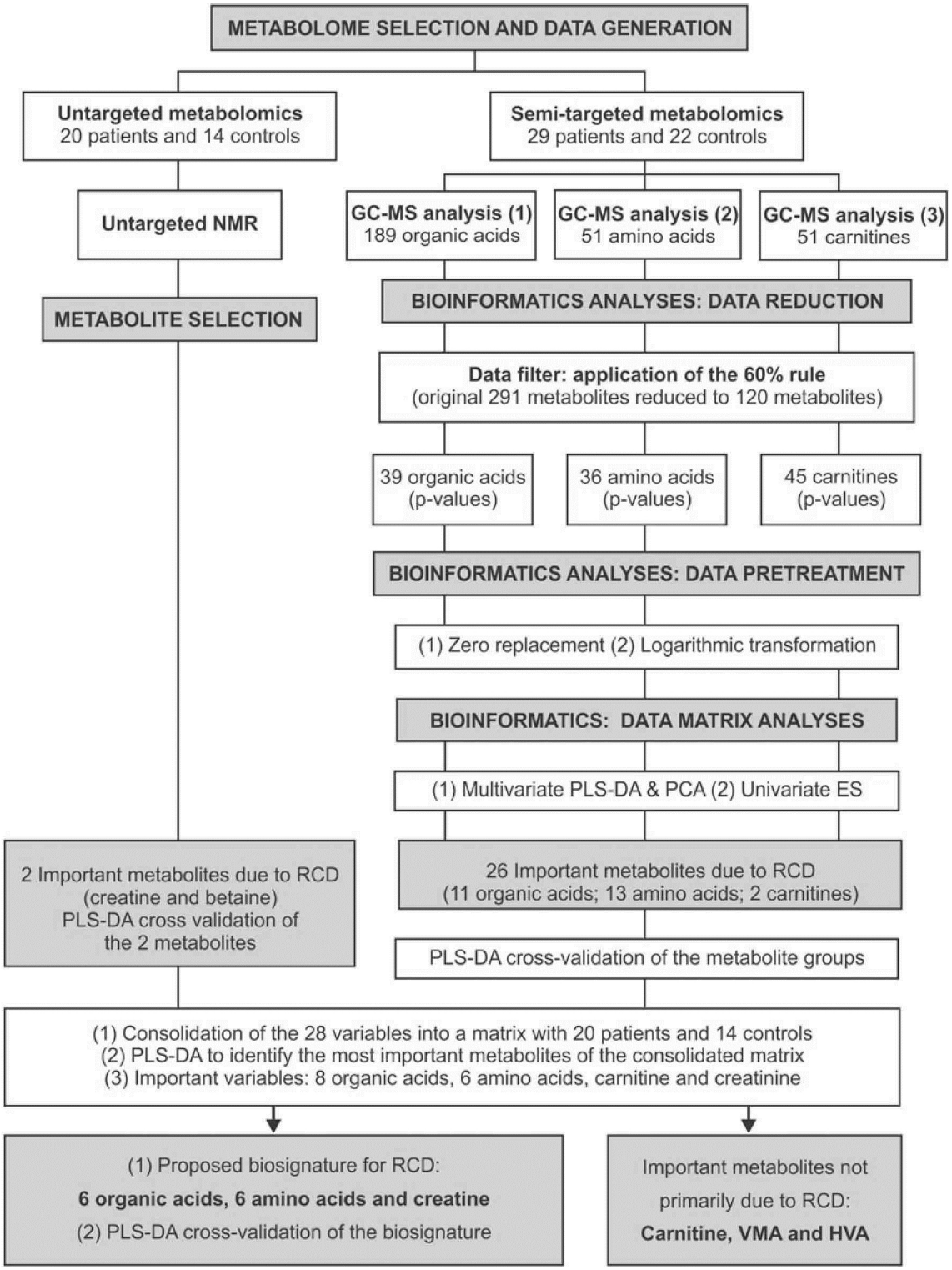

Fig. 2 Metabolomics work-flow and cross-validation of metabolite groups and the biosignature

defect, asin methylmalonyl-CoA mutase (EC 5.4.99.2) deficiency (Morath et al. 2008). Disrupted ATP supply leads to renal dysfunction as part of the pathophysiological profile seen in these metabolic diseases. A related perturbation might exist in the present patient group, reflected by the presence of carnitine as well as the increased

