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Targeted metabolomic analysis of serum phospholipid and acylcarnitine in the adult Fontan patient with a dominant left ventricle

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Abstract

Background: Patients with a Fontan circulation have altered cholesterol and lipoprotein values. We analysed small organic molecules in extended phopsholipid and acylcarnitine metabolic pathways ('metabolomes') in adult Fontan patients with a dominant left ventricle, seeking differences between profiles in baseline and Fontan circulations.

Methods: In an observational matched cross-sectional study, we compared phosphatidylcholine (PC), sphingomyelin (SM), and acylcarnitine metabolomes (105 analytes; AbsoluteIDQ® p180 kit (Biocrates Life Sciences AG, Innsbruck, Austria) in 20 adult Fontan patients having a dominant left ventricle with those in 20 age- and sex-matched healthy controls.

Results: Serum levels of total PC (q-value 0.01), total SM (q-value 0.0002) were significantly lower, and total acylcarnitines (q-value 0.02) were significantly higher in patients than in controls. After normalisation of data, serum levels of 12 PC and 1 SM Fontan patients were significantly lower (q-values <0.05), and concentrations of 3 acylcarnitines were significantly higher than those in controls (q-values <0.05).

Conclusion: Metabolomic profiling can use small specimens to identify biomarker patterns that track derangement in multiple metabolic pathways. The striking alterations in the phospholipid and acylcarnitine metabolome that we found in Fontan patients may reflect altered cell signalling and metabolism as found in heart failure in biventricular patients, chronic low-level inflammation, and alteration of functional or structural properties of lymphatic or blood vessels.

Trial registration number: ClinicalTrials.gov Identifier NCT03886935

Keywords: acylcarnitine, angiogenesis, congenital heart disease, Fontan, heart failure, inflammation, lipid, lymphatic vessel, metabolism, metabolomics, phosphatidylcholine, phospholipid, sphingomyelin, vascular stiffening

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Key summary

What is new?

- In adult Fontan patients with a dominant left ventricle the serum phospholipid and acylcarnitine profile is altered, possibly reflecting altered cell signalling and metabolism as found in heart failure, inflammation, or alteration of vascular properties.

What are the clinical implications?

- Metabolic profiling with the help of 'metabolomics' offers promise in understanding Fontan (patho)physiology and may be of interest as a new diagnostic modality that can facilitate minimally intensive Fontan follow-up.

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Introduction

Chronic heart failure is an important cause of morbidity and mortality in adults with congenital heart disease (CHD), in whom its prevalence is underappreciated.[1] The rarity of individual malformations and their often complex anatomy and physiology make the assessment of cardiac function in CHD difficult.[2] Heart failure (HF) symptoms may not always correlate with objective measures like systemic ventricular function or parameters of cardiopulmonary exercise testing, and the established biomarkers B-type natriuretic peptide (BNP) and N-terminal pro-BNP, used in diagnosis and management of HF due to acquired heart disease in a biventricular circulation, still are of controversial value in adults with CHD, as are high-sensitivity troponin, galectin-3, or growth differentiation factor 15, emerging biomarkers in adults with CHD.[3–7] A special subgroup are patients with univentricular circulation after a Fontan operation, which, without a subpulmonary ventricle, provides blood flow in series to the pulmonary and systemic circulatory systems.[8] Elevated systemic venous pressure to approximately 10–16 mm Hg in the Fontan circulation conveys blood passively into the pulmonary bed (total cavopulmonary connection), with low-shear and non-pulsatile blood flow. Only oxygenated blood passes to the single subsystemic ventricle. For Fontan patients especially, early and non-invasive biomarkers are required for the best haemodynamic monitoring and treatment,[9] as with increased numbers of increasingly older patients who have undergone Fontan surgery – in addition to specific cardiac concerns of Fontan pathophysiology such as systolic dysfunction or raised end-diastolic ventricular pressures – we face new situations that present an important combination of chronic Fontan-specific organ system alterations and aging-specific chronic comorbidities. In addition to (subtle) ventricular dysfunction, the central feature determining end-organ damage is a high central venous pressure, 'natural' for these patients, which impedes lymphatic drainage into this high-pressure venous system. In addition to the development of chylothorax, plastic bronchitis, or protein-losing enteropathy (PLE), liver function and structure in particular are progressively affected.[10–12] Usual biomarkers of hepatobiliary injury are often abnormal in Fontan patients but do not reliably track the extent of hepatobiliary disease; the same holds for findings on imaging studies, including elastography.[10] The role of the liver as a central metabolising and synthesising organ implies that profiling metabolic patterns may offer insights into Fontan physiology.[10,11] The last decade saw initial attempts to assess Fontan-patient metabolism with usual clinical-biochemistry testing. Besides hyperuricemia and abnormal glucose handling, alterations in cholesterol and lipoprotein metabolism were reported.[13–17] With that in mind, and extrapolating from findings in HF patients with a biventricular system, where the myocardial energy source switches from metabolism of fatty acids to the metabolism of glucose and ketone bodies,[18] and from findings on the important role as proinflammatory analytes that other lipids metabolised in the liver, especially phospholipids (PL), play in the setting of increased cardiovascular risk,[19] we inferred that patients with a Fontan circulation also would handle lipids anomalously. We thus elected to examine Fontan patients' serum levels of a variety of PL and acylcarnitines (taking serum acylcarnitine levels as tracking intramitochondrial long-chain fatty acids in the form of their activated acyl-coenzyme-A-esters) and attempted in a homogeneous patient group with a dominant left ventricle (LV) to define patterns that reflect metabolic derangement and that offer promise of better understanding of Fontan physiology.

Materials and methods

Patients

At the Centre of Paediatric Cardiology and Congenital Heart Disease, Heart and Diabetes Centre North Rhine-Westphalia, Ruhr-University of Bochum, Germany, between September 2016 and March 2017, we prospectively examined adult Fontan patients with a dominant LV and one age- and sex-matched healthy biventricular control per case (observational matched cross-sectional study). All Fontan patients had undergone two-stage palliation (Norwood II and III) without aortic reconstruction. Age-and sex-matched healthy controls were recruited among staff of the Centre of Paediatric Cardiology and Congenital Heart Disease, Heart and Diabetes Centre North Rhine-Westphalia, Ruhr-University of Bochum, Germany.

Eligibility criteria for all subjects included written informed consent of participants, an age at testing

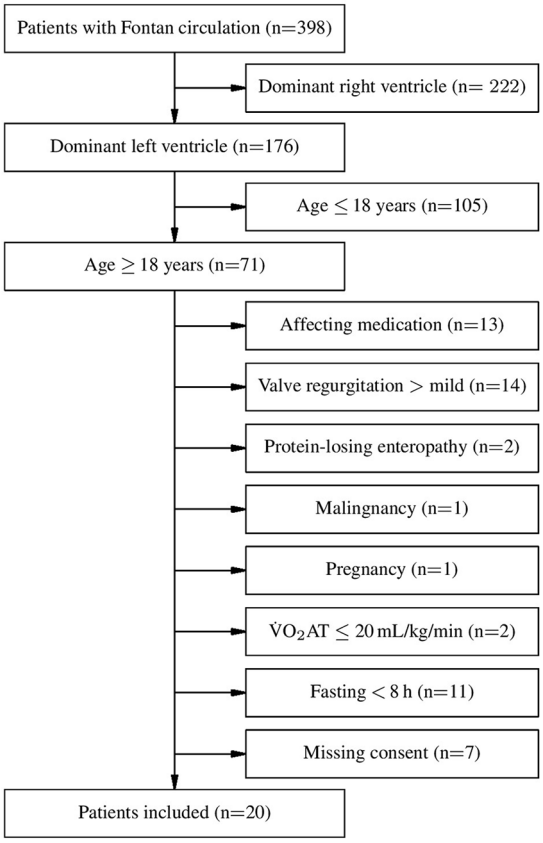

Figure 1. Patient flow chart according to STROBE.
The study population. AT, anaerobic threshold; h, hour; $\dot{V}O_2$, oxygen uptake.

of ⩾18 years, and 8 h fasting before blood sampling. Additional criteria for patients were that the left systemic ventricle be dominant and for controls both that the heart be biventricular without structural or functional abnormality and that they have no systemic, including cardiovascular, disease.

Exclusion criteria were: missing written informed consent; <8 h fasting before blood sampling; medication directly affecting metabolic state, such as cholesterol-lowering agents, or haemodynamic state, such as beta-blockers or sildenafil, with the exceptions of angiotensin converting enzyme inhibitors, diuretics, and anticoagulants; atrial or ventricular arrhythmia; coronary artery disease (history of myocardial infarction, myocardial revascularisation, percutaneous coronary intervention, or coronary artery bypass surgery); failure of the systemic ventricle as assessed by echocardiography; valvular heart disease with stenosis or with worse than mild atrioventricular or aortic regurgitation as assessed by echocardiography; recurrent effusions or PLE; any metabolic disease, such as diabetes mellitus; malignancy or other cachectic disease; liver or renal disease; inflammatory disease such as acute or chronic infection; a myeloproliferative disorder; pregnancy or lactation; multiple organ failure; malnourishment; mental handicap not allowing valid consent to participation in the study or precluding treadmill exercise; physical impairment precluding treadmill exercise; and uptake of oxygen at the anaerobic threshold ($\dot{V}O_2AT$) <20 ml/kg/min (patients) and <25 ml/kg/min (controls) on exercise capacity testing.[20,21] The chart according to STROBE (Figure 1) gives details on the flow of patients through the present study, which is a prespecified subwork of the main study protocol [ClinicalTrials.gov identifier: NCT03886935].

Since in Fontan patients loss of exercise capacity is a powerful predictor of mortality, we correlated metabolic results with routine laboratory parameters and exercise capacity parameters.[15] All patients underwent symptom-limited treadmill exercise capacity testing with expired-gas analysis.[21] A 12-lead electrocardiogram was used to determine heart rate, and oxygen uptake at rest ($\dot{V}O_2$ at rest; ml/kg/min), $\dot{V}O_2AT$ (ml/kg/min), and maximum uptake of oxygen ($\dot{V}O_2max$; ml/kg/min) were measured.

