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ORIGINAL ARTICLE

Pervasive inflammatory activation in patients with deficiency in very-long-chain acyl-coA dehydrogenase (VLCADD)

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Abstract

Objectives. Very-long-chain acyl-CoA dehydrogenase deficiency (VLCADD) is a disorder of fatty acid oxidation. Symptoms are managed by dietary supplementation with medium-chain fatty acids that bypass the metabolic block. However, patients remain vulnerable to hospitalisations because of rhabdomyolysis, suggesting pathologic processes other than energy deficit. Since rhabdomyolysis is a self-destructive process that can signal inflammatory/immune cascades, we tested the hypothesis that inflammation is a physiologic dimension of VLCADD. **Methods.** All subjects ($n = 18$) underwent informed consent/assent. Plasma cytokine and cytometry analyses were performed. A prospective case analysis was carried out on a patient with recurrent hospitalisation. Health data were extracted from patient medical records. **Results.** Patients showed systemic upregulation of nine inflammatory mediators during symptomatic and asymptomatic periods. There was also overall abundance of immune cells with high intracellular expression of IFNγ, IL-6, MIP-1β (CCL4) and TNFα, and the transcription factors p65-NFκB and STAT1 linked to inflammatory pathways. A case analysis of a patient exhibited already elevated plasma cytokine levels during diagnosis in early infancy, evolving into sustained high systemic levels during recurrent rhabdomyolysis-related hospitalisations. There were corresponding activated leukocytes, with higher intracellular stores of inflammatory molecules in monocytes compared to T cells. Exposure of monocytes to long-chain free fatty acids recapitulated the cytokine signature of patients. **Conclusion.** Pervasive plasma

cytokine upregulation and pre-activated immune cells indicate chronic inflammatory state in VLCADD. Thus, there is rationale for practical implementation of clinical assessment of inflammation and/or translational testing, or adoption, of anti-inflammatory intervention(s) for personalised disease management.

Keywords: fatty acid oxidation, inflammation, lymphocytes, monocytes, rhabdomyolysis, very-long-chain acyl-CoA dehydrogenase deficiency

INTRODUCTION

Very-long-chain acyl-CoA dehydrogenase deficiency (VLCADD) is an autosomal disorder of fatty acid β-oxidation.[1,2] It is traditionally considered a disease of energy deficit because patients generally present with hypoglycaemia as the prominent symptom in early infancy.[3] Multiple *ACADVL* mutations have been described,[4,5] all of which result in the failure to catabolise acyl-CoAs of 12–20 carbon-chain lengths. Symptoms may manifest at any age. Without newborn screening, presentation of VLCADD in early childhood has a 75% risk of early death because of hypoketotic hypoglycaemia and cardiomyopathy or arrhythmia. Symptoms in later childhood or adulthood are dominated by rhabdomyolysis and cardiomyopathy; hypoglycaemia is rare.[6] Current treatment is limited to dietary supplementation with medium-chain (6C–12C) fatty acids that bypass the metabolic block to provide fuel for skeletal muscle and heart. In spite of this alternative fuel, most patients still experience recurrent rhabdomyolysis that can require hospitalisation care.[7–9] These acute events reportedly occur after strenuous physical activity or fasting and during febrile periods of common infections, but these are largely anecdotal and exact triggers are yet to be proven.[10,11] While the frequency of major medical events is reduced by a novel odd-, medium-chain triglyceride, they nonetheless continue to occur, and rhabdomyolysis is the least improved clinical symptom in treated patients.[12] These observations suggest additional pathologic process(es) besides energy deficit.

Regardless of the triggering event, muscle destruction, as in the case of rhabdomyolysis, is an alert signal for the immune system to clear damaged cells and debris. Hence, we examined the role of inflammation because clinical presentation of acute rhabdomyolysis among VLCADD patients is accompanied by highly elevated blood levels of creatine phosphokinase (CPK) and secondary myoglobinuria,[6,13] two well-recognised characteristics of inflammatory muscle diseases.[14,15] Idiopathic myopathies and sarcopaenia are examples of other chronic diseases where muscle damage/loss/repair have accompanying immune cell activation, and local or systemic inflammation.[16–18] A magnetic resonance imaging study of patients with various fatty acid oxidation disorders including VLCADD reported muscle T1-weighted changes akin to muscle inflammation typical for paediatric myopathies.[19–21] From a broader scientific standpoint, lipids/fatty acids are known to directly affect immune function and modulate inflammatory processes. On the one hand, free fatty acids (FFA) enhance various immune effector activities.[22,23] On the other hand, excessive amounts of FFA, as seen in VLCADD in metabolic crisis,[10,24] have been shown in experimental and observational studies to cause persistent stimulation of immune cells and other cell types and contribute to various chronic pathologies.[2,25–28]