Table 3 Cross-validation of individual metabolite groups and of the biosignature

Number of cases (validation size)	Metabolite class (number)	Cut-off points	Sensitivity mean (SD)	Specificity mean (SD)	% Mis-classification (SD)
Cross-validation of individual groups of metabolites					
51 ($n_C = 7$, $n_P = 9$)	Organic acids (11)	0.11	100 (0)	100 (0)	0 (0)
51 ($n_C = 7$, $n_P = 9$)	Amino acids (13)	0.17	96.19 (6.67)	97.83 (5.67)	3.09 (4.02)
51 ($n_C = 7$, $n_P = 9$)	Carnitines (2)	0.06	73.78 (14.19)	72.79 (16.30)	26.64 (9.25)
34 ($n_C = 6$, $n_P = 8$)	Creatine and betaine	−0.01	72.95 (16.11)	97.39 (6.26)	16.58 (9.01)
Cross-validation of the biosignature					
34 ($n_C = 6$, $n_P = 8$)	Organic acids (6), amino acids (6) and creatine	0.23	98.12 (4.88)	97.96 (6.40)	1.95 (3.67)

n_C and n_P are respectively the sample sizes from the controls and patients used for validation

aminoaciduria, which resembles a Fanconi–Bickel excretion pattern of these metabolites (Odièvre et al. 2002). However, some amino acids may also increase as a response to other primary and secondary abnormalities due to RCDs. These amino acids include alanine, which follows from an increase in pyruvate and its consequent transamination, as well as glutamic acid that results from elevated amino acid catabolism (indicating also the possible hyperammonemia in RCD disorders) and tyrosine due to underlying liver damage (Levine and Conn 1967). Among the amino acids, α-aminoadipic acid has not been described for RCDs before, and clearly reflects a deficiency in lysine catabolism due to high FADH concentrations.

We did not compare biomarkers that discriminate between mitochondrial and other myopathies. Urinary glycine ($P < 0.001$), creatine ($P < 0.0001$) and betaine ($P = 0.047$) were significantly elevated in our patient group relative to controls, and were reported also to be significantly increased [in the case of glycine ($P < 0.01$), creatine ($P < 0.001$) and betaine ($P = 0.001$)] in juvenile idiopathic inflammatory myopathy patients relative to control subjects (Chung et al. 2005). Elevated creatine in plasma was recently described for RCDs, by using the phosphocreatine shuttle, as a consequence of tissues in a low energy state (Shaham et al. 2010). Furthermore, several of the metabolites that can be attributed to increased

Table 4 The proposed biosignature

Metabolite	VIP	ES	C[mean]	SD	*P*[mean]	SD	*P*/*C*	*t* Value	*P* value
Lactic acid	1.15	2.2	3.2	2.7	65.1	95.2	20	+3.51	<0.001
Succinic acid	1.29	2.1	6.0	5.9	97.5	108.6	16	+4.53	<0.001
2-OH-glutaric acid	1.26	2.6	1.5	1.3	15.3	13.1	10	+5.65	<0.001
3-OH-isobutyric acid	1.39	2.3	2.8	2.9	26.8	17.5	10	+7.27	<0.001
3-OH-valeric acid	1.35	2.5	3.7	2.8	42.1	51.1	11	+4.03	<0.001
3-OH-3-me-glutaric acid	1.18	1.8	1.4	2.4	17.8	17.4	13	+5.02	<0.001
Alanine	1.05	1.9	22.9	11.3	196.9	213.4	9	+4.38	<0.001
Glycine	1.06	1.9	93.6	45.6	638.8	622.1	7	+4.70	<0.001
Glutamic acid	1.01	1.8	4.7	1.9	38.7	39	8	+4.69	<0.001
Serine	1.00	2	35.9	11	216	188.1	6	+5.14	<0.001
Tyrosine	1.04	2	14.5	5.6	64.8	45.6	4	+5.89	<0.001
α-aminoadipic	1.04	1.7	2.5	1.5	34.6	42.7	14	+4.04	<0.001
Creatine	1.11	0.94	0.12	0.15	1.08	1.02	9	+4.11	<0.001

P/*C* designates *P*[mean]/*C*[mean], that is, the mean values per metabolite for the patients and controls, respectively; *ES* effect sizes. *VIP* variables important in projection, derived from the partial least-square discriminant analyses. The *P* values of the *t* test are shown in the table; all *P* values of the Mann–Whitney analyses for the metabolites of the biosignature were below 0.0001 and are not included in the table

catabolism of fatty acids and amino acids share a bioenergetics-sensing (hormone-modulated) induction pathway with FGF-21, which is also associated with a muscle disease phenotype response (Suomalainen et al. 2011).