Age, sex, weight, body mass index, vital parameters, cardiac risk factors, history of cardiac disease, and cardiac medications, with blood sampling for routine haematological and biochemical profiling, were assessed during an outpatient-clinic visit. Fasting patients underwent phlebotomy while recumbent. Echocardiography followed, and, after a defined snack rich in carbohydrates, exercise capacity was tested. Blood studies required samples 0.5 ml greater than those for routine assessments to permit determinations of concentrations of lipid-metabolism analytes. For serum preparation, the blood sample was directly drawn into a tube containing a clotting activator. To separate serum, the sample was centrifuged within 20 min (15°C, 10 min at 2500*g*). Serum aliquots were immediately frozen and stored at −80°C for further analyses (maximum storage time 4 months). Frozen samples were transported on dry ice to the

analysing laboratory. Analyses were performed in batches of 10 samples.

Sample preparation and method

Before analysis, all serum samples were processed as described, with samples thawed on ice and centrifuged; the supernatant was subjected to further analyses.[22] The AbsoluteIDQ® p180 kit assay (Biocrates Life Sciences AG, Innsbruck, Austria) permitted targeted, fully automated quantification of 188 metabolites (142 lipids comprising PC, SM, and acylcarnitines) based on phenylisothiocyanate derivatisation in the presence of internal standards followed by flow injection analysis tandem mass spectrometry using a SCIEX 4000 QTRAP® instrument (SCIEX, Darmstadt, Germany) with electrospray ionisation.

The SCIEX 4000 QTRAP instrument was calibrated periodically after each cleaning cycle, at least once a year, using a chemical standards kit with low/high concentrations of polypropylene glycol polymers (SCIEX, MS Chemical Kit 1, Low-High Conc. PPGs, part number 4406127). The solutions were measured according to the kit instructions and mass calibration and resolution were manually adjusted to meet the specifications of the manufacturer. Measurements were validated by meeting criteria specified by the manufacturer.

Statistics

To exclude metabolites below the limit of detection (LOD), the raw data (µmol/l) were cleaned applying a modified 80% rule; thus, for statistical analysis, at least 80% valid values above LOD needed to be available per analyte in the samples for each group. This reduced the dataset to 143 analytes, including 97 phosphatidylcholines (PC) and sphingomyelins (SM) and 23 acylcarnitines. Remaining values below LOD were imputed applying a log-spline method with values between LOD and LOD/2. After log2 transformation of metabolomics data as well as of 'routine analytes' and clinical data, the dataset was used for multivariate (hierarchical cluster analysis) and univariate statistical analyses. Student's *t*-tests with a Benjamini–Hochberg correction identified significant metabolite (and clinical routine-parameter) differences between patients and controls and p values were calculated, with values <0.05 considered statistically significant. Correlations between the concentration values of the metabolites (and metabolite sums and ratios) with the clinical variables of the participants were evaluated by calculating the Pearson's correlation coefficient r, with r a measure of linear correlation that can have values between -1 and 1, where 1 indicates total positive linear correlation, 0 indicates no linear correlation, and -1 indicates total negative linear correlation. In general, r values >0.5 (< -0.5) indicate a moderate correlation and r values >0.8 (or < -0.8) indicate a strong correlation.[23] To interpret the correlation, the significance of the correlation was calculated from r and the degrees of freedom (a variable dependent on the sample number). The resulting p value was adjusted for multiple testing, or false discovery rate (FDR), according to Benjamini and Hochberg.[24] Correlations with FDR-adjusted p-values <0.05 were considered statistically significant. To differentiate between general lipid mass changes and species-specific alterations the data were normalised in a class- and samplewise manner and were subjected to statistical analysis (*t*-tests with Benjamini–Hochberg correction, $p<0.05$). Values below LOD were not considered for normalisation and were removed before hypothesis testing. For calculation of the ratio between total PC and SM LOD values were treated similarly. Statistical analysis was performed using R version 3.5.1 or 3.6.1 (R Core Team, 2019).[25]

Ethics

The study conformed to the principles outlined in the Declaration of Helsinki and was approved by the local ethics committees of the Medical University of Innsbruck, Austria (AN2015-0303 357.43), and of the Heart and Diabetes Centre North Rhine-Westphalia, Ruhr-University of Bochum, Germany (AZ 52/2016).

Results

Of the 398 Fontan patients registered at the Centre of Paediatric Cardiology and Congenital Heart Disease, Heart and Diabetes Centre North Rhine-Westphalia, Ruhr-University of Bochum, Germany, 176 patients were potentially eligible for our study by morphology (dominant LV). Applying the criterion of ≥ 18 years of age, 71

patients were eligible. After all exclusion criteria were applied, 20 Fontan patients and 20 age- and sex-matched controls were enrolled (Figure 1). Data were complete for all of them. Table 1 lists clinical and echocardiographic parameters of patients and controls.

Exercise capacity testing

In Fontan patients, $\dot{V}O_2$ at the anaerobic threshold and at maximum was significantly lower than in controls (Table 2).

Routine analytes

Haemoglobin and haematocrit, partial thromboplastin time, international normalised ratio (INR), triglyceride, gamma glutamyl transferase activity, alanine aminotransferase activity, total bilirubin, creatinine, urea, and uric acid values were significantly higher, and high density lipoprotein-cholesterol (HDL-C) values and thrombocyte counts were significantly lower in patients than in controls (Table 3).

Metabolomic examination of serum

Serum concentrations of 14 lyso-PC (12 above LOD), 76 PC (71 above LOD), and 15 SM (14 above LOD) were determined. Among the 97 analytes above LOD, serum concentrations of 60 (62 %), including analytes in all three subgroups and fatty acids with mostly higher carbon numbers, were significantly lower in Fontan patients than in controls (q-values <0.05) (Figure 2, Figure 3). Values for no single analyte were higher in patients than in controls. Serum concentrations of 40 acylcarnitines were determined; 23 with values above LOD, 3 were significantly higher in patients than in controls (C0, C18:2, C3; q-values < 0.05). Summed by subgroups – all PC, all lyoPC, all SM, and all acylcarnitines – all lipids were significantly decreased in patients. Table 4 shows concentrations and statistical results are shown for all metabolites determined.

Normalisation of metabolomics data by lipid class reduced the 60 (lyso)PC and SM with significant LODs to just 13 (PC aa C30:0, C32:2, C32:3, C34:3, C34:4, C36:6, C40:4; PC ae C30:0, C38:0, C40:1, C40:3, C42:3; SM (OH) C22:1). All these are low in relative abundance (<5% of total lipid mass). With normalisation all the 3 acylcarnitines with significant LODs lost significance.

Correlations of routine biochemical and clinical parameters with metabolomic parameters

Among routine biochemical analytes, the variables INR, alanine aminotransferase activity, haemoglobin, and haematocrit displayed significantly negative correlations with metabolic parameters. Significantly positive correlations existed between metabolomic parameters and minimum and maximum oxygen saturations. No other correlations could be established, especially none with respect to platelet count, total protein, albumin, CRP, NT-proBNP, and any exercise capacity parameter, including peak $\dot{V}O_2$ (Online Supplemental Table 1).

Discussion

To the best of our knowledge, ours is the first clinical metabolomics study focussing on Fontan patients' serum PL and acylcarnitine levels. Its main result is the observation that PC, SM, and acylcarnitine values differed significantly between Fontan patients and controls: Fontan patients had lower total PC and SM values and higher total acylcarnitine values.

A general decrease in total PL mass in Fontan patients accords with the reduction that we observed in HDL-C, a major lipid constituent of serum. To differentiate effects on lipid mass in general from particular changes in individual lipid species, we normalised our metabolomics data by lipid class. Following normalisation, statistical significance in LOD was lost for $>80\%$ of analyte comparisons, with significance persisting for some low-abundance lipid species (13 PL, <5% of lipid mass). This indicates that in Fontan patients the imbalance in the metabolism of polar lipids is not limited to a subset of distinct lipids, constituting a lipid profile, but instead quantitatively affects all classes of PL and acylcarnitines.

PC and SM are PL; SM also is a sphingolipid (a phosphosphingolipid). PL typically are major participants in structural-lipid metabolism, in concert with cholesterol and steroids forming the extracellular lipid bilayer of cells and located in all tissues.[26] While cholesterol and steroids are

Table 1. Participants' clinical characteristics (mean values ± standard deviation).

	Fontan patients (%)	Controls (%)	*p*-value
Malformation	Double inlet left ventricle: 50		
	TA+PS/PA: 45		
	TA+PS+VSD: 5		
Extracardiac Fontan	80		
Open fenestration (time of study)	15		
Female sex	35	35	
After TCPC (years)	18.8 ± 5.2		
Age (years)	23.1 ± 5.1	24.7 ± 6.6	0.28
Weight (kg)	69.8 ± 13.2	73.3 ± 11.7	0.17
Height (cm)	171.3 ± 7.4	174.5 ± 8.7	0.04
Body mass index (kg/m²)	23.8 ± 4.1	22.5 ± 3.3	0.05
Further cardiac procedures	LPA dilation/stent: 20	-	
	Tunnel dilation/stent: 30	-	
	Closure of fenestration: 5	-	
	Closure of vv collateral: 15	-	
	Electrophysiologic exam: 10	-	
Further extracardiac disease	Celiac disease: 5	-	
	Atopic dermatitis: 5	-	
Dietary intake	Gluten-free diet: 5	-	
Medication			
Aspirin	5	-	
Phenprocoumon	80	-	
ACE inhibitor	20	-	
Diuretic	20	-	
Aldosterone antagonist	20	-	
Echocardiography			
Good ventricular function	100	100	
Absent or mild atrioventricular valve regurgitation	100	100	
Absent or mild aortic valve regurgitation	100	100	

ACE, angiotensin-converting-enzyme; exam, examination; LPA, left pulmonary artery; PA, pulmonary atresia; pts, patients; PS, pulmonary stenosis; TA, tricuspid atresia; TCPC, total cavopulmonary connection; VSD, ventricular septal defect; vv, veno-venous. Patients with celiac disease were on gluten-free diets.

Table 2. Participants' exercise capacity testing results.