Therefore, we tested the hypothesis that pervasive systemic inflammation is a signature of VLCADD. We screened for both humoral and cellular arms of inflammation. Because of genetic and clinical heterogeneity of VLCADD,[24,29,30] we followed one particular patient with frequent episodes of rhabdomyolysis to determine the evolution of an inflammatory condition.[31]

RESULTS

VLCADD patient cohort

Table 1 summarises the characteristics of 18 patients (10 females, eight males) seen at our clinic. Most were Caucasians, ranging from the age of 4 months to 31 years during their initial visit. They had varying mutations in *ACADVL*,

except for two which have not been determined. All patients fit the diagnostic criteria and clinical manifestations of VLCADD. During outpatient visits, they all had CPK levels (expressed as $\mu g\ L^{-1}$), a classic indicator of muscle injury frequently seen in VLCADD,[32] that were already significantly elevated over the normal clinical range. Depending on the patient, CPK levels rose to an average of 79.7–812.7 $\mu g\ L^{-1}$ or the equivalent of ~22% to ~642% during repeated hospitalisations because of acute rhabdomyolysis.

Cytokine/chemokine profiles

Cross-sectional multiplex analyses were performed for 17 inflammatory molecules. There was global systemic inflammatory upregulation in both outpatient (asymptomatic) and inpatient (hospital-collected) samples as shown by Spearman correlations among the cytokines and chemokines in Supplementary tables 1 and 2, respectively. IFNγ, a prototypic Th1 cytokine derived from and utilised by the immune system,[33] was notably correlated significantly with majority of the cytokines/chemokines examined in both patient groups. However, there were unusual direct correlations that do not follow the TH paradigms. IFNγ had significant statistical correlation with another Th1 cytokine IL-12p70, but it also had statistically significant correlation with Th2 cytokines IL-5, IL-6 and IL-13 in both outpatient and inpatient groups. Furthermore, there was not a significant correlation between IFNγ and IL-17, the latter Th17 cytokine being normally co-expressed and/or expressed downstream of IFNγ signalling.[34] In addition, MCP1 (also known as CCL2) and MIP-1β (also known as CCL4), two macrophage/monocyte cytokines, were also not correlated with each other. TNFα, IL-1β, and IL-6, three classic cytokines implicated in many chronic diseases including inflammatory myopathies and sarcopaenia,[18,35,36] were sporadically correlated with the other cytokines/chemokines. There was no significant correlation of cytokine/chemokine levels with CPK in both patient groups.

Compared to healthy controls, however, VLCADD patients had highly elevated levels of 12 of the 17 cytokines/chemokines. Figure 1 shows that nine of these molecules were significantly upregulated ($P < 0.05$, ANOVA) in both outpatient and inpatient groups. Increases over the controls

Table 1. Demographic and clinical characteristics of VLCADD patients

Patient #	Sex	Race/Ethnicity	*ACADVL* mutation	Age at 1st visit	Average CPK, outpatient[a]	Average CPK, admission
1	Female	Caucasian	T1372C (F548L); 1668 ACAG 1669 splice site	19 years	812.7	36 816
2	Female	Caucasian	c.1182+1G>A; c.566T>C (p.I189T)	15 months	122	31 570.5
3[b]	Female	Caucasian	T1619C and 9-bp insertion; duplication of bp 1707-1716 in exon 18, duplicates amino acids 530-532 DGA	4 years (new diagnosis)	97.5	28 933.8
4	Female	Caucasian	848T>C (V283A); 1182(+3)G>T	12 months	79.7	2740
5	Male	Caucasian	1322G>A (p.G401D); 1837C>T (p.R573W)	31 years	244.1	8444.4
6	Female	Caucasian	Deletion 887-888 exon 10; G-6A intron proceeding exon 18	16 years	129	27 227.3
7	Male	Hispanic	N/A[c]	16 years	N/A	16 474
8	Male	Caucasian	A770Del; G1613C	13 years	441.5	25 054.5
9	Male	Caucasian	N/A[c]	12 years	893	31 325
11	Male	Caucasian	A770Del; G1613C	9 years	247.3	5417.5
12	Female	Caucasian	G1406A (R429Q); splice site mutation exon 11, G +1A	13 years		52 293
13	Male	Caucasian	605T>C; 1837C>T	9 years	266.0	72 743
14	Female	African American	*Not done*			76 615
15	Female	Caucasian	1316G>A (G439D); 1328T>G (M443R)	21 months	306	35 542
16	Male	Caucasian	c.343delG p.Glu115LysfsX2; c.1198G>A, p.Val400Met	7 years	163.5	N/A
17	Female	Caucasian	c.343delG p.Glu115LysfsX2; c.1198G>A, p.Val400Met	10 years	265.5	N/A
18	Female	Caucasian	N/A[c]	17 years	252	N/A

