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Prediction of VLCAD deficiency phenotype by a metabolic fingerprint in newborn screening bloodspots

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ABSTRACT

Purpose: Newborns who test positive for very long-chain acyl-CoA dehydrogenase deficiency (VLCADD) in newborn screening may have a severe phenotype with early onset of life-threatening symptoms but may also have an attenuated phenotype and never become symptomatic. The objective of this study is to investigate whether metabolomic profiles in dried bloodspots (DBS) of newborns allow early phenotypic prediction, permitting tailored treatment and follow-up.

Methods: A metabolic fingerprint was generated by direct infusion high resolution mass spectrometry in DBS of VLCADD patients (n = 15) and matched controls. Multivariate analysis of the metabolomic profiles was applied to differentiate subgroups.

Results: Concentration of six acylcarnitine species differed significantly between patients and controls. The concentration of C18:2- and C20:0-carnitine, 13,14-dihydroretinol and deoxycytidine monophosphate allowed separation between mild and severe patients. Two patients who could not be prognosticated on early clinical symptoms, were correctly fitted for severity in the score plot based on the untargeted metabolomics.

Conclusion: Distinctive metabolomic profiles in DBS of newborns with VLCADD may allow phenotypic prognostication. The full potential of this approach as well as the underlying biochemical mechanisms need further investigation.

1. Introduction

Very long-chain acyl-CoA dehydrogenase deficiency (VLCADD) (OMIM 201475) is an autosomal recessive inborn error of long-chain fatty acid oxidation (lcFAO) caused by pathogenic mutations in the *ACADVL* gene (OMIM 609575) [1]. Prominent biochemical features of VLCADD are a disturbed energy homeostasis [2] and accumulation of long-chain acylcarnitines in plasma. Clinical symptoms include hypoglycemia, rhabdomyolysis and cardiomyopathy, potentially leading to early demise. The inclusion of VLCADD in many newborn screening (NBS) programs worldwide [3] has led to identification of patients for whom the clinical significance of the defect is yet unknown [4]. Despite decades of research there are no markers yet for early prognostication. The currently used biomarker used for NBS for VLCADD, C14:1-carnitine and the ratio of C14:1 to C2-carnitine, are not well correlated with disease severity [5], especially in patients with a residual enzyme activity of > 10% [6]. While patients homozygous for two null mutations will likely develop a severe phenotype, prediction of phenotype based on genotype is complicated by a high prevalence of compound heterozygosity for missense mutations and newly identified mutations with unknown pathogenicity [7,8]. It is possible to determine phenotypic severity by measuring lcFAO flux in cultured skin fibroblasts [9]. Although this measurement correlates well with disease severity, the method is invasive (skin biopsies), is costly and has a long turn-around time. While individuals with a benign phenotype do not require strict treatment, early identification of neonates at risk of developing early

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and life-threatening symptoms is critical in order to allow immediate treatment and follow-up in time [10].

The clinical heterogeneity of VLCADD suggests that different pathogenic mechanisms play a role. Small signaling molecules hold potential as powerful biomarkers as they may identify subtle changes in the metabolome. Metabolomics, the study of small molecule metabolites, focuses on the measurement of the relative concentrations of endogenous small molecules (< 600 Da) in different biological matrices (e.g. plasma, dried blot spots (DBS), urine or cerebrospinal fluid). This technology has become useful for clinical practice [11] including screening for inborn errors of metabolism [12–15] and offers a promising alternative to the traditionally used electrospray tandem mass spectrometry (ESI-MS/MS) approach at NBS [16].

The objective of the present study is to investigate whether metabolomic profiles in NBS cards can provide additional information, potentially allowing early prognostication and thus tailored therapeutic interventions. Therefore, metabolomic profiles in DBS from VLCADD patients with severe and mild clinical phenotypes were compared.

2. Materials and methods

2.1. Sample cohort for study

The retrospective cohort was drawn from the NBS program in the Netherlands. Screening for VLCADD is performed within 72–168 h after birth by measuring C14:1-carnitine and C2-carnitine concentrations using 3.2 mm punches from NBS cards, in a quantitative ESI-MS/MS-method using the Neobase 1 assay (PerkinElmer, Turku, Finland - extraction and sample preparation performed according to kit insert) and a Waters micro tandem mass spectrometer (Waters, Milford, USA). All legal guardians of patients with VLCADD approved the use of the remnant samples for this project, in agreement with institutional and national legislation. The biochemical and clinical characteristics of the patients are shown in Tables 1 and 2 respectively. VLCAD enzyme activity and measurement of the lcFAO flux measurements were performed for all patients as standard of care. Unless indicated otherwise, enzyme activity was measured in lymphocytes using palmitoyl-CoA as a substrate as described previously [17]. The lcFAO flux in fibroblasts was measured as the production of ^{3}H-labeled water from [9,10-^{3}H(N)]-oleic acid as described previously [18] and defined as percentage of the mean flux of two controls measured in the same experiment [10]. For one patient (Table 1, PS2), the NBS card was no longer available (cards are legally discarded after five years of storage). For this patient, we included a DBS that was taken on day six of life when he was admitted to our hospital because of hypoglycemia. PS4 was referred because of symptoms on day two after birth before NBS was performed. From this patient an additional DBS was taken on day seven of life, marked in our cohort as PS4b (Figs. 1–4). Controls (three control samples per case) were from children with negative newborn screening results and matched to the cases for storage time and conditions. The use of these anonymized NBS cards (parental approval for anonymized use for research given) was approved by the Working group Scientific Research Newborn Screening of the Dutch screening organization and provided by the National Institute for Public Health and Environment (RIVM). All analytical and experimental procedures were in accordance with the ethical standards of the University Medical Centre Utrecht and with the Helsinki Declaration of 1975, as revised in 2000.