	Fontan patients	Controls	*p*-value
$\dot{V}O_2$ at rest, ml/kg/min	5.6 ± 1.7	5.8 ± 1.1	0.03
$\dot{V}O_2$AT, ml/kg/min	24.5 ± 4.9	30.1 ± 3.6	<0.00001
$\dot{V}O_2$max, ml/kg/min	28.8 ± 10.1	45.7 ± 6.4	<0.00001
Heart rate at rest, 1/min	83 ± 17	86 ± 19	0.08
SpO_2 at rest, %	93 ± 3	99 ± 1	<0.00001
SpO_2 at exercise, %	90 ± 3	98 ± 1.4	<0.00001
BP systolic at rest, mmHg	123 ± 10	119 ± 12	0.04
BP diastolic at rest, mmHg	68 ± 8	71 ± 8	0.46

Values are given as mean ± standard deviation. AT, anaerobic threshold; BP, blood pressure; RR, Riva Rocci; SpO_2, pulsoxymetric oxygen saturation; $\dot{V}O_2$, oxygen uptake.

Table 3. Values, routine analytes.

	Fontan patients	Controls	p-value
Total cholesterol, mg/dl	145.3 ± 26.5	149.0 ± 34.2	0.77
HDL-C, mg/dl	42.5 ± 15.9	51.3 ± 12.3	0.03*↓
Non-HDL-C, mg/dl	85.2 ± 24.8	73.1 ± 20.8	0.2
Triglycerides, mg/dl	128.6 ± 86.5	47.3 ± 22.8	0.0003*↑
Lipoprotein (a), mg/dl	12.4 ± 15.5	10.9 ± 6.6	0.47
Total protein, g/dl	7.2 ± 0.5	7.0 ± 0.7	0.31
Albumin, mg/dl	4145 ± 492	4215 ± 208	0.64
Uric acid, mg/dl	5.9 ± 1.4	3.9 ± 1.3	0.0003*↑
Urea, mg/dl	32.6 ± 7.4	22.8 ± 8.9	0.0008*↑
Creatinine, mg/dl	0.8 ± 0.12	0.53 ± 0.18	<0.00001*↑
Total bilirubin, mg/dl	1.22 ± 0.67	0.3 ± 0.29	<0.00001*↑
AST, U/l	35.3 ± 7.7	31.6 ± 8.4	0.12
ALT, U/l	39.4 ± 11.4	31.9 ± 10.1	0.04*↑
gGT, U/l	86.5 ± 43.6	35.1 ± 19.4	0.00002*↑
Alkaline phosphatase, U/l	103.3 ± 53.2	99.1 ± 72.6	0.20
GLDH, U/l	3.7 ± 1.9	3.5 ± 1.5	0.26

(Continued)

Table 3. (Continued)

	Fontan patients	Controls	p-value
CRP, mg/dl	0.18 ± 0.2	0.16 ± 0.14	0.47
Creatine kinase, U/l	128.7 ± 65.5	94.8 ± 37.7	0.09
NT-proBNP, pg/ml	52.4 ± 69.2	39.3 ± 30.4	0.88
INR	2.1 ± 0.76	1.02 ± 0.04	<0.00001*↑
PTT, sec	35.2 ± 5.7	27.0 ± 3.9	0.00004*↑
Fibrinogen, mg/dl	239.2 ± 78.4	258.7 ± 57.3	0.17
Antithrombin III, %	101.6 ± 10.5	104.5 ± 8.5	0.38
Leucocytes, 1/nl	6.7 ± 3.2	7.2 ± 2.6	0.23
Thrombocytes, 1000/nl	171.3 ± 73.2	279.8 ± 88.5	0.0002*↓
Haemoglobin, g/dl	16.4 ± 2.1	12.7 ± 1.4	<0.00001*↑
Haematocrit, %	47.8 ± 5.6	39.3 ± 4.2	<0.00001*↑

Values are given as mean ± standard deviation. ALT, alanine aminotransferase activity; AST, aspartate aminotransferase activity; C, cholesterol; CRP, C-reactive protein; gGT, gamma glutamyl transferase activity; GLDH, glutamate dehydrogenase activity; HDL, high density lipoprotein; INR, international normalised ratio; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; PTT, partial thromboplastin time; *↑, statistically significant higher serum concentration in Fontan patients than in controls; *↓, statistically significant lower serum concentration in Fontan patients than in controls.

stabilising and stiffening components, PL confer fluidity upon polar-bilayer cell membranes. They also are important components of HDL.[27] In addition to their function in structural metabolism, PL are importantly involved in signalling and they can affect synthesis of prostaglandins and leukotrienes and thus inflammation.[28] Moreover, like fatty acids, PL are involved in energy metabolism. Of the 13 PL that we still found to be decreased in Fontan patients' serum after normalisation of data, 12 are PC. Aside from pregnancy and obesity, no distinct conditions and disorders, especially no cardiovascular disorders, are associated with altered concentrations of these analytes.

A further PL for which we still found decreased serum concentrations in Fontan patients after normalisation of data is SM (phosphosphingolipid). On hydrolysis into ceramides, SM like the one that we identified are generally involved in necroptosis (as long chain fatty acids are), a type of regulated cell death characterised by plasma membrane rupture and inflammatory response.[29] Tissues are enriched in HDL-SM in Niemann–Pick disease, which is caused by an acid sphingomyelinase deficiency due to mutations in *SMPD1*,[30] and SM-depleted HDL was observed in patients with diabetes.[31] Furthermore, SM is substantially involved in the development and progression of the atherosclerotic plaques of coronary artery disease (CAD): human atherosclerotic lesions are enriched in SM and ceramide species,[32] and elevated SM serum levels are independent predictors of CAD in humans.[33,34]

An increase in acylcarnitine blood concentration can mirror an imbalance of mitochondrial fatty acid degradation, as found in patients with pathogenic mutations in genes encoding enzymes involved in long-chain fatty acid oxydation.[35] Interestingly, one important feature of mitochondrial disorders can be the development of a cardiomyopathy. Of further note is that, like SM, long-chain fatty acids are involved in necroptosis, with increased acylcarnitine cell concentrations.[29] Possibly underlying the changes that we saw are shifts in cellular signalling associated with HF, chronic low-level inflammation, and alteration of

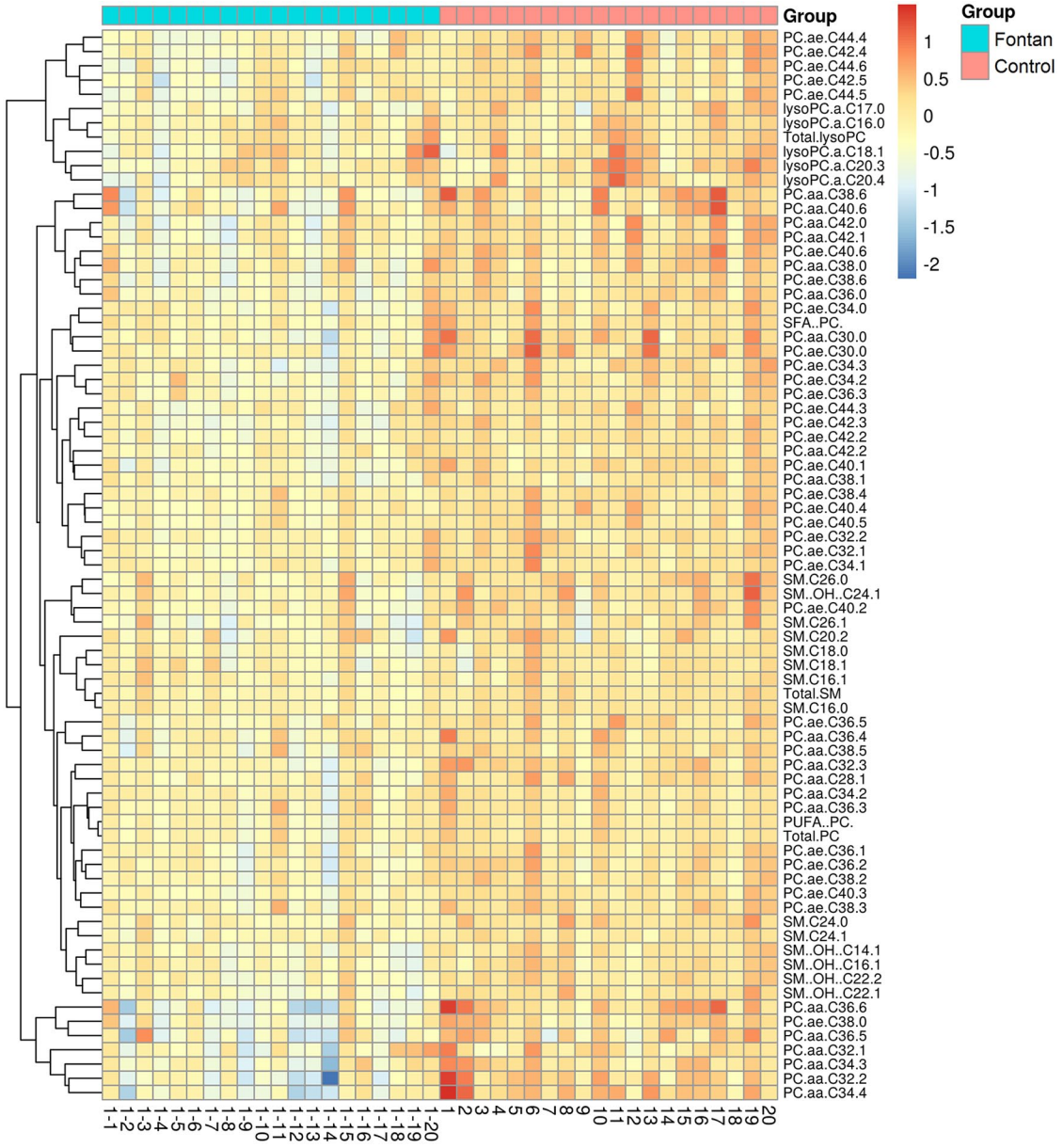

Figure 2. Heat map of phospholipid serum concentrations.
Heat map of serum concentrations of all phospholipids that were significantly different between Fontan patients (green boxes) and matched healthy biventricular controls (red boxes). The colours in the heat map represent the fold changes (Fontan patients *versus* controls) of the univariate statistic: negative values represent a decrease in concentration. PC, phosphatidylcholine; SM, sphingomyelin.

functional or structural properties of lymphatic or blood vessels.