[a]Creatine phosphokinase (CPK) values ($\mu g\ L^{-1}$); normal range = 10–120 $\mu g\ L^{-1}$.
[b]Patient with highly recurrent rhabdomyolysis, data on multiple samples shown in Figures 2 and 4.
[c]N/A, not available.

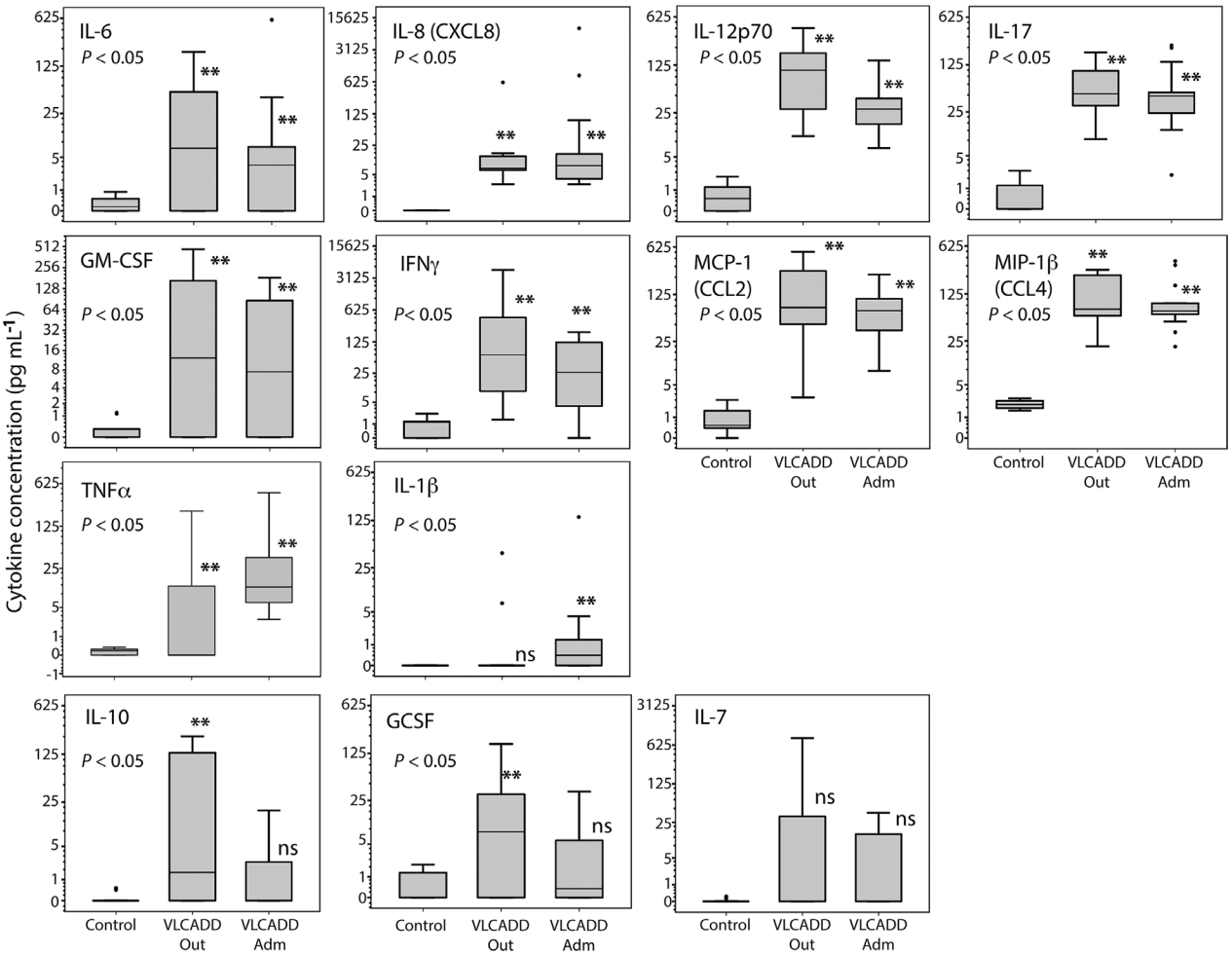

Figure 1. Both symptomatic and asymptomatic patients with VLCADD have an upregulated systemic cytokine profile. Data shown are 12 of 17 cytokines examined that were significantly higher for patients (n = 13 outpatient samples [Out]; n = 10 samples at hospital admission [Adm]) than for healthy controls (n = 9). Box plots indicate the 25th and 75th percentile of values with the solid line inside the box representing the median, the whiskers represent 5th and 95th percentiles, and black circle dots were outliers. The *P*-values indicated were determined by Kruskal–Wallis ANOVA. *Post hoc* paired comparisons between each of the two patient groups and the controls are indicated by ** ($P < 0.005$, Tukey) or ns (not significant). The two patient groups were not significantly different from each other.

