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ORIGINAL ARTICLE

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Cardiometabolic risk factor clustering in patients with deficient branched-chain amino acid catabolism: A case-control study

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Abstract

Classical organic acidemias (OAs) result from defective mitochondrial catabolism of branched-chain amino acids (BCAAs). Abnormal mitochondrial function relates to oxidative stress, ectopic lipids and insulin resistance (IR). We

Abbreviations: AHA, American Heart Association; ANOVA, analysis of variance; BCAA, branched-chain amino acid; CKD, chronic kidney disease; HCL, hepatocellular lipids; HDL, high-density lipoprotein; IDF, International Diabetes Federation; IMCL, intramyocellular lipids; IR, insulin resistance; IVA, isovaleric acidemia; MRS, magnetic resonance spectroscopy; MetS, metabolic syndrome; MMA, methylmalonic acidemia; MMA CBL, methylmalonic acidemia due to cobalamin-synthesis defect; mtDNA, mitochondrial DNA; OA, organic acidemia; OGIS, oral glucose insulin sensitivity; OGTT, oral glucose tolerance test; OxRedox, oxidation reduction; PA, propionic acidemia; PCr, phosphocreatine; Pi, inorganic phosphate; QUICKI, quantitative insulin-sensitivity check index; ROS, reactive oxygen species; SD, standard deviation; TBARS, thiobarbituric acid reactive substances; TCA, tricarboxylic acid; TE, echo time; TG, triglycerides; TR, repetition time.

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investigated whether genetically impaired function of mitochondrial BCAA catabolism associates with cardiometabolic risk factors, altered liver and muscle energy metabolism, and IR. In this case-control study, 31 children and young adults with propionic acidemia (PA), methylmalonic acidemia (MMA) or isovaleric acidemia (IVA) were compared with 30 healthy young humans using comprehensive metabolic phenotyping including in vivo $^{31}P/^{1}H$ magnetic resonance spectroscopy of liver and skeletal muscle. Among all OAs, patients with PA exhibited abdominal adiposity, IR, fasting hyperglycaemia and hypertriglyceridemia as well as increased liver fat accumulation, despite dietary energy intake within recommendations for age and sex. In contrast, patients with MMA more frequently featured higher energy intake than recommended and had a different phenotype including hepatomegaly and mildly lower skeletal muscle ATP content. In skeletal muscle of patients with PA, slightly lower inorganic phosphate levels were found. However, hepatic ATP and inorganic phosphate concentrations were not different between all OA patients and controls. In patients with IVA, no abnormalities were detected. Impaired BCAA catabolism in PA, but not in MMA or IVA, was associated with a previously unrecognised, metabolic syndrome-like phenotype with abdominal adiposity potentially resulting from ectopic lipid storage. These findings suggest the need for early cardiometabolic risk factor screening in PA.

KEYWORDS
cardiometabolic, fatty liver, metabolic syndrome, mitochondria, organic acidemia, oxidative stress

1 | INTRODUCTION

Organic acidemias (OAs) are caused by deficiencies of mitochondrial enzymes or co-factors involved in branched-chain amino acid (BCAA) catabolism.[1] Accumulation of specific metabolites in propionic acidemia (PA), isovaleric acidemia (IVA) and methylmalonic acidemia (MMA) have been suggested to exert 'toxic' effects.[2-4] Isolated MMA is due to either a deficiency of methylmalonyl-CoA mutase, a defect in the transport or synthesis of its cofactor adenosylcobalamin, or a deficiency of the enzyme methylmalonyl-CoA epimerase.[5] In PA and MMA, accumulation of propionyl-CoA inhibits tricarboxylic acid (TCA) and urea cycle activities,[6] but also methylmalonic acid and isovaleric acid may impair mitochondrial morphology and function.[7-10] In addition, the reduced flux of substrates to the TCA cycle may potentially contribute to abnormal mitochondrial functionality.

Abnormal mitochondrial function associates with the production of reactive oxygen species (ROS). Postmortem studies in brain synaptosomes from patients with PA and MMA showed that methylmalonic acid, but not propionic acid, induces lipid peroxidation and protein oxidative damage due to generation of ROS.[11] Exposure to isovaleric acid reduces Na^+/K^+-ATPase activity in synaptic membranes of rat cerebral cortex, likely via free radical formation.[9] Furthermore, fibroblasts of patients with PA exhibit increased ROS levels, possibly related to the severity of the gene defect.[12] In line, antioxidant treatment can reduce ROS levels and oxidative damage, at least in fibroblasts of patients with PA and in a mouse model of PA.[13]

Common states of insulin resistance (IR), such as obesity and type 2 diabetes, may be also characterised by abnormal mitochondrial function along with oxidative stress and ectopic lipid storage.[14-16] In both adults and children, accumulation of hepatocellular and intramyocellular lipids (HCL, IMCL) is tightly linked to IR.[17,18] Furthermore, metabolites such as free fatty acids and BCAAs can directly induce IR via substrate signalling in liver and skeletal muscle.[17] Thus, one might hypothesise that abnormal mitochondrial function – as observed in patients with branched-chain OAs – might lead to IR or its clinical correlates, the metabolic syndrome (MetS) or cardiometabolic risk factor clustering.[19-22] 'Cardiometabolic risk factor clustering' is defined by the accumulation of risk factors that are closely

related to type 2 diabetes and/or cardiovascular disease and, as recently recommended by the American Academy of Pediatrics, should be in the focus for clinical screening in children and adolescents.[21] Those risk factors comprise central obesity, fasting hyperglycaemia, dyslipidemia (high triglycerides [TG], low HDL cholesterol) and high blood pressure. Interestingly, patients with OAs feature a broad range of symptoms of largely unexplained origin[23-26] and those with PA and MMA appear to have less favourable long-term outcome compared to those with IVA.[27-29]

This study therefore examined whether genetically altered mitochondrial function due to abnormal BCAA catabolism is related to abnormal tissue-specific energy metabolism, IR and MetS by employing in-depth metabolic phenotyping with frequent sampling oral glucose tolerance tests (OGTT) and $^{31}P/^{1}H$ magnetic resonance spectroscopy (MRS) of liver and skeletal muscle.