Altered cell signalling and metabolism as indicators of subtle HF

That decreased PL concentrations of PL in our Fontan patients accompany increased acylcarnitine concentrations agrees with our second hypothesis that in addition to structural homeostasis, energy metabolism also might be perturbed, marking early, subtle changes in (myocardial) cell metabolism. The normal heart primarily utilises free fatty acids as a source of energy and switches to glucose metabolism during stress. In biventricular patients with HF, changes in myocardial mitochondrial function result in preferential use of glucose and ketone

Table 4. Metabolite concentrations.

Metabolite	HMBD ID	Patients	Controls	*p*	*q*	FC	Patients°	Controls°	*p*°	*q*°
C0	**HMDB00062**	**45.2 ± 10.3**	**36.8 ± 9.4**	**0.01**	**0.02*↑**	**1.23**	85.5 ± 2.9	83.8 ± 4.4	0.14	0.27
C10	HMDB00651	0.17 ± 0.06	0.22 ± 0.15	0.07	0.11	−1.37	0.34 ± 0.13	0.55 ± 0.45	0.06	0.16
C10:1	HMDB13205	NA	NA	NA	NA	NA	NA	NA	NA	NA
C10:2	HMDB0013325	NA	NA	NA	NA	NA	NA	NA	NA	NA
C12	HMDB02250	NA	NA	NA	NA	NA	NA	NA	NA	NA
C12:1	HMDB0013326	NA	NA	NA	NA	NA	NA	NA	NA	NA
C12-DC		NA	NA	NA	NA	NA	NA	NA	NA	NA
C14	HMDB05066	NA	NA	NA	NA	NA	0.07 ± 0.02	0.09 ± 0.03	0.04	0.12
C14:1	HMDB0002014	0.09 ± 0.03	0.09 ± 0.02	0.67	0.77	−1.03	0.17 ± 0.05	0.21 ± 0.06	0.03	0.1
C14:1-OH	HMDB0013330	NA	NA	NA	NA	NA	NA	NA	NA	NA
C14:2	HMDB13331	0.02 ± 0.01	0.02 ± 0.01	0.15	0.21	1.15	0.05 ± 0.02	0.06 ± 0.01	0.01	0.06
C14:2-OH		NA	NA	NA	NA	NA	NA	NA	NA	NA
C16	HMDB00222	0.11 ± 0.03	0.1 ± 0.03	0.27	0.35	1.11	0.21 ± 0.05	0.24 ± 0.07	0.23	0.37
C16:1	HMDB06317 HMDB13207	NA	NA	NA	NA	NA	NA	NA	NA	NA
C16:1-OH	HMDB13333	NA	NA	NA	NA	NA	NA	NA	NA	NA
C16:2	HMDB13334	NA	NA	NA	NA	NA	NA	NA	NA	NA
C16:2-OH	HMDB13335	NA	NA	NA	NA	NA	NA	NA	NA	NA
C16-OH	HMDB13336	NA	NA	NA	NA	NA	NA	NA	NA	NA
C18	HMDB00848	0.05 ± 0.02	0.04 ± 0.02	0.12	0.19	1.18	0.1 ± 0.03	0.1 ± 0.04	0.65	0.74
C18:1	HMDB0006351 HMDB0005065 HMDB06464 HMDB0013338	0.16 ± 0.09	0.11 ± 0.03	0.04	0.07	1.4	0.3 ± 0.12	0.27 ± 0.08	0.41	0.52

(Continued)

Table 4. (Continued)

Metabolite	HMBD ID	Patients	Controls	*p*	*q*	FC	Patients°	Controls°	*p*°	*q*°
C18:1-OH	HMDB13339	NA	NA	NA	NA	NA	NA	NA	NA	NA
C18:2	**HMDB0006469 HMDB0006461**	**0.05 ± 0.02**	**0.04 ± 0.12**	**0.01**	**0.02*↑**	**1.42**	0.1 ± 0.03	0.09 ± 0.03	0.17	0.31
C2	HMDB00201	5.9 ± 2.1	5.3 ± 1.9	0.3	0.38	1.11	11.0 ± 2.5	12.2 ± 3.3	0.21	0.35
C3	**HMDB00824**	**0.43 ± 0.15**	**0.33 ± 0.17**	**0.02**	**0.04*↑**	**1.29**	0.8 ± 0.19	0.73 ± 0.21	0.27	0.4
C3:1	HMDB0013124	NA	NA	NA	NA	NA	NA	NA	NA	NA
C3-DC (C4-OH)	HMDB02095	0.05 ± 0.04	0.04 ± 0.01	0.17	0.24	1.3	0.09 ± 0.05	0.09 ± 0.03	0.99	0.99
C3-OH	HMDB0013125	NA	NA	NA	NA	NA	NA	NA	NA	NA
C4	HMDB02013 HMDB00736	0.25 ± 0.13	0.19 ± 0.07	0.06	0.1	1.33	0.46 ± 0.17	0.43 ± 0.13	0.49	0.61
C4:1	HMDB0013126	NA	NA	NA	NA	NA	NA	NA	NA	NA
C5	HMDB13128 HMDB0000378 HMDB00688 HMDB0041993	0.18 ± 0.08	0.14 ± 0.07	0.07	0.11	1.26	0.34 ± 0.14	0.31 ± 0.09	0.48	0.61
C5:1	HMDB0002366	NA	NA	NA	NA	NA	NA	NA	NA	NA
C5:1-DC	HMDB0013129	NA	NA	NA	NA	NA	NA	NA	NA	NA
C5-DC (C6-OH)	HMDB0013130	NA	NA	NA	NA	NA	NA	NA	NA	NA
C5-M-DC	HMDB0000552	NA	NA	NA	NA	NA	NA	NA	NA	NA
C5-OH (C3-DC-M)	HMDB0013132 HMDB0013133	NA	NA	NA	NA	NA	NA	NA	NA	NA
C6 (C4:1-DC)	HMDB00756 HMDB0000705	NA	NA	NA	NA	NA	NA	NA	NA	NA
C6:1	HMDB0013161	NA	NA	NA	NA	NA	NA	NA	NA	NA
C7-DC	HMDB0013328	NA	NA	NA	NA	NA	0.16 ± 0.1	0.16 ± 0.08	0.91	0.97
C8	HMDB0000791 HMDB00834	0.16 ± 0.06	0.24 ± 0.21	0.06	0.11	−1.52	0.37 ± 0.13	0.64 ± 0.66	0.11	0.23
C9	HMDB06320 HMDB0013288	0.03 ± 0.01	0.04 ± 0.02	0.03	0.06	−1.32	0.06 ± 0.01	0.09 ± 0.05	0.006	0.05

(Continued)

Table 4. (Continued)

Metabolite	HMBD ID	Patients	Controls	*p*	*q*	FC	Patients°	Controls°	*p*°	*q*°
lysoPC a C14:0	HMDB10379	NA	NA	NA	NA	NA	NA	NA	NA	NA
lysoPC a C16:0	HMDB10382 HMDB0061702	82.2 ± 17.9	91.5 ± 17.0	0.08	0.13	−1.11	6.3 ± 0.88	6.2 ± 0.98	0.54	0.64
lysoPC a C16:1	HMDB0010383	2.6 ± 0.8	2.8 ± 0.5	0.21	0.29	−1.07	0.2 ± 0.04	0.19 ± 0.03	0.3	0.43
lysoPC a C17:0	**HMDB12108**	**1.6 ± 0.4**	**2.1 ± 0.5**	**0.002**	**0.007*↓**	**−1.29**	0.13 ± 0.02	0.14 ± 0.03	0.08	0.19
lysoPC a C18:0	HMDB10384 HMDB0011128	24.1 ± 6.1	27.3 ± 7.0	0.15	0.22	−1.14	1.8 ± 0.3	1.8 ± 0.43	0.98	0.99
lysoPC a C18:1	HMDB02815 HMDB0010385 HMDB0061701	22.3 ± 8.7	25.5 ± 7.5	0.13	0.2	−1.15	1.7 ± 0.55	1.7 ± 0.49	0.94	0.99
lysoPC a C18:2	HMDB10386 HMDB0061700	35.1 ± 14.4	40.6 ± 14.2	0.17	0.24	−1.16	2.7 ± 1.05	2.7 ± 0.91	0.96	0.99
lysoPC a C20:3	HMDB10394 HMDB0010393	2.0 ± 0.5	2.5 ± 0.8	0.03	0.06	−1.24	0.16 ± 0.03	0.17 ± 0.05	0.35	0.48
lysoPC a C20:4	HMDB10395 HMDB0010396	5.7 ± 1.4	7.0 ± 2.1	0.02	0.05	−1.24	0.44 ± 0.13	0.47 ± 0.14	0.51	0.62
lysoPC a C24:0	HMDB10405	NA	NA	NA	NA	NA	NA	NA	NA	NA
lysoPC a C26:0	HMDB0029205	0.39 ± 0.25	0.35 ± 0.2	0.86	0.91	1.09	0.03 ± 0.02	0.02 ± 0.015	0.32	0.45
lysoPC a C26:1	HMDB0029220	0.22 ± 0.13	0.23 ± 0.12	0.8	0.9	−1.01	0.02 ± 0.01	0.02 ± 0.01	0.51	0.62
lysoPC a C28:0	HMDB29206	0.35 ± 0.18	0.35 ± 0.14	0.58	0.68	−1.01	0.03 ± 0.01	0.02 ± 0.01	0.47	0.6
lysoPC a C28:1	HMDB0029221	0.46 ± 0.18	0.52 ± 0.17	0.18	0.26	−1.14	0.04 ± 0.01	0.04 ± 0.01	0.99	0.99
PC aa C24:0		0.13 ± 0.07	0.12 ± 0.06	0.78	0.86	1.08	0.01 ± 0.005	0.01 ± 0.004	0.23	0.37
PC aa C26:0		NA	NA	NA	NA	NA	0.1 ± 0.03	0.08 ± 0.02	0.18	0.31
PC aa C28:1	**HMDB0007867 HMDB0007899**	**2.3 ± 0.5**	**3.1 ± 0.6**	**0.00008**	**0.0005*↓**	**−1.33**	0.18 ± 0.03	0.21 ± 0..03	0.008	0.06
PC aa C30:0	**HMDB0007934 HMDB0007869 HMDB0007965**	**3.2 ± 0.9**	**4.9 ± 1.5**	**0.00004**	**0.0004*↓**	**−1.52**	**0.25 ± 0.05**	**0.33 ± 0.08**	**0.001**	**0.01*↓**

(Continued)

Table 4. (Continued)