2.2. Classification based on lcFAO flux

Whole cell residual lcFAO flux measured in cultured fibroblast was used to categorize patients in subgroups. Previous studies show a clear correlation with a flux ≤10% and development of severe symptoms [9,10], hence we defined these patients as 'severe' (PS). Patients identified by NBS with a higher lcFAO flux (> 20%) are often asymptomatic at presentation [19], we defined these patients as 'mild' (PM). For two patients, PI5 and PI6, lcFAO was flux 13.5% and 19% respectively (Table 1), for which the clinical implication is yet less clear. We defined these two patients as 'intermediate' (PI).

2.3. Non-targeted metabolomics analysis in DBS

Samples were prepared and analyzed using direct infusion high resolution mass spectrometry (DI-HRMS) as described previously [13]. In short, metabolites were extracted from the DBS with methanol. Batch variability was monitored by addition of 20 stable isotope-labeled internal standards [13]. After dilution with 0.3% formic acid and filtration, filtrated extracts were measured in positive and negative mode (mass range 70–600 *m/z*, resolution of 140.000) using chip-based nanoESI-HRMS [13,20]. To check the instrument performance a set of quality control DBS samples (sample of a patient with propionic aciduria (for propionylcarnitine, glycineand propionylglycine), sample of a patient with phenylketonuria (for phenylalanine) and a sample of a patient with lysinuric protein intolerance (for citrulline, glutamine, and lysine) were included. Metabolomic profiles of VLCADD patients and their corresponding control subjects were performed in one analytical run.

2.4. Data processing

Signals in the mass spectra were processed to corresponding mass peaks as described previously [13]. Briefly, detected mass peaks were annotated to the corresponding metabolites using the Human Metabolome Database version 4.0 (HMDB) [21]. Metabolite annotations without adduct ions or with a single adduct ion (Na^+, K^+ or Cl^-) were summed, resulting in one mass peak per annotation and 16,668 features in total. Exogenous and drug metabolite annotations were excluded resulting in ~2200 mass peaks (~3900 biologically relevant metabolite annotations including isomers) that were used for further analysis.

2.5. Data analysis

Metabolites were filtered on variance by interquantile range leaving ~1300 metabolites for statistical analysis. Data was log transformed and autoscaled. All statistical analyses were performed using R (https://www.r-project.org) version 3.5.3 and MetaboAnalyst 4.0 (https://www.metaboanalyst.ca). Group differences were tested with *t*-tests and Mann Whitney *U* test (W) in case of failed normality. Acquired p-values were corrected for multiple testing (false discovery rate (FDR), a q-value < 0.05 was considered significant [22]). Principal component analysis (PCA) showed the distribution of the original data for all samples. To separate the severe and mild VLCADD and understand variables responsible for the discrimination, Partial Least Squares Discriminant Analysis (PLS-DA) was used. Variable importance in projection (VIP) scores was extracted to visualize the most important metabolites contributing to the differentiation. Fold changes (FC) were calculated as the ratio of the average responses of (sub)groups. Metabolites with FC > 2, VIP scores > 1 and q-values < 0.05 were considered most responsible for the differentiation between groups. Pathway analyses of the metabolites were performed to identify the top altered pathways [23]. KEGG metabolic pathways (Kyoto Encyclopedia of Genes and Genomes) were used as reference pathways. Patients with an intermediate flux (PI5 and PI6) were not included in the PLS-DA model of mild and severe patients; rather, their metabolomic profile was predicted into the model.

3. Results

3.1. Patient characteristics

DBS samples from a retrospective cohort of 18 VLCADD patients and 54 age- and sex-matched controls were analyzed. PCA of patients and

Table 1
Biochemical characteristics of VLCADD patients.