2 | MATERIALS AND METHODS

2.1 | Participants

This case-control study enrolled patients with OAs and sex-, age- and BMI-matched healthy humans serving as controls (ClinicalTrials.gov registration number NCT03917212). Inclusion criteria for patients were age ≥ 5 years and diagnosis of OA based on biochemical, enzymatic and/or molecular genetic findings. The group of patients with OAs consisted of 9 patients with PA, 6 with IVA, 10 with classical MMA and 6 with an adenosylcobalamin synthesis defect comprising cobalamin A or B deficiency (MMA CBL) based on cellular functional (CBL A/B n = 4) or molecular genetic analyses (CBL B n = 2). Exclusion criteria for the OGTT comprised chronic gastrointestinal diseases, which could affect glucose absorption, and allergy to red currant, which is contained in the glucose test solution. Patients regularly attending the Division of Metabolic Diseases at the University Children's Hospital Düsseldorf were recruited by phone call. In total, 31 patients with OAs, comprising 23 minors and 8 adults were included. Due to low compliance, OGTT and MRS measurement could not be performed in four and three patients with OAs, respectively (OGTT: PA n = 3, MMA n = 1; MRS: PA n = 1, MMA n = 1, MMA CBL n = 1). Additionally, OGTT and MRS data were not available from three and three patients with OAs, respectively, because of technical reasons (OGTT: n = 1 patient each with PA, MMA and IVA; MRS: n = 1 patient each with PA, MMA CBL and IVA).

Healthy individuals were recruited among the patients' siblings and through convenience sampling. Twenty-one healthy children, five of whom were siblings of patients, and 9 healthy adults underwent MRS measurements as a control group. In addition, 11 healthy children of comparable age, sex and BMI from the Yale Pathophysiology of Type 2 Diabetes in Youth Study[30] and 5 healthy adults from a previous study at the German Diabetes Center[31] served as controls for OGTT data. The study was approved by the ethics board of the Medical Faculty, Heinrich Heine University Düsseldorf (protocol no. 3778). Written informed consent was obtained from the participants and their parents, when participants were minors.

2.2 | Study design

The study was performed at the Department of General Pediatrics, Neonatology and Pediatric Cardiology of the University Children's Hospital Düsseldorf for clinical and metabolic examinations and at the German Diabetes Center for MRS measurements. The study took place between August 2015 and February 2017. Participants were admitted to the hospital 1 day prior to measurements and advised to avoid exhaustive physical activity for two days before admission.

2.3 | Clinical parameters

Height and weight were measured in underwear, and BMI values of children and adolescents were plotted on age- and sex-specific percentiles[32] and defined as "high normal" if values were ≥75th percentile.[33] Blood pressure and waist circumference were determined in children and adults and defined as high according to national and international reference values,[34,35] respectively. The absolute daily intake of energy and macronutrients of patients with PA and MMA, who were on a defined and controlled dietary therapy (Table 1), was extracted from their medical records. MetS was defined for children according to Goodman et al[20] and for adults according to Alberti et al[19] and diagnosed, if any 3 or more of 5 MetS components were present (Table S1). For children, MetS components were: fasting glucose ≥ 100 mg/dL, systolic blood pressure ≥ 90th percentile and/or diastolic blood pressure ≥ 90th percentile, waist circumference ≥ 90th percentile, HDL cholesterol ≤ 10th percentile and TG ≥ 110 mg/dL. For adults, MetS components were: fasting glucose ≥ 100 mg/dL; systolic blood pressure ≥ 130 mm Hg and/or diastolic blood pressure ≥ 85 mm Hg; waist circumference ≥ 94 cm for men, ≥80 cm for women; HDL cholesterol < 40 mg/dL in men, <50 mg/dL in women; TG ≥ 150 mg/dL.

TABLE 1 Daily energy and macronutrient intake in patients with PA and MMA at the time of study

Disease	Age years	Weight kg	Sex	Protein g/kg d	Carbohydrate g/kg d	Carbohydrate % energy[37]	Fat g/kg d	Fat % energy[37]	Energy kcal/kg d	Age- and sex-specific recommendation for energy intake[36 a] kcal/kg d
PA[b]	12	61.6	f	0.7	4.7	49.6	1.8	43.1	37.5	40.4
	12	56.5	m	0.9	3.9	51.8	1.2	36.2	29.2	46.3
	13	38.5	m	0.9	6.6	59.3	1.6	32.6	44.9	41.4
	16	44.4	m	1.2	6.4	57.0	1.6	32.3	44.6	37.6
	17	58.1	f	1.1	4.2	50.9	1.3	35.7	32.1	33.6
	17	57.8	m	0.7	5.1	58.3	1.3	33.7	35.1	37.6
	18	69.1	m	n/a[c]	n/a[c]	n/a[c]	n/a[c]	n/a[c]	n/a[c]	37.6
	23	71.3	m	n/a[c]	n/a[c]	n/a[c]	n/a[c]	n/a[c]	n/a[c]	33.9
MMA	6	20.0	f	1.0	10.1	59.4	2.6	34.7	68.1	64.7
	9	43.5	m	1.0	6.7	58.0	1.7	33.4	46.1	58.0
	10	35.0	m	0.9	8.0	58.6	2.1	34.9	54.6	46.3
	11	30.8	f	0.8	10.3	67.8	1.8	26.9	59.4	40.4
	11	29.2	m	1.2	11.6	57.8	3.2	36.2	80.1	46.3
	12	38.9	f	0.8	6.0	57.5	1.6	34.8	40.8	40.4
	12	43.0	m	0.9	7.6	58.3	2.0	34.8	51.9	46.3
	14	42.2	f	0.8	6.6	58.6	1.7	34.3	45.2	35.2
	14	50.6	f	0.7	n/a[c]	n/a[c]	n/a[c]	n/a[c]	39.5	35.2
	19	50.0	f	0.9	5.8	53.8	1.8	37.9	43.1	31.4

Abbreviations: MMA, methylmalonic acidemia; n/a, not available, PA, propionic acidemia.

[a]Guiding values based on lowest physical activity level (PAL) 1.4: absolute values were divided by reference body weights of the respective 50th percentile based on the German KiGGS study.[32,38]

[b]Data was available from 8 of 9 patients with PA.