were uniformly > 5 pg mL^{-1} and up to 125-fold for IL-6, IL-12p70, IL-17, GM-CSF, MCP1 (CCL 2), MIP-1β (CCL4) and TNFα compared to controls. For IL-8 (also known as CXCL8) and IFNγ, values were elevated up to 3125-fold over the controls. There was also upregulation of IL-1β in inpatient VLCADD samples, but the median concentration was < 5 pg mL^{-1}. IL-10 and GCSF were significantly elevated in the outpatient VLCADD samples, but not in the inpatient samples.

Figure 2 shows additional specific cytokine values for three patients, for which there were three samples available for cytokine analysis. As depicted, Patients 1 and 2 had varying but significant upregulations in 12 of 17 molecules examined. Notably, IL-12p70, IL-17, IFNγ, MCP1 (CCL2) and MIP-1β (CCL4) remained elevated at levels > 5 pg mL^{-1} during in all three outpatient samples. For Patient 3, the samples analysed included one outpatient and two inpatient samples. IL-12p70, IL-17, MCP1 (CCL2) and MIP-1β (CCL4) were already at elevated levels > 5 pg mL^{-1} at an outpatient visit and then rose further at the two subsequent inpatient visits. Five other molecules, namely, IL-6, IL-17, IL-10, GM-CSF, TNFα, were negligible at the outpatient visit but were elevated up to ~125-fold in the two subsequent inpatient visits.