PID	Acylcarnitines in NBS			Genetic variants		VLCAD activity	lcFAO flux
	C14:1(μmol/L) normal < 0.6	C2 (μmol/L)	C14:1/C2 normal < 0.023	Allele 1	Allele 2	Lymphocytes (nmol/min/mg) normal: 2.15–3.79	Skin fibroblasts (% of control)
PS1	4.46	14	0.319	c.1183-15A > G (splicing defect)	c.1183-15A > G (splicing defect)	< 0.18[a]	4
PS2	4.91	10	0.505	c.1322G > A (p.Gly441Asp)	c.1322G > A (p.Gly441Asp)	< 0.18	5
PS3	4.93	11	0.448	c.104delC (p.Pro35Leufs*26)	c.104delC (p.Pro35Leufs*26)	< 0.18	6
PS4	1.64	6	0.273	c.643T > C (p.Cys215Arg)	c.643T > C (p.Cys215Arg)	< 0.18	10
PI5	2.47	11	0.225	c.520G > A (p.Val174Met)	c.622+1G > T (splicing defect)	0.25	13.5
PI6	1.29	8	0.161	c.848T > C (p.Val283Ala)	c.1322G > A (p.Gly441Asp)	< 0.18	19
PM7	1.68	6	0.260	c.104delC (p.Pro35Leufs*26)	c.848T > C (p.Val283Ala)	0.18	30.5
PM8	1.46	11	0.133	c.848T > C (p.Val283Ala)	c.1366C > G (p.Arg456Gly)	0.28	49
PM9	0.17	4	0.043	c.848T > C (p.Val283Ala)	c.848T > C (p.Val283Ala)	0.53	55.5
PM10	0.57	23	0.025	c.277G > A (p.Val93Met)	c.848T > C (p.Val283Ala)	0.39	74.5
PM11	0.21	8	0.026	c.848T > C (p.Val283Ala)	c.1700G > A (p.Arg567Gln)	0.52	76.5
PM12	0.25	8	0.031	c.848T > C (p.Val283Ala)	c.848T > C (p.Val283Ala)	0.45	78
PM13	0.44	10	0.044	c.848T > C (p.Val283Ala)	c.1081G > A (p.Asp361Asn)	1.18	88.5
PM14	0.28	12	0.023	c.829_831delGAG (p.Glu277del)	c.848T > C (p.Val283Ala)	0.95	100
PM15	0.37	14	0.026	c.541dup (p.His181Profs*72)	c.1040C > T (p.Ala347Val)	0.82	100

lcFAO flux: long-chain fatty acid β-oxidation flux. PID: patient identity, PI: patient with intermediate phenotype, PM: patient with mild phenotype, PS: patient with severe phenotype.
[a] 0.18 (nmol/min/mg) is limit of quantitation of VLCAD enzyme assay

their corresponding controls showed clear outliers for nine controls (three times set of three) (data not shown). These outliers were the oldest cards (stored for a period of six to seven years). Therefore, these DBS samples (controls and their corresponding patients) were removed from further analysis. The remaining 15 patients (eight females and seven males) and their corresponding control subjects (n = 45) were included for further analysis. All patients classified as severe developed clinical symptoms in the first year of life, first presentation ranged from one day to seven months. Two patients in the severe group deceased during follow-up. Cardiomyopathy was only seen in the severe group. In the group classified as mild eight out of nine patients did not develop any signs of symptoms associated with VLCADD until present. The only patient who did develop symptoms, PM7, was asymptomatic until age of four, at which she had an episode of hypoglycemia triggered by viral

Table 2
Clinical characteristics patients.

PID	Current age	Age at 1st VLCADD symp	Clinical manifestations during decompensation (onset age)			
			Hypoglycemia (< 2.5 mmol/L)	Rhabdomyolysis CK > 1000 U/L	Heart failure	Arrhythmia
PS1	2 y	1.5 m	–	–	1 m	–
PS2	9 y[a]	1 w	1 w	1 w	9 y	9 y
PS3	7 m[a]	7 m	7 m	7 m	7 m	–
PS4	15 m	1 d (pre-NBS)	1 d	1 d	8 m	8 m
PI5	17 m	–	–	–	–	–
PI6	10 m	1 w	–	1 w	–	–
PM7	4 y	4 y	4 y		–	–
PM8	2 y	–	–	–	–	–
PM9	2 y	–	–	–	–	–
PM10	3 y	–	–	–	–	–
PM11	3 y	–	–	–	–	–
PM12	3 y	–	–	–	–	–
PM13	5 y	–	–	–	–	–
PM14	5 y	–	–	–	–	–
PM15	4 y	–	–	–	–	–

PID: patient identity, PI: patient with intermediate phenotype, PM: patient with mild phenotype, PS: patient with severe phenotype.
[a] Age patient deceased.