[c]Patients did not follow a calculated dietary therapy.

2.4 | Oral glucose tolerance test

Eight hours prior to their first blood draw, all patients were given a standardised night meal (200 kcal) to prevent metabolic decompensation that may occur after prolonged fasting. Adults received 75 g glucose and children received 1.75 g/kg body weight glucose (Accu-Check O.G.T Dextrose, Roche Diagnostics, Mannheim, Germany) orally or through a feeding tube between 08:30 and 9:00 AM. Blood sampling was done before (0) and 30, 60 and 120 minutes after glucose intake. Fasting insulin sensitivity was assessed from the quantitative insulin-sensitivity check index, whereas dynamic insulin sensitivity was assessed from the oral glucose insulin sensitivity index (OGIS).[39] Insulin secretion was described by fasting beta-cell function, adaptation index, disposition index, ratio of change in C-peptide and glucose at 30 minutes ($\Delta Cp_{30}/\Delta G_{30}$), while insulin kinetics were assessed from hepatic insulin extraction.[39]

2.5 | Biochemical analyses

Glucose, total cholesterol, LDL and HDL cholesterol, glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase, gamma-glutamyl transferase, C-reactive protein, creatinine, uric acid and cystatin C were measured photometrically (Cobas 8000 c702 chemistry analyzer, Roche Diagnostics, Mannheim, Germany), and haemoglobin A1c was analysed by a turbidimetric inhibition immunoassay (Cobas 6000 c501 analyzer). For TG measurements, blood samples were drawn into a vial containing orlistat to prevent in vitro lipolysis and immediately chilled on ice for 30 minutes, as described.[31] Insulin and C-peptide were analysed by radioimmunoassays (Millipore, St. Charles, CO). Serum thiobarbituric acid reactive substances (TBARS), oxidation reduction (OxRedox) potential and antioxidative capacity were measured to estimate oxidative stress.[40] Details on the biochemical analyses of the Yale cohort were reported previously.[30]

TABLE 2 Participants' clinical and biochemical characteristics, insulin sensitivity and secretion, liver and muscle energy metabolism according to disease group and respective control group

Parameter	PA	CON PA	MMA	CON MMA	MMA CBL A/B[a]	CON MMA CBL A/B	IVA	CON IVA
n	9	9	10	10	6	6	6	6
Sex (male)	6 (67)	6 (67)	4 (40)	4 (40)	5 (83)	5 (83)	2 (33)	2 (33)
Age (years)	15.7 ± 3.6	17.2 ± 4.0	11.8 ± 3.5	12.4 ± 3.9	14.8 ± 7.4	14.5 ± 7.7	19.0 ± 11.9	18.8 ± 11.2
BMI (kg/m^2)	23.7 ± 3.7	20.4 ± 3.8	19.6 ± 2.7	19.2 ± 2.6	20.3 ± 4.0	19.7 ± 4.3	21.2 ± 6.8	21.3 ± 8.8
Overweight or high normal weight (≥P75)[b]	5 (56)	2 (22)	3 (30)	2 (20)	2 (33)	2 (33)	2 (33)	1 (17)
Normal weight[c]	4 (44)	7 (78)	7 (70)	7 (70)	4 (67)	4 (67)	4 (67)	4 (67)
Underweight[d]	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	1 (17)
Fasting glucose (mg/dL)	98.2 ± 19.1	84.4 ± 20.8	95.3 ± 12.4	92.8 ± 12.0	85.8 ± 13.7	85.8 ± 18.5	86.2 ± 7.7	85.1 ± 19.6
HOMA-IR (mg/dL)	3.4 ± 1.8	2.1 ± 1.8	3.2 ± 0.2	3.6 ± 1.0	2.1 ± 1.3	2.8 ± 1.7	1.0 ± 0.1	2.5 ± 2.0
QUICKI	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
Matsuda index	3.1 ± 2.0	8.0 ± 7.1	4.3 ± 3.4	2.3 ± 0.4	6.4 ± 4.8	5.5 ± 5.6	9.1 ± 6.0	7.1 ± 7.7
OGIS (mL min^{-1}/m^2)	**364 ± 83**	488 ± 83	403.4 ± 34.5	389.6 ± 36.9	408.8 ± 71.4	470.0 ± 75.9	421.3 ± 62.3	438.5 ± 106.5
Fasting beta-cell-function	170.8 ± 40.1	121.8 ± 64.5	234.4 ± 131.2	113.6 ± 39.2	154.8 ± 91.2	119.8 ± 57.4	80.9 ± 49.1	105.4 ± 23.6
$\Delta Cp_{30}/\Delta G_{30}$	0.6 ± 0.4	1.0 ± 0.5	1.78 ± 1.05	0.70 ± 0.53	0.67 ± 0.22	0.85 ± 0.47	**0.38 ± 0.20**	0.75 ± 0.19
Adaptation index	0.4 ± 0.1	0.4 ± 0.2	0.6 ± 0.2	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
Disposition index	2.9 ± 1.0	2.9 ± 1.5	3.4 ± 1.6	4.1 ± 0.8	2.3 ± 1.2	3.4 ± 1.3	2.4 ± 1.4	3.4 ± 1.8
Hepatic extraction (%)	58.5 ± 8.7	66.0 ± 7.8	**61.8 ± 6.7**	42.3 ± 5.3	61.0 ± 1.1	65.7 ± 9.0	69.2 ± 12.1	62.0 ± 14.2
Liver γ-ATP (mmol/L)	2.63 ± 0.58	2.61 ± 0.41	2.74 ± 0.42	2.96 ± 0.32	3.43 ± 1.77	3.88 ± 1.09	2.47 ± 0.48	2.88 ± 0.75
Liver Pi (mmol/L)	2.19 ± 0.60	1.53 ± 0.23	2.47 ± 0.55	2.14 ± 0.62	2.78 ± 1.45	2.70 ± 1.23	1.88 ± 0.50	2.09 ± 0.63
Muscle γ-ATP/TP	0.17 ± 0.01	0.16 ± 0.01	**0.16 ± 0.01**	0.17 ± 0.01	0.17 ± 0.03	0.17 ± 0.01	0.16 ± 0.01	0.16 ± 0.01
Muscle PCr/TP	0.78 ± 0.01	0.77 ± 0.03	0.76 ± 0.01	0.76 ± 0.01	0.77 ± 0.04	0.74 ± 0.02	0.76 ± 0.02	0.77 ± 0.02
Muscle Pi/TP	**0.06 ± 0.01**	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.02	0.09 ± 0.02	0.08 ± 0.01	0.07 ± 0.02