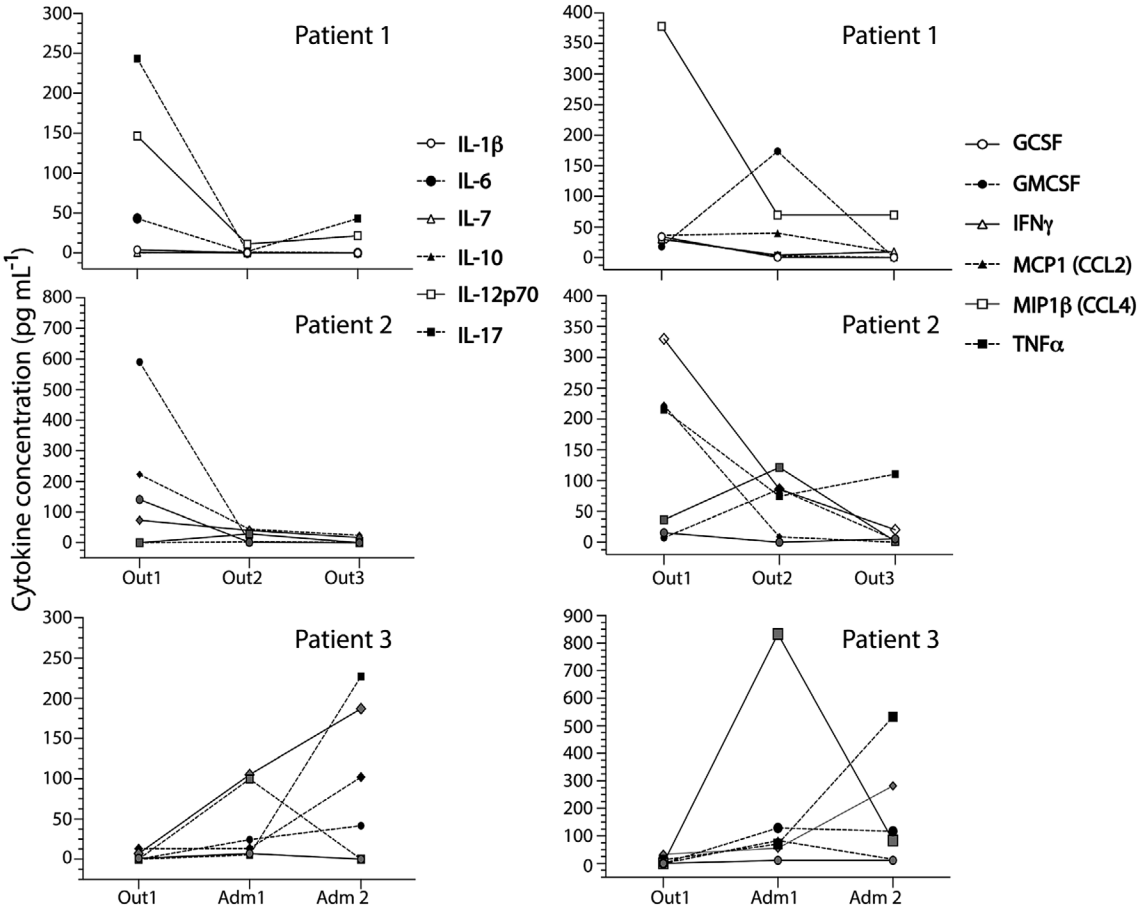

Figure 2. Fluctuations of cytokine concentrations in VLCADD patients at multiple visits. Data shown are raw measurements of plasma cytokines from three patients with multiple evaluable samples during routine outpatient [Out] and/or at hospital admission [Adm]. Cytokine measurements were determined as in Figure 1.

Activated leukocyte profiles

Because of a limited number of evaluable banked PBMC samples, cytometry focused on intracellular stores of inflammatory factors rather a broad characterisation of memory, naïve or discrete functional subsets (e.g. regulatory, cytolytic, phagocytic) of immune cells. We focused on cytoplasmic IL-6, IFNγ, TNFα and MIP-1β (CCL4), all of which were upregulated in plasma of VLCADD patients (as shown in Figure 1). This cytometric strategy included detection of phosphorylated forms of STAT1 and p65-NFκB, which are known transcriptional regulators of IFNγ and TNFα signalling, respectively.[37,38] Figure 3 shows the cross-sectional cytometric analyses for seven evaluable VLCADD samples from three asymptomatic and four symptomatic patients and from five healthy controls. As indicated, all cytometry data generated from six immune cell subsets were statistically significant ($P < 0.0001$, ANOVA). Patients generally had higher levels of intracellular stores of IL-6, IFNγ, and MIP-1β (CCL4) and TNFα than the controls. Symptomatic and asymptomatic patients had equivalent intracellular levels of these molecules in their $CD14^+$ and $CD16^+$ monocytes, NK cells, and $CD4^+$ and $CD8^+$ T cells that were statistically significant from controls (determined by *post hoc* pairwise comparisons). The levels of these same molecules in B cells of patients also trended higher but were not statistically significant than corresponding B cells of controls. Similarly, intracellular levels of p-p65-NFκB and p-STAT1 were significantly higher (*post hoc* pairwise comparisons) for the patients than for the controls. However, only p-p65-NFκB levels in $CD14^+$ monocytes, and $CD4^+$ and $CD8^+$ T cells of