Metabolite	HMBD ID	Patients	Controls	*p*	*q*	FC	Patients°	Controls°	*p*°	*q*°
PC aa C30:2	HMDB0007999	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC aa C32:0	HMDB00564 HMDB0007871 HMDB0008031	9.7 ± 2.0	10.4 ± 2.0	0.25	0.33	−1.07	0.75 ± 0.11	0.7 ± 0.08	0.08	0.19
PC aa C32:1	**HMDB0007872 HMDB0007873 HMDB0008097 HMDB0007969**	**9.9 ± 3.7**	**12.4 ± 3.5**	**0.01**	**0.03*↓**	**−1.26**	0.75 ± 0.21	0.83 ± 0.18	0.18	0.31
PC aa C32:2	**HMDB0008002 HMDB0007874**	**2.1 ± 0.7**	**3.7 ± 1.2**	**<0.00001**	**0.0001*↓**	**−1.75**	**0.16 ± 0.05**	**0.25 ± 0.06**	**<0.00001**	**0.0006*↓**
PC aa C32:3	**HMDB0007876**	**0.29 ± 0.05**	**0.41 ± 0.08**	**<0.00001**	**0.00008*↓**	**−1.41**	**0.02 ± 0.003**	**0.03 ± 0.005**	**0.0004**	**0.007*↓**
PC aa C34:1	HMDB0007971 HMDB08100 HMDB0008035 HMDB0007972 HMDB0008003	141.2 ± 35.5	152.8 ± 23.7	0.14	0.21	−1.08	10.8 ± 1.3	10.3 ± 1.0	0.14	0.27
PC aa C34:2	HMDB07973 HMDB0008101 HMDB0008133 HMDB0008005 HMDB0008004	220.9 ± 36.2	249.0 ± 41.7	0.03	0.05	−1.13	17.1 ± 1.6	16.7 ± 1.2	0.36	0.49
PC aa C34:3	**HMDB08006 HMDB0007974 HMDB0007975**	**10.2 ± 3.1**	**14.4 ± 3.5**	**0.0003**	**0.001*↓**	**−1.42**	**0.77 ± 0.16**	**0.97 ± 0.18**	**0.0009**	**0.01*↓**
PC aa C34:4	**HMDB0007883 HMDB0007976**	**0.9 ± 0.27**	**1.6 ± 0.54**	**<0.00001**	**0.00004*↓**	**−1.83**	**0.07 ± 0.02**	**0.11 ± 0.03**	**<0.00001**	**0.0004*↓**
PC aa C36:0	**HMDB0008265 HMDB0008036 HMDB0008525 HMDB0007886**	**1.8 ± 0.47**	**2.3 ± 0.46**	**0.0007**	**0.003*↓**	**−1.3**	0.14 ± 0.03	0.15 ± 0.03	0.07	0.18
PC aa C36:1	HMDB08038 HMDB0008069 HMDB0007978 HMDB0008102	41.5 ± 12.2	43.9 ± 7.7	0.3	0.38	−1.06	3.2 ± 0.53	3.0 ± 0.5	0.24	0.38
PC aa C36:2	HMDB08039 HMDB0008070 HMDB08135 HMDB0007979	156.8 ± 32.2	166.1 ± 23.4	0.25	0.34	−1.06	12.0 ± 1.24	11.2 ± 0.89	0.01	0.07
PC aa C36:3	HMDB08105 HMDB0007980 HMDB0007981 HMDB0008040	80.9 ± 18.6	92.9 ± 16.5	0.03	0.05	−1.15	6.2 ± 0.58	6.2 ± 0.61	0.88	0.94
PC aa C36:4	**HMDB07982 HMDB0008107 HMDB0008138 HMDB0008234 HMDB0008429 HMDB0008170 HMDB0008138 HMDB0008042 HMDB0008203 HMDB0008106**	**110.6 ± 24.0**	**134.8 ± 30.0**	**0.005**	**0.01*↓**	**−1.22**	8.5 ± 1.3	9.0 ± 1.2	0.25	0.38

(Continued)

Table 4. (Continued)

Metabolite	HMBD ID	Patients	Controls	*p*	*q*	FC	Patients°	Controls°	*p*°	*q*°
PC aa C36:5	**HMDB07984 HMDB0008015**	**11.8 ± 5.3**	**17.6 ± 5.0**	**0.0005**	**0.002*↓**	**−1.49**	0.89 ± 0.32	1.18 ± 0.32	0.007	0.05
PC aa C36:6	**HMDB0008206 HMDB0007892**	**0.4 ± 0.17**	**0.78 ± 0.26**	**<0.00001**	**0.00004*↓**	**−1.92**	**0.03 ± 0.01**	**0.05 ± 0.01**	**<0.00001**	**0.0004*↓**
PC aa C38:0	HMDB0007893 HMDB0008528 HMDB0008043 HMDB0008755 HMDB0008267 HMDB0007985	2.1 ± 0.67	2.5 ± 0.6	0.03	0.05	−1.21	0.16 ± 0.05	0.17 ± 0.04	0.66	0.75
PC aa C38:1	**HMDB0007894 HMDB0008269 HMDB0007986 HMDB0008268 HMDB0008109 HMDB0008044**	**0.74 ± 0.16**	**0.93 ± 0.16**	**0.0005**	**0.002*↓**	**−1.25**	0.06 ± 0.01	0.06 ± 0.01	0.11	0.23
PC aa C38:3	HMDB0008020 HMDB0008046 HMDB0008047	33.0 ± 10.5	33.3 ± 5.5	0.61	0.7	−1.01	2.5 ± 0.5	2.2 ± 0.28	0.04	0.11
PC aa C38:4	HMDB0008048 HMDB0008112 HMDB0008113 HMDB0007988	68.6 ± 20.0	71.9 ± 11.0	0.3	0.38	−1.05	5.3 ± 1.1	4.8 ± 0.45	0.11	0.23
PC aa C38:5	HMDB0007990 HMDB0007989 HMDB0008050 HMDB0008114	33.4 ± 9.2	40.7 ± 6.4	0.005	0.01*↓	−1.22	2.6 ± 0.4	2.7 ± 0.32	0.13	0.25
PC aa C38:6	HMDB0008725 HMDB0008116 HMDB0008434 HMDB0007991 HMDB0008147 HMDB0008083 HMDB0008499	38.0 ± 15.8	56.4 ± 18.7	0.0005	0.002*↓	−1.48	2.9 ± 0.98	3.75 ± 1.0	0.01	0.06
PC aa C40:1	HMDB0008084 HMDB0008052 HMDB0008117 HMDB0007993 HMDB0008275	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC aa C40:2	HMDB0008308 HMDB0008276	0.25 ± 0.05	0.26 ± 0.04	0.47	0.55	−1.03	0.02 ± 0.002	0.02 ± 0.003	0.03	0.12
PC aa C40:3	HMDB0008278 HMDB0008277 HMDB0008086 HMDB0008119	0.42 ± 0.08	0.44 ± 0.05	0.32	0.4	−1.04	0.03 ± 0.003	0.03 ± 0.004	0.007	0.05
PC aa C40:4	**HMDB0008279 HMDB0008628 HMDB0008054**	**2.6 ± 0.72**	**2.6 ± 0.34**	**0.93**	**0.96**	**1.02**	**0.2 ± 0.04**	**0.17 ± 0.02**	**0.005**	**0.047*↓**
PC aa C40:5	HMDB0008056 HMDB0008055 HMDB0008120	6.3 ± 2.0	6.7 ± 1.2	0.33	0.41	−1.05	0.49 ± 0.1	0.45 ± 0.07	0.22	0.36
PC aa C40:6	**HMDB08057 HMDB0008089**	**14.9 ± 5.6**	**19.0 ± 6.4**	**0.02**	**0.049*↓**	**−1.27**	1.1 ± 0.3	1.3 ± 0.36	0.2	0.34

(Continued)

Table 4. (Continued)

Metabolite	HMBD ID	Patients	Controls	*p*	*q*	FC	Patients°	Controls°	*p*°	*q*°
PC aa C42:0	**HMDB0008537 HMDB0008760 HMDB0008282 HMDB0008058**	**0.4 ± 0.1**	**0.55 ± 0.14**	**0.0005**	**0.002*↓**	**−1.39**	0.03 ± 0.01	0.04 ± 0.01	0.31	0.11
PC aa C42:1	**HMDB0008762 HMDB0008124 HMDB0008283 HMDB0008538 HMDB0008059**	**0.19 ± 0.05**	**0.25 ± 0.06**	**0.001**	**0.004*↓**	**−1.32**	0.02 ± 0.004	0.02 ± 0.004	0.12	0.24
PC aa C42:2	**HMDB0008157 HMDB0008795 HMDB0008125 HMDB0008763 HMDB0008092 HMDB0008316 HMDB0008794 HMDB0008602 HMDB0008284 HMDB0008348 HMDB0008539 HMDB0011330 HMDB0008570**	**0.19 ± 0.04**	**0.22 ± 0.04**	**0.008**	**0.02*↓**	**−1.16**	0.01 ± 0.002	0.02 ± 0.002	0.79	0.85
PC aa C42:4	HMDB0008191	0.15 ± 0.03	0.17 ± 0.03	0.09	0.14	−1.1	0.01 ± 0.001	0.01 ± 0.001	0.17	0.31
PC aa C42:5	HMDB08287	0.26 ± 0.05	0.29 ± 0.05	0.12	0.18	−1.1	0.02 ± 0.003	0.02 ± 0.003	0.4	0.52
PC aa C42:6	HMDB0008288	0.36 ± 0.07	0.41 ± 0.09	0.12	0.17	−1.13	0.03 ± 0.004	0.03 ± 0.003	0.96	0.99
PC ae C30:0	**HMDB0013341**	**0.34 ± 0.09**	**0.5 ± 0.13**	**0.00004**	**0.0004*↓**	**−1.44**	**0.03 ± 0.006**	**0.03 ± 0.008**	**0.005**	**0.048*↓**
PC ae C30:1	HMDB0013402	0.22 ± 0.14	0.24 ± 0.11	0.26	0.34	−1.08	0.02 ± 0.01	0.02 ± 0.01	0.55	0.65
PC ae C30:2	HMDB0013410	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC ae C32:1	**HMDB0013404 HMDB0007896 HMDB0007994**	**1.8 ± 0.32**	**2.3 ± 0.44**	**0.0001**	**0.0007*↓**	**−1.26**	0.14 ± 0.03	0.16 ± 0.03	0.17	0.31
PC ae C32:2	**HMDB0013411**	**0.46 ± 0.08**	**0.56 ± 0.1**	**0.0009**	**0.003*↓**	**−1.21**	0.04 ± 0.006	0.04 ± 0.007	0.35	0.48
PC ae C34:0	**HMDB0013405**	**0.84 ± 0.21**	**1.1 ± 0.23**	**0.0003**	**0.001*↓**	**−1.33**	0.07 ± 0.01	0.08 ± 0.01	0.04	0.11
PC ae C34:1	**HMDB0013426**	**7.2 ± 1.4**	**8.7 ± 1.5**	**0.002**	**0.004*↓**	**−1.2**	0.56 ± 0.08	0.58 ± 0.08	0.39	0.51
PC ae C34:2	**HMDB0011151**	**8.0 ± 2.2**	**10.3 ± 2.1**	**0.0008**	**0.003*↓**	**−1.28**	0.62 ± 0.15	0.7 ± 0.11	0.13	0.25
PC ae C34:3	**HMDB0013413**	**5.0 ± 1.2**	**6.7 ± 1.4**	**<0.00001**	**0.0005*↓**	**−1.36**	0.39 ± 0.09	0.46 ± 0.08	0.01	0.07
PC ae C36:0	HMDB13406 HMDB0013417	0.61 ± 0.18	0.63 ± 0.11	0.45	0.54	−1.03	0.05 ± 0.01	0.04 ± 0.006	0.08	0.19