Note: Data are mean ± SD or n (%); unpaired Student's *t* test, Mann-Whitney *U* test or Fisher's exact test; bold value indicates statistical significance ($P < .05$ vs CON group). ATP, adenosine triphosphate; BMI, body mass index; CON, control; ΔCp30/ΔG30, early phase of C-peptide secretion function; HDL, high density lipoprotein; HOMA-IR, homeostatic model assessment insulin resistance; IVA, isovaleric acidemia; MMA, methylmalonic acidemia; MMA CBL, adenosylcobalamin synthesis defect; OGIS, oral glucose insulin sensitivity index; PA, propionic acidemia; PCr, phosphocreatine; Pi, inorganic phosphate; QUICKI, quantitative insulin-sensitivity check index; TP, total phosphorus. Fasting glucose was available in PA n = 6, MMA n = 9, MMA CBL n = 6, IVA n = 6 and respective matched controls. HOMA-IR, OGIS, QUICKI and Matsuda index were available in PA n = 5, MMA n = 7, MMA CBL n = 6, IVA n = 5 and respective matched controls. Fasting β-cell function, adaptation and disposition index were available in PA n = 5, MMA n = 7, MMA CBL n = 6, IVA n = 5 and respective matched controls. Hepatic insulin extraction was available in PA n = 4, MMA n = 4, MMA CBL n = 3, IVA n = 3 and respective matched controls. ΔCP30/ΔGlu30 was available in PA n = 5, MMA n = 6, MMA CBL n = 3, IVA n = 5 and respective matched controls. Liver γATP and Pi were available in PA n = 4, MMA n = 5, MMA CBL n = 4, IVA n = 5 and respective matched controls. Muscle γATP/TP, PCr/TP and Pi/TP were available in PA n = 6, MMA n = 7, MMA CBL n = 3 IVA n = 5 and respective matched controls.

[a]Cobalamin A/B defect (n = 4), cobalamin B defect (n = 2).

[b]BMI ≥ 25 kg/m^2 (≥18 years) or BMI ≥ P75 (<18 years).[33]

[c]BMI 18.5-24.9 kg/m^2 (≥18 years) or BMI P3 ≤ P75 (<18 years).

[d]BMI < 18.5 kg/m^2 (≥18 years) or BMI < P3 (<18 years).[32]

2.6 | Liver ultrasound

All patients underwent liver ultrasound by experienced radiologists on an Aplio 400, Aplio XG, Xario (all from Canon Medical Systems, Neuss, Germany) or a S2000 (Siemens AG, Erlangen, Germany). For children, liver size was measured in the anterior axillary line and compared to standard percentiles.[41] Steatosis was defined by hyperechogenicity of liver tissue and increased posterior beam attenuation.

2.7 | Liver and skeletal muscle ^{1}H- and ^{31}P-MRS

Four hours prior to measurements, patients received a meal according to their dietary protocol, and controls were provided an age-appropriate meal of similar energy content (280-460 kcal) to ensure comparable nutritional conditions. All measurements were performed in a 3-T magnet (Achieva, Philips Heathcare, Best, The Netherlands). IMCL was measured in vastus lateralis muscle by ^{1}H-MRS using the point resolved spectroscopy sequence from a localised volume of interest (VOI: $1 \times 1 \times 2$ cm^3) with a repetition time of 2000 ms and an echo time of 32 ms. The signal averages of non-water-suppressed and water-suppressed MRS were 16 and 96, respectively. Spectra were analysed using LCmodel with build-in eddy-current correction to obtain a measure of IMCL relative to tissue water content. HCL was measured with ^{1}H-MRS using the stimulated echo acquisition mode sequence. Briefly, non-water-suppressed spectra were acquired from a VOI of $3 \times 3 \times 2$ cm^3 with water and fat peaks integrated using the jMRUI v4.0 software.

Liver and skeletal muscle ATP and inorganic phosphate (Pi) contents were measured using ^{31}P-MRS.[42]