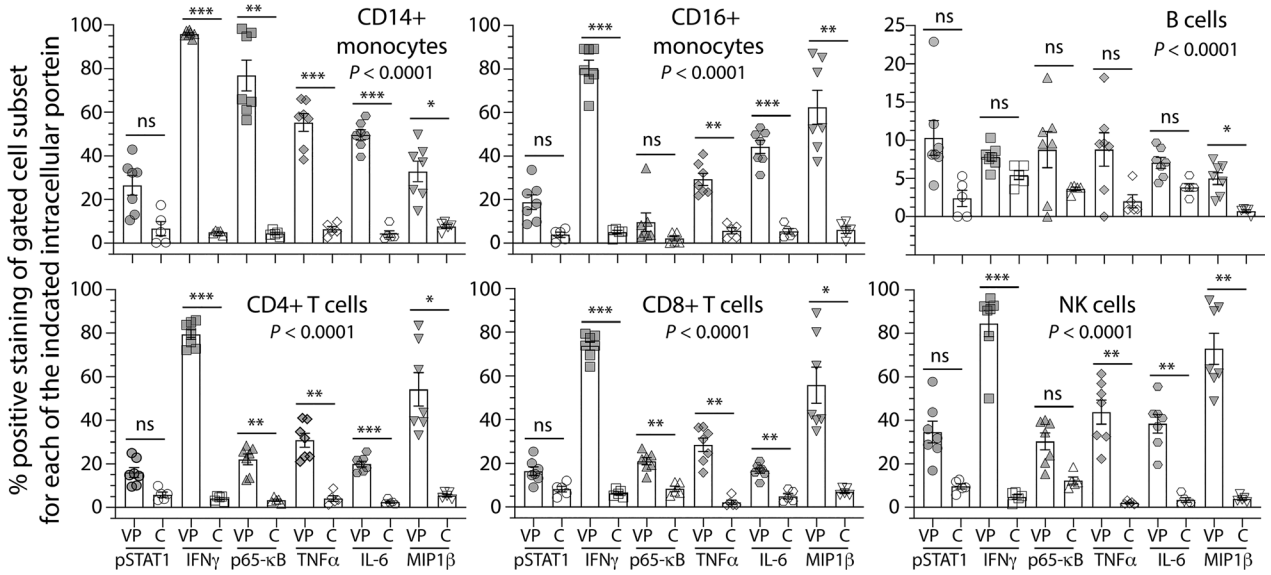

Figure 3. Circulating immune cell subsets of VLCADD patients have highly activated phenotypes. Data shown are cross-sectional cytometric analyses for intracellular expression levels of four cytokines (IFNγ, IL-6, MIP-1β [CCL4], TNFα] and the phosphorylated forms of two transcription factors (p65-NFκB, STAT1). As indicated, six immune cell subsets were examined ($CD14^+$ and $CD16^+$ monocytes; $CD4^+$ and $CD8^+$ T cells; NK cells). The bar–whisker plots were means ± SEM, with the superimposed measurements from individual subjects (five controls [C], 7 VLCADD patients [V]) represented by polygons. The indicated *P*-values were determined by Brown–Forsythe and Welch ANOVA. *Post hoc* pairwise comparison between controls and patients is indicated by * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0001$), ns (not significant).

patients were statistically significant when compared to levels of similar cells of controls.

Evolution of inflammatory profile in a VLCADD patient

The above cross-sectional data indicate an inflammatory state in VLCADD patients even when asymptomatic. In order to examine the evolution of an inflammatory state, we followed Patient 3 (refer to Table 1) who was diagnosed with VLCADD by newborn screening and had her first clinical symptom of hypoglycaemia at 4 months of age. Recurrent rhabdomyolysis became evident by the age of 6 years when treatment with triheptanoin was begun. As shown in Figure 2, this patient already had some degree of cytokine upregulation during an early visit at 4 years of age. Figure 4 shows an extended humoral profiling over the course of 2 years for 12 successive hospital admissions because of acute rhabdomyolysis. There was an overall pattern of waves of changes in cytokine levels. Notably, IL-12p70, IL-17, MCP1 (CCL2), and MIP-1β (CCL4) were all upregulated during the outpatient visit and persisted at high levels up to ~32-fold of baseline (~8 pg mL^{-1} baseline versus maximum of ~256 pg mL^{-1} at hospitalisation). IL-6, IFNγ and TNFα were negligible initially and then rose to high levels (up to ~625 pg mL^{-1}) at the first hospitalisation and remained at high levels through subsequent admissions. IL-7, IL-10 and GCSF were initially low (< 5 pg mL^{-1}) and then spiked during later admissions. In contrast, IL-1β was also initially low, but was undetectable through subsequent admissions.

Table 2 shows the correlation matrix CPK and the same set of cytokines/chemokines as shown in Figure 4. The data show statistically significant correlation of CPK independently with IL-12p70 (Th1) and IL-17 (Th17). There was also significant correlation between these two cytokines, but neither of them was correlated with IFNγ (Th1). IL-12p70 was further correlated with the two macrophage chemokines MCP1 (CCL2) and MIP-1β (CCL4), but the latter two were not correlated with each other. IL-12p70, IL-17 and IFNγ were correlated with IL-10. The three other classic inflammatory cytokines IL-1β, IL-6 and TNFα were not correlated with each other and the other cytokines examined.