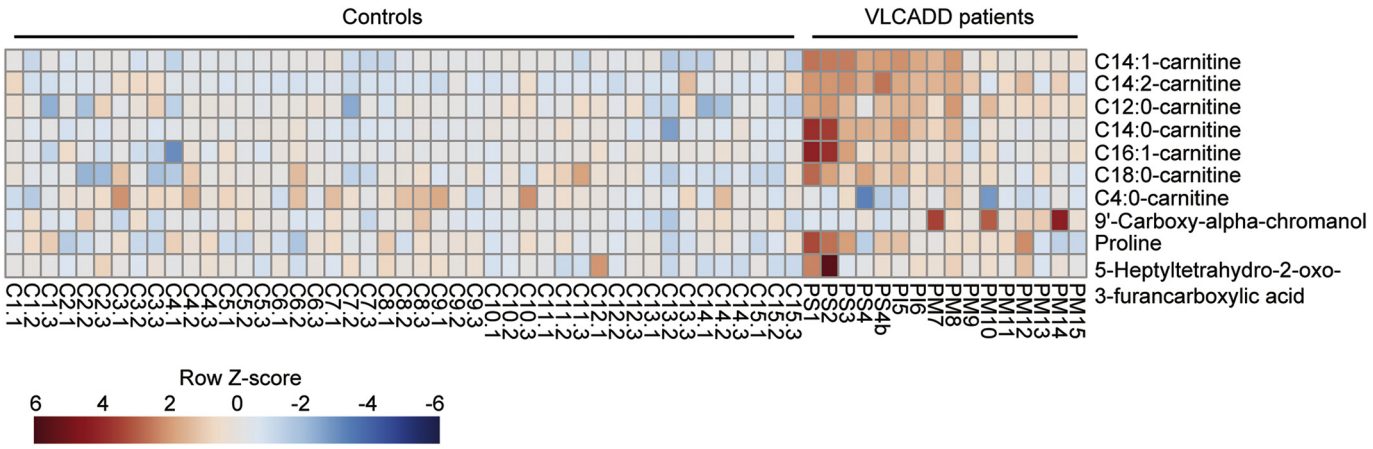

Fig. 1. Heatmap of top 10 changes in identified metabolites in dried blood spots between VLCADD patients and controls. Metabolites are ranked on VIP scores extracted from the PLS-DA model, the top row contains the highest VIP score. Data were logarithmically transformed. The colors of the heatmap represent the relative abundance, red indicates a higher value and blue indicates a lower value. Individual samples of VLCADD patients and controls are depicted in the columns. See also Table 3 for p-values.

infection.

3.2. Metabolite profiles in VLCADD patients compared to controls

Metabolite annotations of a total of ~1300 mass peaks from DI-HRMS were used for univariate and multivariate analyses to reveal most differentiating metabolites between groups. First, we compared the VLCADD patient group with the matched controls using PLS-DA. A satisfactory separation was achieved between the groups (Fig. S1). We extracted VIP scores to rank the annotated metabolites based on their contribution to the separation of VLCADD patients and controls. Top 10 contributing metabolites are presented in Fig. 1 and Table 3. Univariate