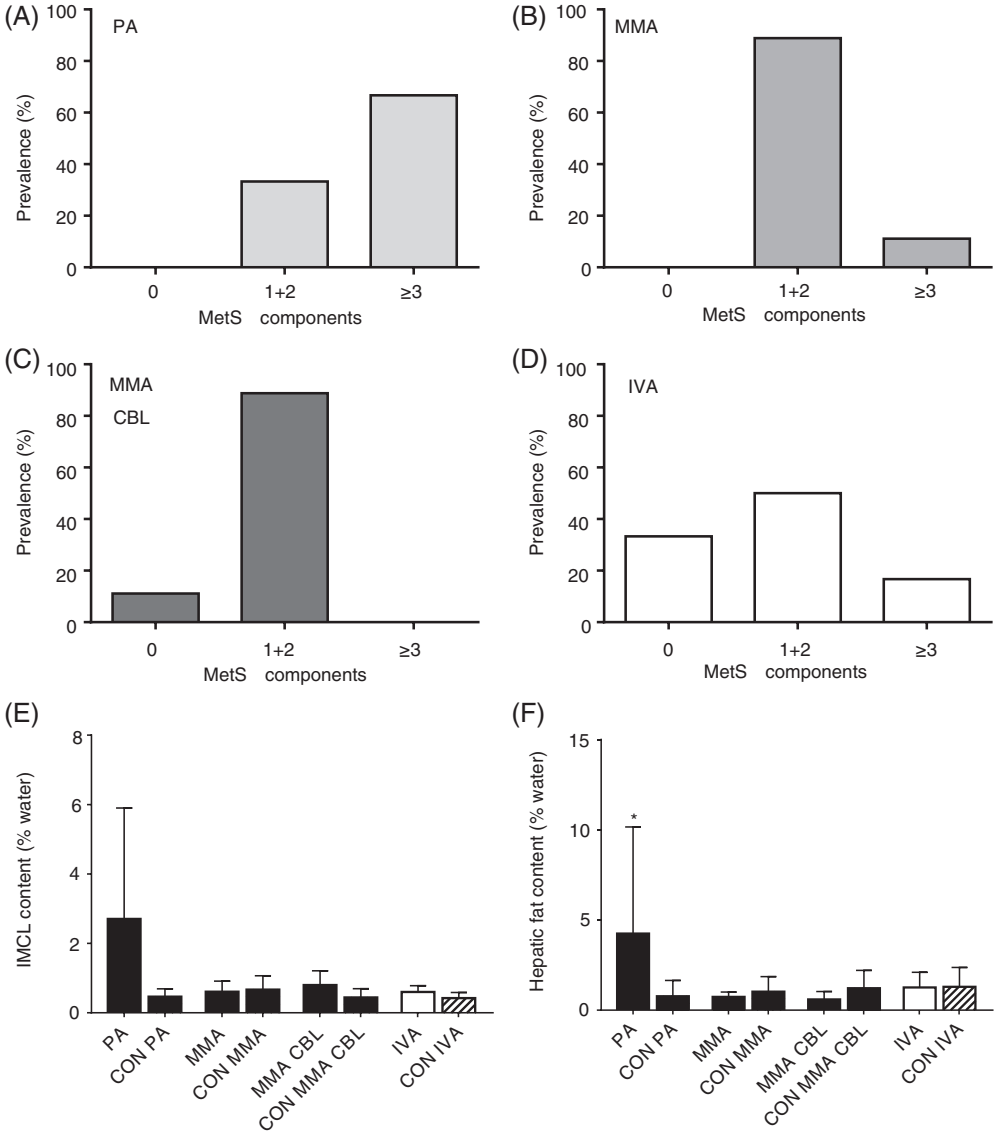

FIGURE 1 Prevalence of the MetS and MetS components as well as ectopic lipid storage in patients with organic acidemias. A-D, Prevalence of MetS components, according to Goodman et al[20] for children and according to Alberti et al[19] for adults. E, Intramyocellular lipid content and F, hepatic fat content. Data are mean ± SD, $^{*}P < .05$ vs CON. CON, control; IMCL, intramyocellular lipids; IVA, isovaleric acidemia; MetS, metabolic syndrome; MMA, methylmalonic acidemia; MMA CBL, adenosylcobalamin synthesis defect; PA, propionic acidemia. MetS components available in PA n = 7, MMA n = 10, MMA CBL n = 6, IVA n = 6 and respective controls. IMCL available in PA n = 7, MMA n = 5, MMA CBL n = 4, IVA n = 6 and respective controls. Liver fat content available in PA n = 7, MMA n = 6, MMA CBL n = 4, IVA n = 5 and respective controls

Briefly, liver spectra were acquired using a 14-cm circular ^{31}P surface coil (Philips Healthcare), using the ^{1}H body coil for ^{1}H-decoupling and nuclear Overhauser enhancement. Localised liver spectra were obtained with VOIs of $4 \times 4 \times 4$ cm^3 for children and $6 \times 6 \times 6$ cm^3 for adolescents and adults. The resulting spectra were analysed for absolute concentration of ATP and Pi using the jMRUI v4.0 software. For skeletal muscle, a 6-cm surface coil (PulseTeq Ltd, Chobham, UK) was positioned on 2 cm into the medial head of the right gastrocnemius muscle. From non-localised ^{31}P spectra, muscle ATP, Pi and phosphocreatine (PCr) content were assessed and expressed relative to the total phosphorus content (TP = ATP + Pi+PCr).

2.8 | Statistical analysis

Descriptive statistics are reported as mean ± SD for normally distributed continuous variables and median (interquartile range) for skewed data. Categorical variables are expressed as proportions above defined cut-off values.[19,20] Differences between patients and respective control groups were analysed using unpaired Student's *t* test for normally distributed continuous variables and the Mann-Whitney *U* test for skewed data. Categorical data was compared using Fisher's exact test. OGTT data was subjected to two-way analysis of variance (ANOVA) with repeated-measures factors time and group, and *P*-values were multiplicity-adjusted for each comparison using Sidak's multiple comparisons test. One-way ANOVA was used for comparisons between OA patient groups for serum TBARS, serum OxRedox potential and serum antioxidative capacity data.

3 | RESULTS

3.1 | Patients' clinical characteristics

Table 2 shows the clinical and biochemical data of the groups of patients with PA, MMA, MMA CBL, IVA and the control groups matched to the respective disease groups. The prevalence of MetS and its components for the various OA groups are presented in Figure 1A-D. MetS defined as having ≥3 of 5 components[19,20] was present in nearly 70% of patients with PA (Figure 1A). The majority had an increased waist circumference and dyslipidemia, 50% showed fasting hyperglycaemia (≥100 mg/dL),[19,20] and approximately one-third had arterial hypertension (Table 3). Four out of five PA patients with a severe phenotype (Table S1) presented with MetS and the same

TABLE 3 Metabolic syndrome components in patients according to disease group

Parameter	PA	MMA	MMA CBL A/B[a]	IVA
Increased waist circumference[b]	5 (71)	4 (50)	2 (33)	2 (33)
Fasting glucose (mg/dL)	98.2 ± 19.1	95.3 ± 12.4	85.8 ± 13.7	86.2 ± 7.7
Fasting hyperglycaemia[c]	3 (50)	3 (33)	0 (0)	0 (0)
Triglycerides (mg/dL)	262.9 ± 234.4	263.6 ± 121.5	97.3 ± 24.8	75.0 ± 23.9
Hypertriglyceridemia[d]	4 (67)	9 (100)	0 (0)	0 (0)
HDL cholesterol (mg/dL)	33.3 ± 16.4	30.7 ± 7.7	48.6 ± 10.3	51.0 ± 15.3
Low HDL cholesterol[e]	5 (83)	8 (89)	1 (17)	2 (40)
SBP (mm Hg)	112.9 ± 23.5	110.5 ± 11.5	128.5 ± 28.6	116.8 ± 12.0
Systolic hypertension[f]	2 (29)	4 (40)	4 (67)	2 (33)
DBP (mm Hg)	68.4 ± 16.7	69.4 ± 10.8	78.3 ± 29.9	63.2 ± 7.3
Diastolic hypertension[g]	2 (29)	3 (30)	3 (50)	0 (0)