During the same 2-year period follow-up of Patient 3, there were six evaluable banked PBMC samples that were suitable for flow cytometry.

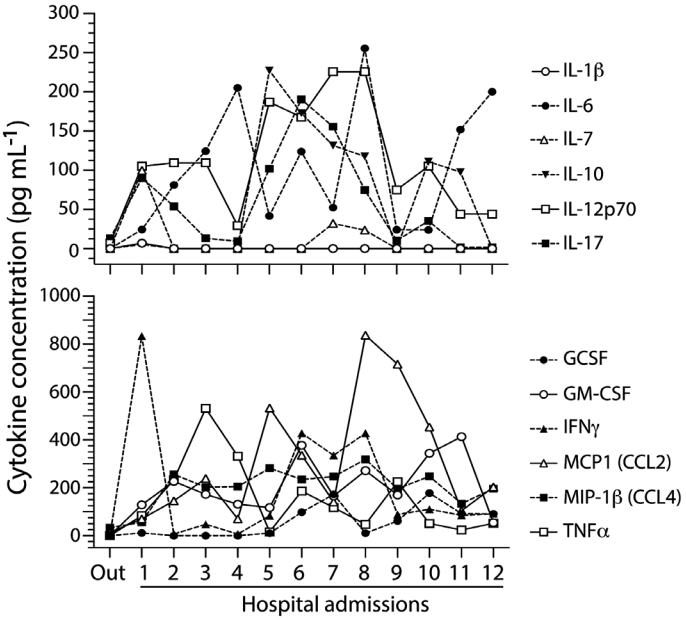

Figure 4. Changes in cytokine profile of a VLCADD patient over successive hospitalisation: a case analysis. Plasma samples from a patient with highly recurrent rhabdomyolysis (Patient 3 in Table 1) that were banked over time were examined for cytokine content. As indicated, a sample from an outpatient visit [out] was compared with similar samples banked from 12 successive hospitalisation over a period of 2 years. Data shown are raw measurements of the same 12 cytokines measured in Figure 1.

Table 2. Correlation coefficient matrix of CPK and cytokine/chemokine levels of a VLCAD patient[a]

	CPK	IL-1β	IL-6	IL-7	IL-10	1L-12p70	IL-17	GCSF	GM-CSF	IFNγ	MCP1 (CCL2)	MIP-1β (CCL4)	TNFα
CPK	1	0.154	0.148	0.476	0.283	0.618*	0.638*	0.237	0.368	0.548**	0.236	0.44	0.5275
IL-1β		1	−0.231	0.626	0	0	0.232	0	−0.231	0.464	−0.309	−0.386	0
IL-6			1	0.03	−0.02	0.138	−0.223	−0.159	0.258	0.072	0.071	0.259	0.2637
IL-7				1	0.332	0.492	0.491	0.183	−0.093	0.665*	−0.074	0.063	−0.074
IL-10					1	0.692*	0.725*	0.552	0.318	0.571*	0.393	0.5266	−0.413
IL-12p70						1	0.823*	0.2	0.292	0.509	0.566*	0.7307*	0.08
IL-17							1	0.204	0.118	0.494	0.272	0.5138	−0.003
GCSF								1	0.396	0.678*	0.259	0.0531	−0.215
GM-CSF									1	0.292	0.357	0.3274	0.1813
IFNγ										1	0.369	0.1434	−0.083
MCP1 (CCL2)											1	0.6245	−0.011
MIP-1β (CCL4)												1	0.066
TNFα													1

[a]Data shown are Spearman correlation coefficients between each indicated molecular variables for Patient 3 referred to in Table 1.
*Indicates statistically significant correlation at $P < 0.05$.
**Indicates $P = 0.055$.