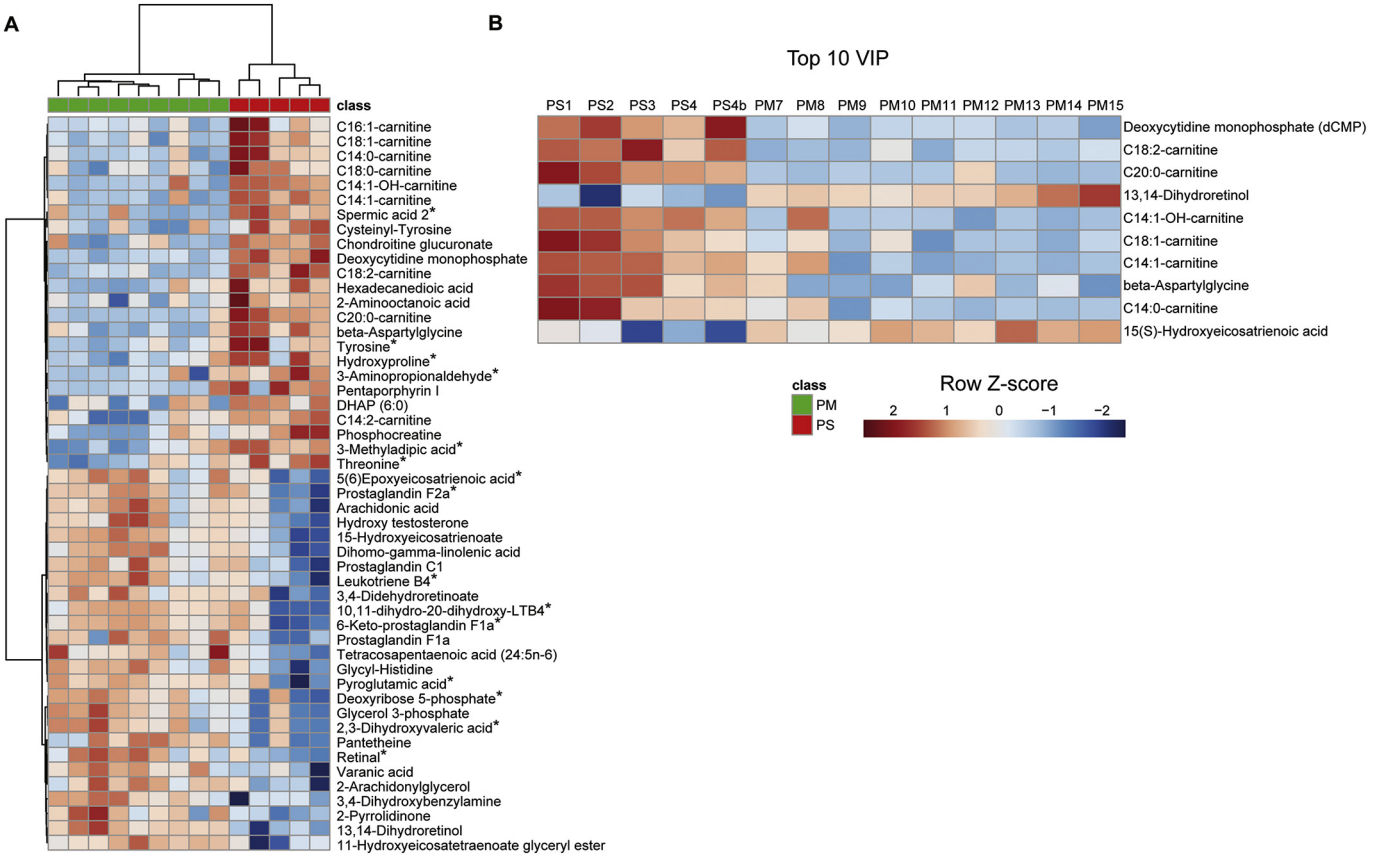

Fig. 2. Discriminating metabolites in dried blood spots between mild and severe patients. a) Unsupervised hierarchical clustering of identified metabolites in mild patients (PM) and severe patients (PS). Data were logarithmically transformed. The colors of the heatmap represent the relative abundance, red indicates a higher value and blue indicates a lower value. The columns represent individual samples. Two clusters and their shorter Euclidean distances indicate high similarity within the two subgroups. *Metabolite annotations were obtained based on *m/z* values. The isomeric compounds of metabolite annotations reported here are reported in Supplementary Table S1. b) Heatmap demonstrating the top 10 discriminating metabolites between PS and PM ranked on VIP scores extracted from PLS-DA. See Table 4 for p-values.

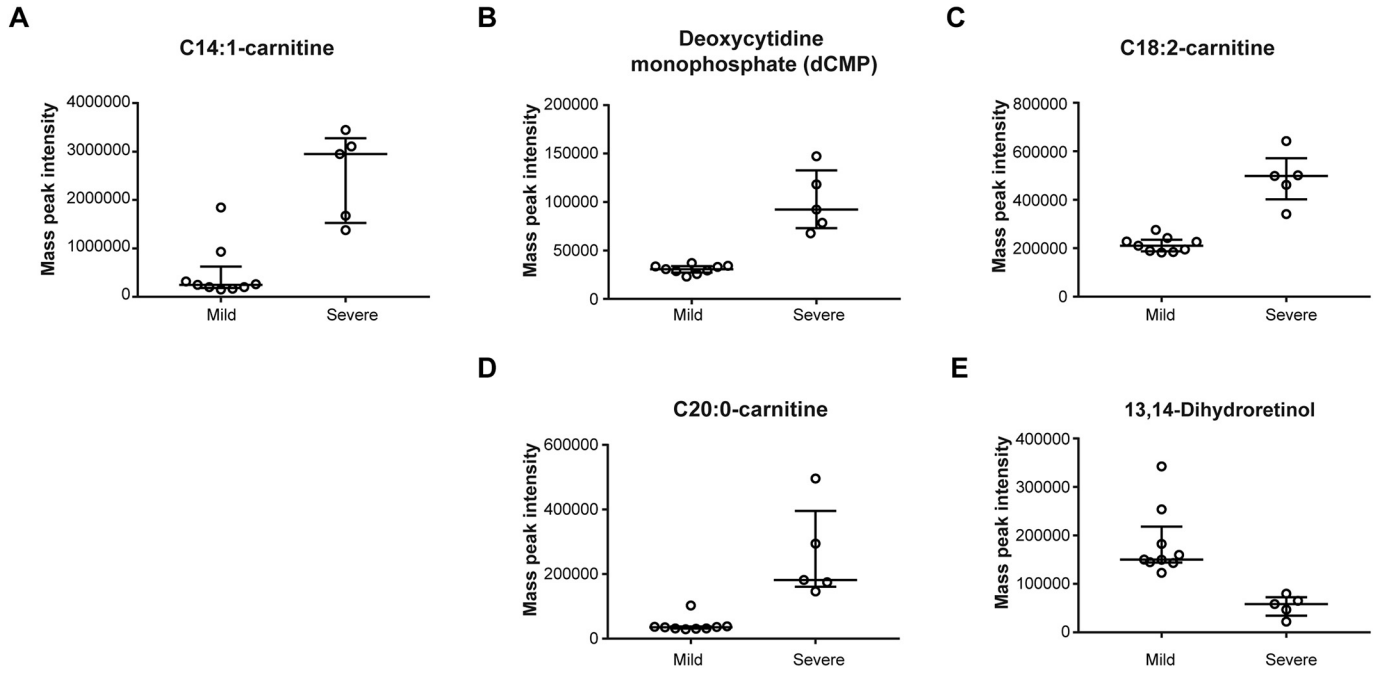

Fig. 3. Scatter plots showing the signal intensity of the most differentiating metabolites between the severe and mild patient subgroups. a–e) Horizontal lines show the median, error bars show the interquartile range. See also Table 4 for p-values.