Note: Data are mean ± SD or n (%). DBP, diastolic blood pressure; HDL, high density lipoprotein; IVA, isovaleric acidemia; MMA, methylmalonic acidemia; MMA CBL, adenosylcobalamin synthesis defect; PA, propionic acidemia; SBP, systolic blood pressure. **Respective reference values for healthy children are given in the table legend**. Reference values established in populations of German or European children were used for HDL cholesterol (≤P10) and TG.[43] Weight circumference was available in PA n = 7, MMA n = 8, MMA CBL n = 6, IVA n = 6. Triglycerides were available in PA n = 6, MMA n = 9, MMA CBL n = 6, IVA n = 5. Fasting glucose was available in PA n = 6, MMA n = 9, MMA CBL n = 6, IVA n = 6. Systolic and diastolic blood pressure was available in PA n = 7, MMA n = 10, MMA CBL n = 6, IVA n = 6.

[a]Cobalamin A/B defect (n = 4), cobalamin B defect (n = 2).

[b]≥94 cm for males (≥16 years), ≥80 cm for females (≥16 years)[19] or ≥P90 (<16 years, according to age, sex, race/ethnicity).[20]

[c]Elevated ≥100 mg/dL.[19,20]

[d]Elevated ≥150 mg/dL (≥16 years)[19] or ≥110 mg/dL (<16 years).[20]

[e]Decreased <40 mg/dL for males (≥16 years), <50 mg/dL for females (≥16 years)[19] or ≤P10 (<16 years, according to sex and race).[20]

[f]Systolic hypertension ≥130 mm Hg (≥16 years)[19] or ≥P90 (<16 years, according to age, sex, height).[20]

[g]Diastolic hypertension ≥85 mm Hg (≥16 years)[19] or ≥P90 (<16 years, according to age, sex, height).[20]

proportion of them exhibited pre-diabetes or overt type 2 diabetes. Dyslipidemia was found in the majority and hypertension in more than one-third of patients with MMA (Table 3). Chronic kidney disease (CKD) stage ≥G3b[44] was present in 89% of patients with MMA (Table S1). But even upon exclusion of hypertension and hypertriglyceridemia, which are also features of CKD,[44] 60% of patients with MMA still showed 2 to 3 MetS components (Table S1). Conversely, no patient with MMA CBL fulfilled the criteria for MetS (Figure 1C and Table S1), but systolic and/or diastolic hypertension was present in 83%, with only one of these patients having CKD stage ≥G3b.

3.2 | Dietary factors

Individual daily energy and macronutrient intake by the calculated dietary treatment prescribed for patients with PA or MMA are presented in Table 1. Compared to age- and sex-specific recommendations for energy intake,[36] only 33% of patients with PA but 80% of patients with MMA were above the recommended energy intakes. Percentages of energy from fat and carbohydrates were within standard recommendations (30%-35% and >50%, respectively)[37] in the vast majority of patients of either group.

3.3 | Glucose metabolism

During OGTT, blood glucose concentrations were markedly higher at 60 and 120 minutes in patients with PA, but lower at 60 minutes in patients with MMA as compared to the respective controls (Figure 2A,B). No differences were found for patients with MMA CBL or IVA (Figure 2C,D). Although fasting insulin sensitivity was not different, OGIS was lower in patients with PA than in controls (Table 2). Hepatic insulin extraction was higher in patients with MMA, and $\Delta Cp_{30}/\Delta G_{30}$ was lower in patients with IVA, compared to the respective controls.

3.4 | Skeletal muscle energy metabolism

Compared to controls, IMCL in the gastrocnemius muscle was not different for all OA groups (Figure 1E). Patients with MMA had slightly lower ATP ($P < .05$), while patients with PA featured slightly lower Pi compared to respective controls ($P < .05$) (Table 2). Muscle phosphocreatine content was comparable between patients with OAs and their controls (Table 2).