(Continued)

Table 4. (Continued)

Metabolite	HMBD ID	Patients	Controls	*p*	*q*	FC	Patients°	Controls°	*p*°	*q*°
PC ae C36:1	**HMDB0013427 HMDB0013414**	**5.5 ± 1.2**	**7.0 ± 1.2**	**0.0005**	**0.002*↓**	**−1.27**	0.43 ± 0.06	0.47 ± 0.06	0.02	0.09
PC ae C36:2	**HMDB0013418 HMDB0013428 HMDB0011243**	**9.2 ± 1.9**	**12.0 ± 2.4**	**0.0005**	**0.002*↓**	**−1.3**	0.72 ± 0.12	0.81 ± 0.12	0.02	0.1
PC ae C36:3	**HMDB0013429 HMDB0013425**	**5.6 ± 1.2**	**6. ± 1.1**	**0.004**	**0.01*↓**	**−1.18**	0.44 ± 0.09	0.45 ± 0.06	0.68	0.75
PC ae C36:4	HMDB0013435 HMDB0013407	12.5 ± 3.0	14.0 ± 2.3	0.05	0.09	−1.12	0.99 ± 0.35	0.94 ± 0.13	0.56	0.65
PC ae C36:5	**HMDB0013415 HMDB0011220**	**7.7 ± 1.5**	**9.8 ± 1.9**	**0.0002**	**0.001*↓**	**−1.28**	0.61 ± 0.18	0.66 ± 0.1	0.25	0.38
PC ae C38:0	**HMDB0013408 HMDB0013419**	**1.1 ± 0.37**	**1.6 ± 0.35**	**0.00006**	**0.0005*↓**	**−1.45**	**0.09 ± 0.02**	**0.11 ± 0.02**	**0.001**	**0.01*↓**
PC ae C38:1	HMDB0013419 HMDB0013408	0.6 ± 0.16	0.68 ± 0.16	0.09	0.14	−1.14	0.05 ± 0.008	0.05 ± 0.009	0.99	0.99
PC ae C38:2	**HMDB0013416 HMDB0013430**	**1.5 ± 0.29**	**1.8 ± 0.33**	**0.001**	**0.004*↓**	**−1.25**	0.11 ± 0.01	0.12 ± 0.02	0.05	0.13
PC ae C38:3	**HMDB0013436 HMDB0013431**	**2.8 ± 0.58**	**3.5 ± 0.57**	**0.0002**	**0.001*↓**	**−1.26**	0.21 ± 0.03	0.23 ± 0.03	0.04	0.11
PC ae C38:4	**HMDB0013420**	**9.1 ± 1.4**	**11.1 ± 1.5**	**0.00005**	**0.0005*↓**	**−1.21**	0.72 ± 0.13	0.75 ± 0.1	0.38	0.51
PC ae C38:5	HMDB11253 HMDB0013432	13.7 ± 3.0	14.5 ± 2.4	0.24	0.33	−1.06	1.1 ± 0.32	0.98 ± 0.15	0.24	0.37
PC ae C38:6	**HMDB0013409**	**4.5 ± 1.2**	**5.7 ± 1.0**	**0.001**	**0.004*↓**	**−1.27**	0.35 ± 0.11	0.38 ± 0.06	0.25	0.38
PC ae C40:1	**HMDB0013433**	**0.85 ± 0.2**	**1.2 ± 0.2**	**<0.00001**	**0.0001*↓**	**−1.38**	**0.07 ± 0.01**	**0.08 ± 0.01**	**<0.00001**	**0.003*↓**
PC ae C40:2	**HMDB0013437**	**1.1 ± 0.25**	**1.5 ± 0.34**	**0.00007**	**0.0005*↓**	**−1.36**	0.09 ± 0.02	0.1 ± 0.02	0.03	0.1
PC ae C40:3	**HMDB0013445 HMDB0013446**	**0.7 ± 0.12**	**0.97 ± 0.13**	**<0.00001**	**0.00004*↓**	**−1.31**	**0.05 ± 0.007**	**0.07 ± 0.008**	**0.003**	**0.03*↓**
PC ae C40:4	**HMDB0013442**	**1.6 ± 0.27**	**2.1 ± 0.4**	**0.00001**	**0.0002*↓**	**−1.31**	0.13 ± 0.02	0.14 ± 0.03	0.03	0.1
PC ae C40:5	**HMDB0013444**	**2.5 ± 0.41**	**3.1 ± 0.5**	**0.0001**	**0.0008*↓**	**−1.25**	0.19 ± 0.03	0.2 ± 0.03	0.17	0.3
PC ae C40:6	**HMDB0013422**	**2.7 ± 0.66**	**3.7 ± 0.8**	**0.0001**	**0.0007*↓**	**−1.37**	0.2 ± 0.04	0.24 ± 0.05	0.01	0.07

(Continued)

Table 4. (Continued)

Metabolite	HMBD ID	Patients	Controls	*p*	*q*	FC	Patients°	Controls°	*p*°	*q*°
PC ae C42:0	HMDB0013423	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC ae C42:1	HMDB0013434 HMDB0013447	0.3 ± 0.06	0.3 ± 0.06	0.4	0.48	−1.05	0.02 ± 0.004	0.02 ± 0.003	0.11	0.23
PC ae C42:2	**HMDB0013438**	**0.4 ± 0.09**	**0.5 ± 0.06**	**0.00005**	**0.0004*↓**	**−1.27**	0.03 ± 0.004	0.034 ± 0.04	0.02	0.08
PC ae C42:3	**HMDB0013458 HMDB0013459**	**0.5 ± 0.12**	**0.7 ± 0.12**	**<0.00001**	**0.0001*↓**	**−1.39**	**0.04 ± 0.008**	**0.05 ± 0.006**	**0.001**	**0.013*↓**
PC ae C42:4	**HMDB0013448 HMDB0013454**	**0.64 ± 0.15**	**0.9 ± 0.23**	**0.00004**	**0.0004*↓**	**−1.43**	0.05 ± 0.01	0.06 ± 0.02	0.01	0.07
PC ae C42:5	**HMDB0013451**	**2.0 ± 0.49**	**2.5 ± 0.44**	**0.001**	**0.003*↓**	**−1.28**	0.17 ± 0.03	0.17 ± 0.03	0.65	0.74
PC ae C44:3	**HMDB0013449 HMDB0013452**	**0.11 ± 0.03**	**0.13 ± 0.02**	**0.008**	**0.02*↓**	**−1.19**	0.008 ± 0.002	0.008 ± 0.001	0.51	0.62
PC ae C44:4	**HMDB0013455 HMDB0013453 HMDB0013460**	**0.3 ± 0.07**	**0.4 ± 0.09**	**0.00009**	**0.0006*↓**	**−1.36**	0.03 ± 0.006	0.03 ± 0.007	0.04	0.11
PC ae C44:5	**HMDB0013456**	**1.8 ± 0.48**	**2.2 ± 0.58**	**0.008**	**0.02*↓**	**−1.25**	0.14 ± 0.04	0.15 ± 0.04	0.33	0.46
PC ae C44:6	**HMDB0013450 HMDB0013457**	**1.0 ± 0.25**	**1.2 ± 0.3**	**0.006**	**0.014*↓**	**−1.26**	0.08 ± 0.02	0.08 ± 0.02	0.31	0.45
SM (OH) C14:1	**HMDB0013462**	**3.9 ± 0.8**	**5.2 ± 0.9**	**0.00006**	**0.0005*↓**	**−1.31**	2.2 ± 0.4	2.4 ± 0.3	0.06	0.16
SM (OH) C16:1	**HMDB0013463**	**2.2 ± 0.44**	**2.8 ± 0.47**	**0.0002**	**0.001*↓**	**−1.27**	1.2 ± 0.16	1.3 ± 0.14	0.11	0.23
SM (OH) C22:1	**HMDB0013466**	**7.6 ± 1.7**	**10.5 ± 1.7**	**<0.00001**	**0.00004*↓**	**−1.38**	**4.2 ± 0.53**	**4.9 ± 0.54**	**0.0002**	**0.005*↓**
SM (OH) C22:2	**HMDB0013467**	**7.8 ± 1.6**	**10.2 ± 1.5**	**0.00001**	**0.0002*↓**	**−1.31**	4.3 ± 0.57	4.8 ± 0.4	0.008	0.06
SM (OH) C24:1	**HMDB0013469**	**0.84 ± 0.2**	**1.1 ± 0.31**	**0.0003**	**0.001*↓**	**−1.36**	0.46 ± 0.05	0.5 ± 0.12	0.04	0.11
SM C16:0	**HMDB0061712 HMDB0010169**	**75.6 ± 9.6**	**87.24 ± 10.1**	**0.0004**	**0.002*↓**	**−1.15**	42.0 ± 1.9	40.6 ± 1.4	0.009	0.06
SM C16:1		10.9 ± 2.0	12.0 ± 1.9	0.06	0.11	−1.1	6.0 ± 0.6	5.6 ± 0.54	0.02	0.07
SM C18:0	**HMDB01348**	**13.3 ± 2.6**	**16.0 ± 2.4**	**0.001**	**0.004*↓**	**−1.2**	7.3 ± 0.7	7.4 ± 0.79	0.68	0.75