From the metabolomics and statistical analyses, as well as from the biochemical considerations discussed here, the proposed biosignature for our experimental group consisted of 6 organic acids, 6 amino acids and creatine, as shown in Table 4.

3.4 Specifications for a biosignature

It has been proposed that the specification for a single metabolite (a biomarker), or a combination of metabolites (a biosignature), is the requirement to assign an individual patient to a unique group with defined characteristics (Jacobsen et al. 2008). The evaluation of a biosignature thus requires the use of a data set to validate the capacity of a putative biosignature to classify individual samples correctly. The data set from which a biosignatureis defined may also be used for the validation, but an independent data set should preferably be used for this purpose. For an inherited metabolic disease, the latter can be generated only over a period of time or by the creation of a data set through information gathered from participants at several medical centres, as was recently reported for FGF-21 as a potential biomarker for an RCD (Suomalainen et al. 2011). In our investigation the original data sets had to be used to validate the RCD biosignature, as an independent data set was not available for this purpose.

The cross-validation procedure described in the statistical section was used for the validation of the biosignature, and the outcome is summarized in Table 3. This validation was conducted for the data set consisting of the 13 metabolites (Table 4) and the 20 patients and 14 controls used in the ^{1}H-NMR analysis. The cut-off point for the cross-validation of the biosignature shown in Table 3 was determined for the consolidated set of variables. The cross-validation of the biosignature indicates its advantage as an indicator of an RCD compared with the use of a limited number of metabolite markers. Using the biosignature for the larger group of patients, a separation between the controls and patients with a CI, CIII or CM-deficiency could be obtained by unsupervised PCA as well as comparison of supervised PLS-DA. Thus, the results presented here give proof-of-concept that metabolomics investigations can include inherited metabolic diseases in their field of investigation.

3.5 From metabolomics to the clinic

According to Mancuso et al. (2009), the requirements for an ideal biomarker for a metabolic disorder such as an RCD are that it should improve the timing and accuracy of diagnosis, minimize the invasive procedure needed for the final diagnosis, and be useful to monitor disease progression and efficacy of treatment. They concluded, however, "that to date, no one can bet on this, but we are all looking forward to find it". The translation of research findings into clinical practice is not straightforward (Hu, 2011) but the criteria to be satisfied for a biomarker or biosignature of an inherited metabolic disease to become a practical and useful instrument in a clinical setting are clear, although complex (Turnbull 2011). For RCDs, the inductive approach (Kell 2004) to define a biosignature for the present experimental group, and its successful validation through the method of cross-validation opens up the possibility of formulating a hypothesis for the further development of a biosignature for RCDs. We postulate that the composite of organic acids $\approx$ amino acids $>$ creatine $>$ betaine $>$ carnitines represents the basic biosignature for RCDs. The experimental approach to test the hypothesis and define the suite of organic and amino acids of a validated and consistent/specific biosignature could be the subject of a future study that includes a cohort of more cases than those used in the present study, as well as additional controls of related but different mitochondrial disorders. The development of a sensitive and specific biosignature may well prove to be an essential step in selecting patients for more invasive and complex diagnostic procedures, and the availability of such a biosignature could influence or even eventually change clinical practice with regard to RCD diagnosis and monitoring of treatment.

Acknowledgments We would like to thank Dr M. Duran from the Laboratory for Genetic and Metabolic Diseases, Academic Medical Centre (AMC), Amsterdam, The Netherlands, for his comments on the original manuscript. This study formed part of BioPAD Project BPP007, funded through the South African Department of Science and Technology. Additional financial support from North-West University is likewise acknowledged. S.W. Mason is a recipient of a Vrije Universiteit (VU) Amsterdam-National Research Foundation (NRF)-Desmond Tutu PhD Fellowship.

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