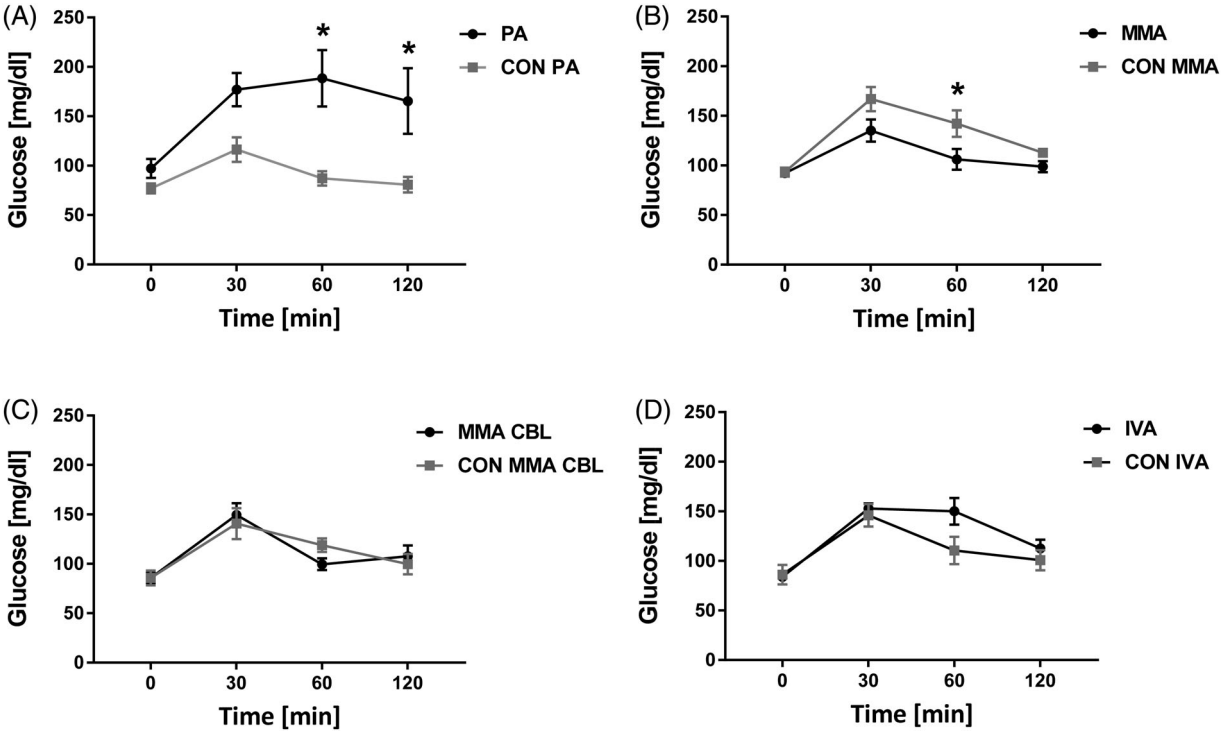

FIGURE 2 Blood glucose concentrations during an oral glucose tolerance test in patients with organic acidemias and controls. A, PA n = 5; B, MMA n = 8; C, MMA CBL n = 6 and D, IVA n = 5. Data are mean ± SD, *$P < .05$ vs CON. CON, control; IVA, isovaleric acidemia; MMA, methylmalonic acidemia; MMA CBL, adenosylcobalamin synthesis defect; PA, propionic acidemia

3.5 | Liver morphology and energy metabolism

Sonographically, 75% (6/8) of PA, 80% (8/10) of MMA and 67% (4/6) of MMA CBL patients, but only 17% (1/6) of IVA patients featured hepatomegaly. While ultrasound suggested hepatic steatosis in 38% (3/8) of patients with PA and in one patient each of the remaining groups (data not shown), two-thirds of them showing hepatomegaly, ^{1}H-MRS identified only one patient with PA and steatosis who also had hepatomegaly (Figure 1F). Of note, neither hepatic ATP nor Pi differed between OA patients and matched controls (Table 2).

3.6 | Oxidative stress

Serum OxRedox potential (Figure S1A) and serum antioxidative capacity (Figure S1B) were similar in all patient groups. Serum TBARS were higher in patients with PA than in those with IVA (Figure S1C).

4 | DISCUSSION

Here, we provide evidence for cardiometabolic risk factor clustering in PA from a case-control study in patients with branched-chain OAs. Patients with PA exhibit an accumulation of cardiometabolic risk factors with abdominal adiposity and IR already at young age predisposing for MetS. On the other hand, the data suggest an early adaptation of substrate fluxes in all OAs as shown by unchanged hepatic ATP levels.

4.1 | Propionic acidemia

Among all patients with OAs, children and adolescents with PA showed the highest percentage of abdominal obesity, postprandial hyperglycaemia, dyslipidemia, and hepatic fat accumulation, along with lower insulin sensitivity. Their daily macronutrient and energy intake was medically controlled by a calculated diet as an essential feature of treatment.[27] Of note, PA participants did not follow a more calorie-dense diet compared to standard recommendations,[36] and both carbohydrate and fat contents were within recommended ranges (Table 1). Therefore, the specific metabolic phenotype of patients with PA is not related to an excessive dietary energy intake.[36] Furthermore, the family histories did not identify a higher incidence and/or other causes of dyslipidemia or MetS in first-degree relatives of our participants, and thus, this factor likely does not affect the findings. Compared to other OAs and controls, high proportions of children and adolescents with PA exhibited fasting hyperglycaemia and dyslipidemia consistent with the definitions of paediatric MetS or cardiometabolic risk factor clustering.[21] Indeed, early-onset PA may manifest with abnormal glucose metabolism, even mimicking diabetic ketoacidosis,[45,46] although patients have so far not been reported to present with forms of diabetes.[47,48] Patients with PA also showed lower insulin sensitivity, which tightly associates with MetS, cardiometabolic risk und ultimately type 2 diabetes, potentially manifesting in childhood or adolescence. In our study, nearly 70% of children and adolescents with PA presented with MetS and the remaining approximately 30% had at least one MetS component.[20] This seems markedly higher than the prevalence of MetS ranging from 1% to 11% in European children aged 2 to 11 years[49] and approximately 30% in obese children and adolescents.[50] Consequently, early screening for MetS components in childhood appears to be of clinical relevance for this group of patients. Notably, the severity of the PA phenotype seems to relate to the prevalence of MetS or pre-diabetes/type 2 diabetes, suggesting a previously unrecognised link between PA and impairment of glucose and lipid metabolism. The strongly increased prevalence of paediatric MetS or cardiometabolic risk factor clustering in PA may further support the hypothesis that severe mitochondrial derangements can indeed lead to abnormalities of glucose and lipid metabolism, as suggested for other mitochondriopathies such as Friedreich's ataxia[51] or mitochondrial abnormalities in first-degree relatives of type 2 diabetes patients.[52] A higher frequency of cardiovascular insults in patients with PA has so far not been described.

Defective mitochondrial catabolism in PA also results in accumulation of propionyl-CoA-related metabolites, potentially leading to hyperammonemia through inhibition of N-acetylglutamate synthetase and inhibition of energy metabolism.[53] This leads to mitochondrial abnormalities including reductions in mitochondrial (mt)DNA, expression of respiratory chain complexes and oxidative phosphorylation in skeletal muscle as reported for PA patients.[54] In the present study, patients with PA showed altered muscle energy metabolism based on mildly reduced Pi levels, further confirming mitochondrial abnormalities in PA using non-invasive in vivo MRS measurements.