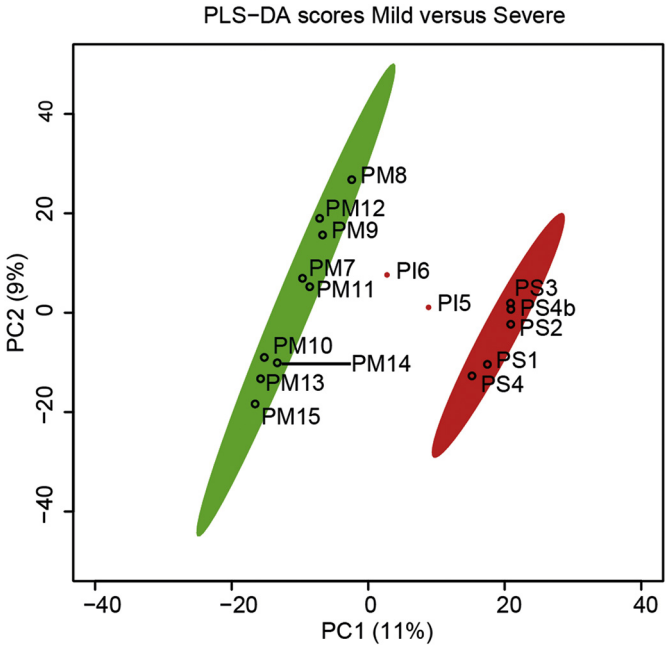

Fig. 4. Score plot of the multivariate PLS-DA model of severe patients (PS) in red versus mild patients (PM) in green. Intermediate patients (PI5 and PI6 indicated by red dots) were predicted in the score plot based on their metabolomics profiles. Ellipses around groups represent 95% confidence intervals of the group. PC: principal component.

statistics revealed that only six metabolites were significantly changed in VLCADD patients compared to controls (q-value < 0.05). These were all elevations in long-chain acylcarnitine species: tetradecenoylcarnitine (**C14:1**), tetradecadiencarnitine (C14:2), dodecanoylcarnitine (C12:0), **tetradecanoylcarnitine** (**C14:0**), **hexadecenoylcarnitine** (C16:1) and stearoylcarnitine (C18:0) (Fig. 1, Table 3). Notable variation in concentration of these metabolites between individual VLCADD patients was present (Fig. 1). Whereas the acylcarnitines are clearly elevated in the severe and intermediate patients, overlap with controls was present in the PM9-PM15. This is in agreement with the initial C14:1-carnitine concentration in NBS with the C14:1-carnitine concentration of these patients being below the cut-off value of 0.6 μmol/L (Table 1).

3.3. Differentiation between mild and severe patients

Because of the clearly different clinical presentation of the mild and severe VLCADD patients (Table 2) we searched for differences in the metabolic profiles of these two subgroups using PLS-DA in order to detect compounds that were not initially considered to generate hypotheses for pathology. Five DBS from severe VLCADD and nine DBS from patients with a mild phenotype were analyzed using a PLS-DA model and univariate statistical analysis. We ranked all metabolites by their contribution to the separation based on VIP scores. Unsupervised hierarchical clustering of the top 50 VIP metabolites showed 24 increased metabolite identifications and 26 decreased metabolite identifications (Fig. 2A). Most discriminating identified metabolites based on VIP scores between the severe and mild group were deoxycytidine monophosphate (dCMP), C18:2-carnitine, C20:0-carnitine and 13,14-dihydroretinol (Fig. 2B and Table 4). In agreement with the initial values in the NBS data in this cohort (Table 1), C14:1-carnitine was higher in the severe subgroup compared to the mild subgroup, although there was overlap (Fig. 3A). C18:2-carnitine was not overlapping between severe and mild patients (Fig. 3B). Retrospective collection of C18:2 carnitine data in plasma (using a validated and targeted technique) of the same patients included in this study confirmed a statistically significant increase in C18:2 carnitine in the group of severe VLCADD patients (median 0.46 μmol/L: range 0.24–0.64) compared to the mild group (median 0.06 μmol/L: range 0.03–0.15). Unfortunately, C20:0-carnitine concentration in plasma was not performed. 13,14-dihydroretinol, involved in the retinol metabolism pathway, was significantly lower in severe patients compared to mild (Fig. 3E and Table 4). To put the metabolite changes between mild and severe into context we used an integrative pathway analysis. Sixty-nine pathways were obtained with importation of the metabolite identifications in the pathway analysis of MetaboAnalyst 4.0 (Metaboanalyst 4.0 [24]) (Fig. 5). Top three most significant pathways were 'Pantothenate and

Table 3
Top 10 metabolite changes patients versus controls.