(Continued)

Table 4. (Continued)

Metabolite	HMBD ID	Patients	Controls	*p*	*q*	FC	Patients°	Controls°	*p*°	*q*°
SM C18:1	HMDB0012100 HMDB0012101	7.2 ± 1.7	8.1 ± 1.5	0.07	0.11	−1.13	3.9 ± 0.52	3.8 ± 0.52	0.27	0.4
SM C20:2		0.25 ± 0.07	0.3 ± 0.08	0.03	0.05	−1.22	0.14 ± 0.03	0.14 ± 0.03	0.68	0.75
SM C22:3		NA	NA	NA	NA	NA	NA	NA	NA	NA
SM C24:0	**HMDB11697**	**13.8 ± 2.6**	**18.0 ± 3.3**	**0.00001**	**0.0002*↓**	**−1.3**	7.6 ± 0.76	8.4 ± 1.2	0.03	0.1
SM C24:1	**HMDB12107**	**36.7 ± 6.5**	**43.2 ± 5.5**	**0.001**	**0.003*↓**	**−1.18**	20.3 ± 1.9	20.1 ± 1.5	0.69	0.75
SM C26:0	**HMDB0011698**	**0.12 ± 0.03**	**0.17 ± 0.04**	**0.00007**	**0.0005*↓**	**−1.35**	0.07 ± 0.01	0.08 ± 0.01	0.02	0.07
SM C26:1	**HMDB0013461**	**0.26 ± 0.08**	**0.35 ± 0.07**	**0.0007**	**0.002*↓**	**−1.33**	0.14 ± 0.03	0.16 ± 0.03	0.05	0.13
Total AC		**52.9 ± 12.1**	**43.8 ± 10.7**	**0.016**	**0.02*↓**					
Total PC		**1299 ± 234**	**1490 ± 189**	**0.007**	**0.01*↓**					
Total SM		**180 ± 26.3**	**215 ± 24.1**	**0.00003**	**0.0002*↓**					
PC/SM		7.3 ± 1.3	7.0 ± 0.2	0.42						

Metabolite concentrations for all metabolites grouped by Fontan patients *versus* controls before (column 3 to 7) and after normalisation of data (column 8–11, tagged with °). Values are given as mean ± standard deviation, unit of values of data before normalisation (µmol/l), unit of values of data after normalisation (lipid classwise percentage). AC, acylcarnitine FC, fold change (Fontan patients *versus* controls); HMBD ID, Human Metabolome Database identification; lysoPC, lysophosphatidylcholine; NA, not available; PC, phosphatidylcholine; SM, sphingomyelin; SM(OH), hydroxysphingomyelin; *↓, statistically significant lower serum concentration in Fontan patients than in controls; *↑, statistically significant lower serum concentration in Fontan patients than in controls.

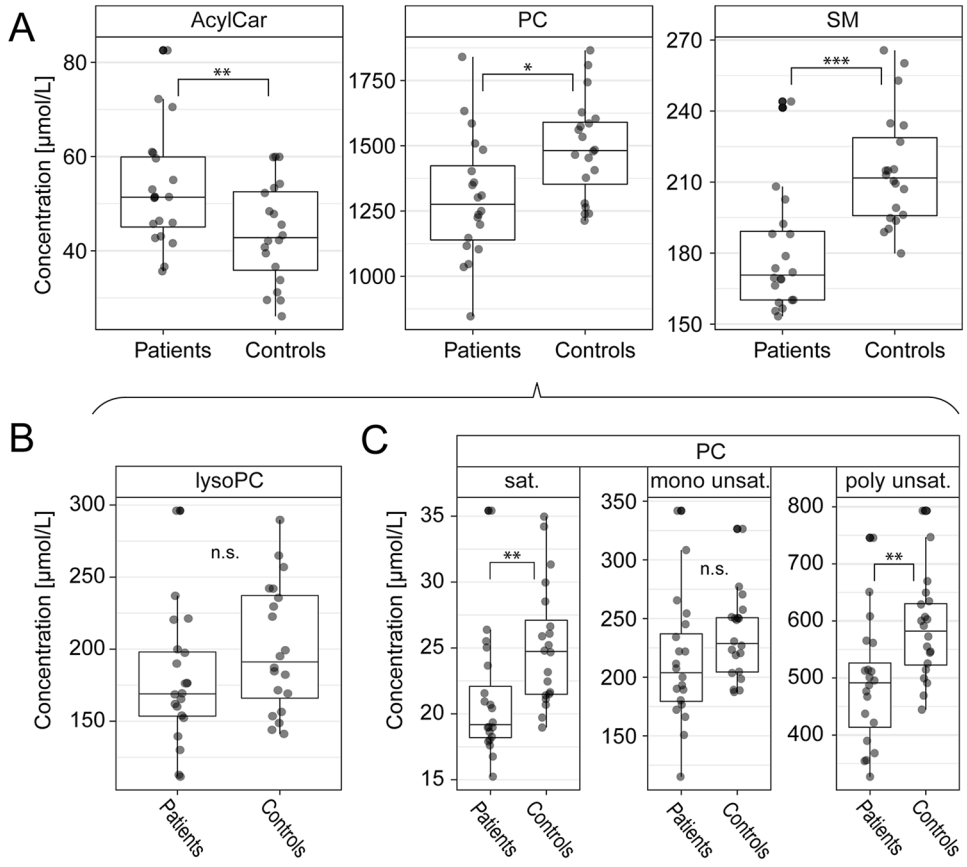

Figure 3. Serum concentrations of phospholipid subgroups.
Box-and-whisker-plots of serum concentrations of phospholipid subgroups that were significantly lower in Fontan patients (grey boxes) than in matched healthy biventricular controls (white boxes). The boxes show the 25th and the 75th percentile, the whiskers the minimum and the maximum. PC, phosphatidylcholine; SM, sphingomyelin.

bodies. When the substrate shifts as HF progresses is still unknown.[36]

PL, especially SM, with their location mainly in the (cell) membrane, serve as signalling molecules in cell metabolism. Sphingolipid content of myocardial tissues is decreased in cardiomyopathic hamster models at the onset of HF.[37,38] Even subtle stages of HF may be indicated, as emphasised by the fact that Fontan patients in our study 'clinically' had sustained haemodynamics with a mean $\dot{V}O_2AT$ of 25 ml/kg/min and $\dot{V}O_2max$ of 29 ml/kg/min, which correlates well with normal values for right-ventricle Fontan patients.[39]

Findings in atrial-fibrillation patients with a biventricular system, in whom blood PL levels rose between closure of left atrial appendage (LAA) and 6 months thereafter, support the assumption that decreased PL levels in our patients indicate subtle stages of HF with slightly raised end-diastolic pressures, hinting at altered diastolic and sustained systolic ventricular function.[40] In these atrial-fibrillation patients and in our patients, values for PL with fatty acids of higher carbon numbers were altered. However, whilst in the atrial-fibrillation patients analyte concentrations were higher than in controls, we found lower analyte concentrations in patients than in controls. This calls attention to the fact that LAA closure leads to a loss of LAA metabolic and endocrinologic functions. In our patients, who had all undergone extracardiac tunnel surgery, these LAA functions should be relatively intact. Typical of the aging Fontan circulation (even of the so-called 'intact' Fontan circulation) is the development of raised end-diastolic ventricular pressures with sustained systolic function, a feature still difficult to assess by conventional echocardiographic measures of diastolic function.[41] Many of our patients (all adult) thus may have experienced slightly raised atrial pressures,

which might explain the studies opposed findings with respect to how analyte values shifted. Haemodynamic measurements of end-diastolic ventricular and atrial pressures might buttress this inference. Moreover, even if our patients did not exhibit differences from controls in NT-proBNP levels, and even if this established marker for HF could not be correlated with the lipidome alterations that we found, measurements in our patients of cardiovascular biomarkers that are established in the normal population and emerging in adults with CHD, such as high-sensitivity troponin, galectin-3, or growth differentiation factor 15, would be interesting.[6,7,42]

Chronic low-level inflammation

Involvement of PL in arachidonic acid metabolism and thus in the production of pro-inflammatory metabolites like leukotrienes agrees with our second hypothesis that our results indicate inflammation in our patients. In addition, sphingomyelinase activation of the SM-ceramide pathway is thought to induce inflammation, adding to oxidative stress, and to induce cell death, resulting in atherosclerosis, aging, and cardiovascular events.[29,32] An activated pathway can be characterised by reduced levels of SM, as seen in our patients.[43] Even if acute inflammation is not to be expected in our patients 18 years after Fontan repair and independent of surgery in general, as supported by the normal CRP levels in patients and controls and by the lack of correlation with lipidomic variables, chronic low-level inflammation might still be present. High-sensitivity C-reactive protein (hsCRP) is a potential biomarker in adult patients with various forms of CHF[44]; assays measuring hsCRP can differentiate concentrations within the normal range and can thus in the normal population indicate chronic low-level inflammation, which is known to reflect atherosclerotic cardiovascular disease. Higher hsCRP levels, even within the normal range, are associated with an increased risk for cardiovascular events. Adults with various forms of CHD (including single-ventricle Fontan circulation) with elevated hsCRP not only have a worse functional status and exercise capacity as measured by peak $\dot{V}O_2$, but also have a greater risk for death or non-elective cardiovascular hospitalisation.[44] The CHD cohort showed no association between hsCRP and history of coronary artery disease or cerebrovascular events, but increased hsCRP levels still indicate chronic low-level inflammation due to non-atherosclerotic causes and higher values might predict adverse outcomes.[44] These findings support our hypothesis that the altered levels of PL observed could be due to inflammation, even if in our study patients (and controls) 'routine' CRP levels could not be correlated with lipidomic variables and even if CRP levels did not differ between Fontan and control cohorts. One reason for a chronic inflammatory status in Fontan patients might be the combination of (intestinal) inflammation and altered mesenteric haemodynamics, both affecting or even provoking PLE.[45,46] In combination, subtle impairment of myocardial (diastolic) function, inflammation in biventricular patients with chronic congestive HF and a chronic low cardiac output, and altered PL levels (with induction of endothelial-cell oxidative stress) in patients with CAD underscore the possibility that chronic low-level inflammation exists in our Fontan patients even without overt HF and without overt PLE.[47,48] Of note is that in addition to decreases in HDL-C values and different structural and functional HDL properties in patients with an acute-phase response 3 days after cardiopulmonary bypass surgery in comparison with healthy controls,[49] patients with rheumatoid arthritis and thus with chronic inflammation also have low values for total cholesterol, low density lipoprotein cholesterol, and HDL-C, similar to the significant decrease in HDL-C values that we found in Fontan patients and similar to low cholesterol metabolism profiles in Fontan patients.[15–17,50] Such alterations in cholesterol profile exist even 5 years before rheumatoid arthritis manifests clinically, and a similar cholesterol profile is associated with an increased risk of cardiovascular disease.[50] We propose that inflammation affects cholesterol and PL metabolism at different stages of liver and/or vessel disease. If so, the combination of cyanosis with a chronic inflammatory state might exacerbate changes like those that we found: adults with cyanotic CHD have hypocholesterolaemia, although their extended lipid status has not been further examined.[51]

Alteration of structural or functional lymphatic-vessel or blood-vessel properties

As with subtle alteration of myocardial function and a chronic inflammatory state, decreased serum concentrations of PL may also indicate alteration of structural or functional vascular properties in our Fontan patients.