4.2 | MMA and MMA CBL

In contrast to the identified metabolic alterations in PA, patients with MMA had even lower post-glucose challenge glycaemia accompanied by altered hepatic insulin kinetics,

but no changes in fasting glucose, insulin sensitivity or secretion. Alterations in glucose regulation have been reported in infants and young children with MMA mimicking diabetic ketoacidosis.[55,56] Overall, 60% of patients with MMA in our study still had 2 to 3 cardiometabolic risk factors even after exclusion of high blood pressure and hypertriglyceridemia from the panel of MetS components for those with CKD ≥ stage G3b.[44] On the other hand, none of the children and adolescents with MMA CBL fulfilled criteria for MetS, and the majority had 1 to 2 risk factors. These findings underscore the differences in clinical presentation between the two entities MMA and MMA CBL despite the common biochemical finding of methylmalonic acid accumulation.[5] Patients with MMA also exhibited hepatomegaly in accordance with previous findings.[25] However, hepatomegaly was present without hepatic steatosis and might be related to abnormal liver regeneration as suggested previously by increased alpha-fetoprotein concentrations in patients with MMA and PA.[25] In this regard, a recent report points to the development of liver neoplasms in patients with MMA as an emerging liver complication.[57] Also, hepatomegaly was not accompanied by changes in ATP concentrations, which differs from a previous study in rodents reporting abnormal hepatic mitochondrial morphology and lower hepatic mitochondrial complex IV activity from ex vivo measurements.[7] The present study measured hepatic ATP concentrations non-invasively in vivo, which reflects resting flux through ATP synthase.[16] Thus, the discrepancies might be due to the methodology and the distinct feature of mitochondrial function measured (ex vivo vs in vivo). Mechanisms of differences in steatosis development in PA and MMA have not been fully elucidated, but toxicity of specific metabolites, besides depletion of coenzyme A pools, might be responsible for the hepatic abnormalities.[25]

Interestingly, we detected slightly lower skeletal muscle ATP content in MMA compared to controls, suggestive of abnormal muscle energy metabolism. Of note, muscle hypotonia and decreased muscle mass have been reported as a common clinical feature.[58] Nevertheless, the absence of any alterations in insulin sensitivity is in line with the concept that abnormal muscle mitochondrial function does not generally relate to IR.[16]

4.3 | IVA and comparison with other OAs

In opposite to PA and MMA, patients with IVA did not show any relevant alterations in energy metabolism, insulin sensitivity or secretion. This possibly reflects the clinically milder and intermittent phenotype of IVA which results from a defect of leucine catabolism that is located more proximally in the BCAA catabolic pathway than the enzymes defective in PA and MMA. In contrast to the impairment of acetyl-CoA formation in IVA, both PA and MMA enzyme defects lead to shortage of succinyl-CoA for TCA cycle function, possibly contributing to the more severe energy deficiency than in IVA.[24,29] It seems likely that mechanisms involving the defective pathways in OAs and/or disease-specific metabolites affect certain features of mitochondrial function, thereby exhibiting different degrees of mitochondrial toxicity and thus influencing metabolic phenotypes. Such differences, for example, in anaplerotic pathways, might underlie the changes in muscle energy metabolism of patients with PA and MMA, but not of those with IVA. Similarly, hepatomegaly was present in the majority of patients with PA, MMA and MMA CBL, potentially due to increased liver regeneration,[25] but only in one patient with IVA. Accumulation of propionate and propionyl CoA-derived metabolites both in PA and MMA and subsequent depletion of coenzyme A pools might possibly explain the observed hepatic involvement, based on data showing direct toxic effects on mitochondrial function in liver.[59-61]

While patients with PA exhibited increased cardiometabolic risk factor clustering and IR, there were no such changes in glucose metabolism or MetS in the groups of MMA and IVA participants. Whether severe impairment of TCA cycle function in PA might lead to reduced rates of glycolysis with lower acetyl-CoA flux to the TCA cycle and might possibly contribute to postprandial hyperglycaemia, remains speculative. However, cardiometabolic risk factor clustering in PA cannot be explained by more frequent anabolic therapies, as we found a nearly 9-fold increase in hospital admissions due to metabolic decompensations in our group of MMA patients compared to PA patients within the last 2 years prior to study.

4.4 | Strengths and limitations

This study has several limitations. The relatively small group size and wide spectrum of clinical presentation within this group of deeply phenotyped patients with OAs results in higher intragroup variation and decreases statistical power. Nevertheless, the group size of these patients with OAs as ultra rare conditions[62] and commonly functional limitations, which could hinder them from participating in research, is larger than in previous studies. The control groups were carefully recruited for each OA, but some variables (serum oxidative stress) are not available in the healthy children due to investigator consideration regarding blood sampling in minors (Figure S1). Also, some control participants were used from a study at a different site and time.[30] While acute effects of physical activity can be largely excluded for all

patients by the recommendation of refraining from exercising for 48 hours prior to admission and for another 24 hours before metabolic tests, habitual physical activity has not been formally assessed, and thereby a possible confounding effect of long-term exercise cannot be excluded. In addition, as 6 out of 9 participants with PA were young adults of 16 years of age and older, puberty may have affected the prevalence of IR in this cohort. All of these factors may introduce some variation and limit the generalisability of the results. On the other hand, our work offers novel data on in vivo organ energy metabolism in children and adolescents and can now serve as a basis for future studies in minors using repetitive measurements in prospective cohorts or intervention trials such as testing adjuvant treatment with antioxidants in PA.[13]

In conclusion, PA increases the risk of MetS and cardiometabolic risk factor clustering potentially suggesting that genetic mitochondrial derangements may contribute to the development of further metabolic abnormalities. Alterations in muscle energy metabolism of patients with PA or MMA might underlie or contribute to their IR and ectopic lipid accumulation. From a clinical perspective, these findings would suggest regular screening for cardiometabolic risk factor clustering particularly in patients with PA to prevent additional disease burden. Nevertheless, prospective studies are needed for identifying the longitudinal time course of alterations and testing treatment strategies addressing liver and muscle energy metabolism.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

S.G. and D.C. collected and analyzed analysed data and wrote the manuscript, A.B. researched and analyzed analysed data, T.J., N.S. and S.C. collected data, Ma.R. collected and researched data, G.P. calculated indices of insulin sensitivity and beta cell function, D.M. and D.H. collected and analyzed analysed data, J-H.H. reviewed and edited the manuscript, S.Ö., J.M., S.v.D., D.K., A.S., E.T., T.M. and E.M. collected data, R.E. and M.R. designed the study, researched data and reviewed and edited the manuscript. All authors reviewed and edited the manuscript, contributed to discussion, and approved the final manuscript. R.E. and M.R. are the guarantors of the work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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