VIP	Fold change	p-Value	q-Value (FDR)	HMDB code	Name
5.57	10.86	0.000 (W)	0.000	HMDB13329	Tetradecenoylcarnitine (C14:1)
5.28	3.04	0.000 (W)	0.000	HMDB13331	Tetradecadiencarnitine (C14:2)
4.39	2.25	0.000 (W)	0.000	HMDB02250	Dodecanoylcarnitine (C12:0)
4.38	4.08	0.000 (W)	0.028	HMDB05066	Tetradecanoylcarnitine (C14:0)
3.66	2.39	0.000 (W)	0.024	HMDB13207	Hexadecenoylcarnitine (C16:1)
3.39	1.98	0.000	0.028	HMDB00848	Stearoylcarnitine (C18:0)
3.12	0.76	0.001	0.091	HMDB00736	(Iso)butyrylcarnitine (C4:0)
3.03	2.29	0.025 (W)	0.696	HMDB12866	9′-Carboxy-alpha-chromanol
2.76	1.40	0.035	0.758	HMDB00162	Proline
2.68	1.51	0.015 (W)	0.696	HMDB30994	5-Heptyltetrahydro-2-oxo-3-furancarboxylic acid

Top 10 discriminative metabolite changes in dried blood spots for VLCADD patients versus controls. p-Values were corrected for false discovery rate (FDR) (q-values). (W): Mann Whitney *U* test was used for univariate statistics because of failed normality.

CoA biosynthesis', 'Arachidonic acid metabolism' and 'Retinol metabolism'. Several derivatives of arachidonic acid (leukotrienes and prostaglandins) were also lower in severe VLCADD patients as shown in Fig. 2A.

3.4. Prediction of intermediate phenotypes in mild-severe model

Because our goal was to use metabolic profiles to estimate clinical severity, we plotted the results of the patients who were not used in the development of the PLS-DA model (PI5 and PI6) in the score plot based on their metabolic profile. Both patients were predicted to end up between the mild and severe (Fig. 4), which agrees with the intermediate lcFAO flux.

4. Discussion

This study shows that a distinctive metabolic profile is present in DBS of newborns with VLCADD, and furthermore points to potential new biochemical biomarkers associated with disease severity and the underlying disease mechanisms. These results hold potential for early prognostication and tailored therapeutic interventions.

As expected, the metabolic profiles in a matched control group were different from the metabolic profiles of VLCADD patients. Analysis of > 1300 assigned metabolites pointed to long-chain acylcarnitines as the most differentiating metabolites, with the primary screening marker C14:1-carnitine as most important. The use of the C14:1-carnitine concentration in NBS as marker for disease severity proved to be not conclusive before [5,6]. This is in agreement with the present data although in our study we did see a trend of higher C14:1 in the severe group (Fig. 3A).

Using untargeted non-quantitative DI-HRMS metabolomics we found several other metabolites that were more contributing to differentiation between severe and mild patients, i.e. deoxycytidine monophosphate (dCMP), C18:2-carnitine, C20:0-carnitine and 13,14-dihydroretinol. Most significantly increased in the severe group was dCMP, which plays a role in DNA synthesis [25]. This increased dCMP might be related to myocardial disease, since dCMP is transmyocardially enriched after induced ischemia [26]. In our study, cardiac symptoms were only observed in patients in the severe VLCADD

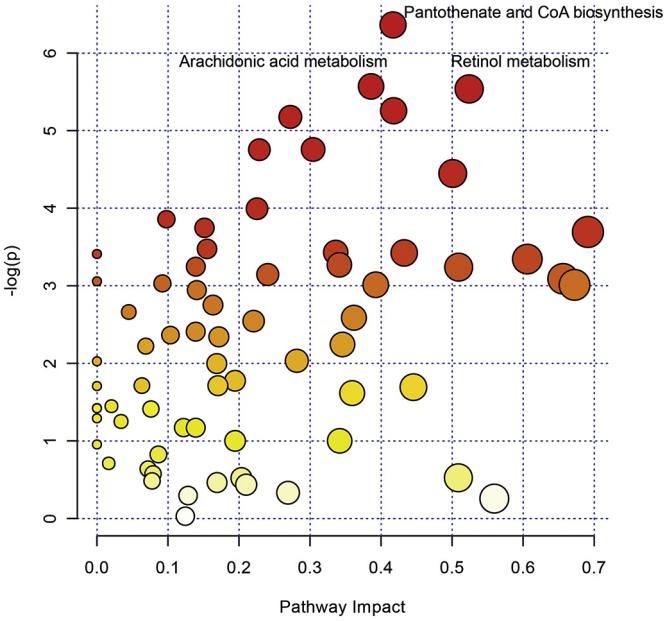

Fig. 5. Pathway analysis summary from MetaboAnalyst 4.0 showing the changed pathways in severe patients vs. mild patients. Pathways are plotted according to significance (y-axis) and pathway impact value (x-axis). Larger circles represent greater pathway enrichment and darker colors represent more significance. Most contributing pathways are in the top right corner.

Table 4
Metabolite changes patient severe versus patient mild.