This might involve lymphatic vessels. Various disorders such as HF and inflammatory diseases disrupt the lymphatic vascular system.[12] Assuming

that the Fontan circulation imposes a state of (subtle) chronic HF associated with chronic low-level systemic inflammation, increased vascular permeability, and increased production of interstitial fluid, the chronic challenge to the lymphatic system is evident, with poor drainage especially into the Fontan-specific high-pressure systemic venous system.[52] Besides lymphangiectasia, lymphatic collaterals form and tortuosity and dilation of the thoracic duct occur in Fontan patients; these interestingly have also been reported in children before Fontan surgery, but might be exacerbated by the Fontan circulation.[52] Lymphatic dysfunction can contribute to deterioration of organ function in Fontan patients, with lymphoedema, plastic bronchitis, PLE, and liver dysfunction. The two latter disorders can be seen as major reasons for the alterations in PL, acylcarnitine, and HDL-C levels that we found; even if our patients exhibited no overt signs of PLE and even if lipidomic variables did not correlate with serum total protein or albumin levels, enteral lipid uptake might be altered, in combination with altered hepatic metabolism of lipids and of lipoproteins, which are important carriers of PL.[15] Whether this mirrors the 'normal' Fontan situation or is already a subtle indication that the Fontan circulatory system is impaired remains to be seen.

This could refer to the alteration of structural or functional properties of blood vessels. Two facts reinforce this assumption. First, SM is important in the development and progression of the atherosclerotic plaques of CAD.[32–34] Second, stiffening of vessels is seen in patients with non-alcoholic fatty liver disease or with hypoplastic left heart syndrome, in the latter group an observation discussed as evidence of ventriculo-vascular or rheo-vascular coupling.[53–55] The Fontan patient with a right systemic ventricle who has undergone extensive aortic surgery is especially likely to exhibit aortic stiffness <5 years after Fontan completion.[54] Young Fontan patients with a dominant LV, like our patient group, show arterial stiffening, and this increased-stiffening process can be seen especially in the ascending aorta.[55] Non-invasive work in a heterogeneous group of young adult Fontan patients (80% showing dominant LV morphology) supports these findings, with elevated global arterial stiffness detected on peripheral-artery sphygmomanometry.[56] Positive correlations also existed between increased arterial stiffness and inflammation, which might be explained by disturbed vitamin D/parathormone interactions frequent in this patient group.[56,57] Vascular stiffening also occurs in cerebral arteries in young Fontan patients, suggesting altered (arterial) vascular properties in general or at least in vessels neighbouring the heart.[58] Local assessment of arterial stiffness would be desirable.[59,60] Might these alterations, which we interpret as altered structural or functional blood vascular properties and regard as adaptation to Fontan physiology, be simultaneously adverse with regard to elevated cardiovascular risk as known in the biventricular patient and favourable in the setting of Fontan physiology? Might altered structural and functional properties of blood vessels, especially vascular stiffening, maintain arterial pulsatility sufficient to support end-organ perfusion and supply a driving force for pulmonary circulation? In histopathologic investigations of Fontan patients with a mean age of 30 years, mural remodelling has been demonstrated in the pulmonary artery and in the inferior and superior vena cava.[61] Moreover, in the course of their signalling function, PL regulate angiogenesis, possibly linking their concentrations in serum with altered collateral-vessel formation.[37] A decrease in PL concentrations as measured might mean that PL are present in another form, an activated or oxidised one, which in our assay was not determined.[37] Further to assess this hypothesis it would be useful in Fontan patients to determine the forms and concentrations of PL in vascular walls and to assess the degree of collateral flow.

Summary

The metabolism of PL and acylcarnitines is distorted in adult Fontan patients with a dominant LV. Three mechanisms may act alone or in combination in altering PL and/or acylcarnitine metabolism: a) altered cell signalling and metabolism as indicators of subtle HF; b) chronic low-level inflammation; d) alteration of functional or structural properties of lymphatic or blood vessels. Our results do not identify single metabolites that may permit differentiation between disease groups and controls, but differences in the content of an entire substance class such as – in our study – serum PL and acylcarnitine content may suggest extended metabolic alterations in LV Fontan patients. These findings may, extended and confirmed, permit better understanding of Fontan physiology, which should be regarded as a complex state involving diverse pathways and (patho)physiological processes.

Limitations

In patients with a Fontan circulation, the underlying (embryologic) cardiovascular malformation, extent of surgical reconstruction of the neoaorta, or loading conditions (also in the case of relevant collateral flow) can differ significantly. To limit variability, we focused on Fontan patients with a dominant LV who underwent Norwood II and III surgery. Our patient group thus is small. This may limit the extent to which our findings can be generalised. So may our choice of a targeted metabolomic approach, as metabolites not included in the commercial kit that we used might be important. That we analysed serum samples precludes direct comparisons between our results and those of studies that used tissue samples. That PL and acylcarnitines are reliably detectable in healthy human serum and plasma lends our data credence.[27] Still, having used not vascular (or myocardial) tissue but serum, we cannot rule out certain system-driven aspects of pathogenesis – unknown confounders that affect metabolic profiles might be the true basis for the observed differences. We strove to lessen the likelihood of such errors by following a strict inclusion and exclusion protocol, especially with regard to (known) comorbidities or medication. Although only fasting serum was tested, differences in body composition or lifestyle might still have influenced our results to an unknown degree. The opportunity to correlate our findings with those on invasive assessment would have been advantageous, especially for the general possible explanation of our findings that they might reflect (slightly) raised end-diastolic ventricular pressure, which our conventional echocardiographic measurements were not able to reveal. Furthermore, to distinguish Fontan patients with preserved haemodynamic status (as suggested by clinical findings, echocardiography results, and routine laboratory biomarker values) from those with impaired haemodynamic status, we might not have focused only on the configuration of the systemic ventricle, oxygen saturation, exercise capacity, and valve regurgitation, but also ideally on haemodynamic parameters like fenestration due to elevated pulmonary vascular resistance and high central venous pressure, as well as on the presence of substantial collateral flow, dilatation of the tunnel, diaphragmatic paresis, or cardiac index. Without data from angiography, our hypothesis that our findings indicate angiogenesis/collateral flow must be considered carefully. Further influencing our findings might be the intake of phenprocoumon, which 80% of our patients received. As an oral anticoagulant inhibitor of vitamin K oxidoreductase, it interacts with hepatic metabolism and can even induce hepatitis. An effect on our results cannot be excluded, a possibility that should be examined in a larger cohort of patients with more patients not taking phenprocoumon.

Finally, to evaluate our hypothesis that chronic low-level inflammation contributes to the alterations in the lipidome that we found, measuring hsCRP levels would be elegant. To demonstrate higher hsCRP levels between in Fontan patients than in controls would support that hypothesis; such a study might even expose correlations with the lipidome that 'routine' CRP levels could not unmask.[44]

Outlook

To use non-invasive haemodynamic MRI studies, assessing end-diastolic ventricular pressures and atrial pressures, flow patterns, and calculating collateral flow, in evaluating our hypotheses regarding raised end-diastolic ventricular pressures, inflammation, and functional or structural alteration of vascular systems is an interesting prospect. To compare and to contrast lipidome-constituent levels in subgroups of Fontan patients stratified by underlying malformation, morphologic features of the systemic ventricle, type of aortic surgery, comorbidities, (semi-)invasive haemodynamic parameters, and particularly by means of evaluation of hepatic function is an immediate aim, as is, in the longer term, follow-up to investigate how rapidly metabolomic changes develop in patients with Fontan circulation.[20] Further, to correlate our results with proteomic studies, especially those that dissect inflammation, will be very satisfying, as will be both histopathologic studies to assess vessel alterations and genomic investigations.

Conclusion

Requiring only minute specimens for simultaneous quantitation of participants in multiple metabolic pathways, lipidomic profiling was useful for assessment of metabolic derangement in LV Fontan patients, in whom we found striking PL and acylcarnitine level alterations that may reflect altered cell signalling and metabolism as found in HF in biventricular patients; inflammation; or

alteration of structural or functional properties of the lymphatic and blood vessel system.

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Author contributions

MM designed the study, examined patients, prepared samples, analysed and interpreted results and wrote the manuscript; KOD supervised lung function and exercise testing; KOD and KTL helped classify the patients; MGA and UM performed biochemical assays and helped with statistical analysis of metabolomic-examination results; SSB, KTL, KOD, MAK, and JK assisted in analysis and interpretation of results; AE, UM and JK provided the figures; all authors proofread the manuscript and read and approved the final version of the manuscript.

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Conflict of interest statement

MM, KOD, MZ, DK, MGA, AE, MK, JK, IOK, RG, CS, CN, UM, SS-B, and KTL declare that they have no financial or non-financial competing interests with respect to the research, authorship, and/or publication of this article.

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Supplemental material

Supplemental material for this article is available online.

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