VIP	p-Value	q-Value (FDR)	Fold change	HMDB code	Name
2.74	0.000	0.001	3.29	HMDB01202	Deoxycytidine monophosphate (dCMP)
2.69	0.000	0.002	2.28	HMDB06469	Linoleylcarnitine (C18:2)
2.66	0.001 (W)	0.132	6.24	HMDB06460	Arachidylcarnitine (C20:0)
2.49	0.000	0.033	0.30	HMDB11618	13,14-Dihydroretinol
2.43	0.007 (W)	0.212	2.99	HMDB13330	3-Hydroxytetradecenoylcarnitine (C14:1-OH)
2.41	0.000	0.056	2.85	HMDB05065	Oleoylcarnitine (C18:1)
2.38	0.004 (W)	0.212	5.21	HMDB13329	Tetradecenoylcarnitine (C14:1)
2.35	0.001	0.072	2.02	HMDB11165	beta-Aspartylglycine
2.33	0.004 (W)	0.216	5.98	HMDB05066	Tetradecanoylcarnitine (C14:0)
2.32	0.016	0.313	0.43	HMDB05045	15(*S*)-Hydroxyeicosatrienoic acid

Top 10 discriminative metabolite changes in dried blood spots for severe patients versus mild patients. (W) Mann Whitney *U* test was used for univariate statistics because of failed normality. FDR: false discovery rate. VIP scores were obtained from PLS-DA model.

subgroup, however, none of these patients had prominent cardiac symptoms at the time the NBS card was obtained.

The elevated C18:2-carnitine concentration in severe patients compared to mild patients was confirmed by analysis of the acylcarnitine profiles measured for confirmatory testing of the screen-positives for VLCADD. This indicates that C18:2-carnitine may be a useful biomarker for predicting disease severity in VLCADD. C18:2-carnitine is not new as marker for VLCADD [27], however, it has to our knowledge not previously been related to disease severity. Also, other long-chain acylcarnitines, i.e. C20:0-carnitine and C18:1-carnitine, were increased in the severe group compared to the mild group. Most likely this is the result of the strongly reduced fatty acid oxidation flux in the severe patients, leaving the long-chain acyl-CoAs un-oxidized leading to accumulation of their corresponding acylcarnitines. In contrast, in patients with a milder phenotype long-chain acyl-CoA esters still undergo a number of cycles of β-oxidation and therefore accumulation is more pronounced at the level of C14:1-carnitine [9,28].

Both in the pathway analysis and in the top VIP score metabolites we found that arachidonic acid and a number of derivatives (prostaglandins and leukotrienes) were lower in severe compared to mild VLCADD patients. Metabolites of arachidonic acid have both pro- and anti-inflammatory properties [29]. Arachidonic acid (C20:4) is produced from (dietary) linoleic acid (C18:2 n-6) by chain elongation and desaturation [30]. A disturbance in arachidonic acid and derivatives may cause pathology in VLCADD, however targeted analysis of arachidonic acid and related metabolites is warranted. 13,14-Dihydroretinol, a metabolite closely linked to vitamin A, was significantly lower in patients with the severe phenotype compared to patients with the mild phenotype. Additionally, in the pathway analysis several metabolites in the retinol metabolism, including retinol (vitamin A_1), showed a similar trend. Even though monitoring of fat-soluble vitamins is recommended for VLCADD patients this finding was surprising, since our study was done in NBS cards, before any dietary fat restriction was implemented.

We used untargeted metabolomics data of ~1300 metabolites to predict severity in two patients we could not prognosticate based on early clinical symptoms (Tables 1 and 2). Correct classification of these two patients between mild and severe (Fig. 4) confirmed the robustness of the obtained mild-severe PLS-DA model. The clinical outcome of these two patients remained to be seen.

We acknowledge several advantages as well as limitations of our study. The main advantage of using DBS from newborns for metabolic fingerprinting is that the influence of external factors (e.g. dietary intake, pharmacological, physical activity) on laboratory results are relatively small. However, the number of residual DBS samples from VLCADD patients is limited. Especially, there are insufficient numbers of well-characterized patients with severe clinical presentation, since disease prevalence for VLCADD is low (estimated at 1:30,000 to 1:100,000 births) [19]. A limitation of DI-HRMS is the inability to discriminate between isomeric compounds based solely on accurate mass (e.g. methylmalonic acid and succinic acid) or geometric isomers (e.g., L-serine and D-serine). Another point of concern is the preprocessing procedure for studying untargeted metabolomics. The fact that samples stored for a longer period showed a different metabolic profiling required exclusion of the oldest DBS. This also advocates that storage conditions need to be tightly controlled.

5. Conclusion

In conclusion, the presence of differential metabolomic profiles between VLCADD patients with a severe and mild phenotype demonstrates that metabolic alterations are already present and detectable in newborns and points to potential new biochemical biomarkers associated with disease severity. It will be interesting and useful to confirm these findings in a larger cohort and to clarify the mechanisms by which these metabolic changes contribute to disease progression later in life. With the aim of direct translation of the metabolomic profiles for prognostication, we identified C18:2-carnitine and C20:0-carnitine as biochemical markers for predicting disease severity. To further assess the predictive value of these and other metabolites, targeted analysis is warranted.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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