Figure 5 shows the data for the intracellular stores of four cytokines and two transcription factors. Patient 3 clearly had persistent intracellular expression of IFNγ, MIP-1β (CCL4) TNFα, p-p65-NFκB and p-STAT1 in the $CD14^+$ and $CD16^+$ subsets of monocytes. IL-6 was characteristically mostly highly expressed in B cells. NK cells and the two T-cell subsets also had increased levels of all the six intracellular molecules examined, with appreciable detection of up to 40% of NK and T-cell subsets for IFNγ and p-p65NFκB. Clinical exome sequencing was

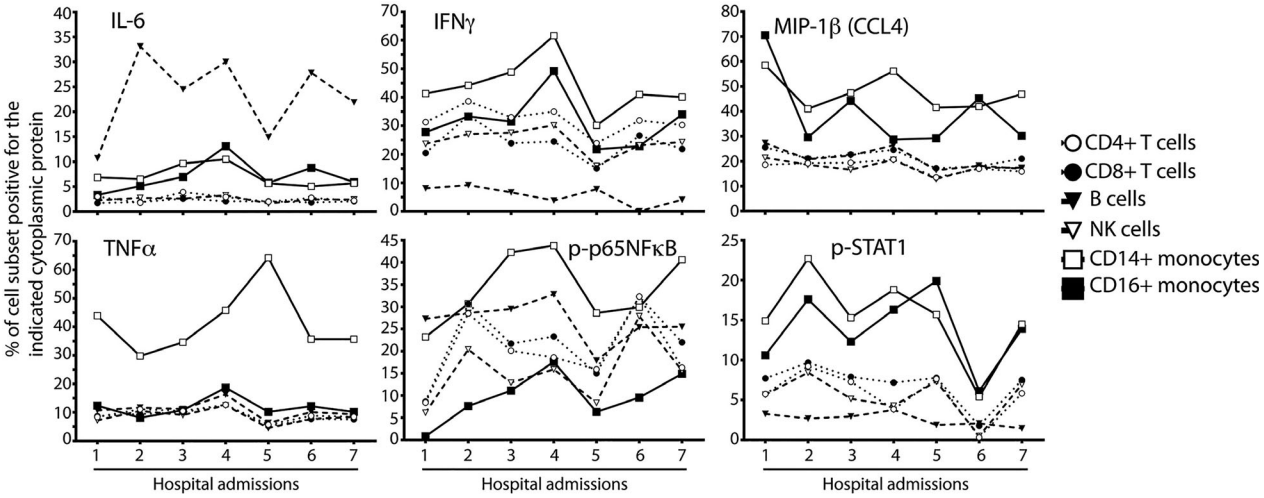

Figure 5. Changes in immune cell-activated phenotypes of a VLCADD patient over successive hospitalisation: a case analysis. Over the same 2-year period as in Figure 4, seven banked PBMC samples from successive hospitalisations of Patient 3 were evaluable for cytometry. As indicated, the intracellular stores of IL-6, IFNγ, MCP1 (CCL2), TNFα, p-p65NFκB and p-STAT were examined in $CD14^+$ and $CD16^+$ subsets of monocytes, $CD4^+$ and $CD8^+$ T cells, NK cells and B cells.

Table 3. Biopsy report for a VLCADD patient with highly recurrent rhabdomyolysis[a]

Histopathological parameter	Description
Glycogen staining	Intense PAS staining
Myofibre atrophy	Focal
Myofibre degeneration	Focal, endomysial
Myofibre regeneration	Focal, endomysial
Myofibrillar disruption	Focal, NADH-positive
Rimmed vacuoles	Negative
Capillary loss	Negative by C5b-9 (MAC) staining
Capillary dilatation	Negative by CD31 staining
T-cell infiltration	Focal CD8 stainingFocal CD3 staining, perivascularWidespread CD3 staining, endomysial
Macrophage infiltration	Focal esterase and CD68 stainingFocal myophagocytosis
B-cell infiltration	Negative for CD20
MHC-1 expression (major histocompatibility complex class I)	Focal staining, ~3% of myofibres
Cytochrome oxidase expression	Negative
Neurogenic atrophy	Negative
Succinic dehydrogenase expression	Negative

[a]Summary of a histopathological report from a biopsy of the left vastus lateralis. Pathologic evaluation was performed by an independent clinical pathologist who reported the findings in the patient medical record. This is a pathology report for Patient 3 (see Table 1).

ultimately performed to look for an alternative cause for rhabdomyolysis, which identified only her already known *ACADVL* mutations shown in Table 1.

Muscle pathology

As a result of highly recurrent rhabdomyolysis, patient 3 (Table 1, Figure 4) underwent a muscle biopsy. Table 3 summarises the most recent muscle biopsy report during hospital admission. Consistent with rhabdomyolysis, there were many foci of muscle degeneration and regeneration. Significantly, there was focal infiltration of T cells and macrophages. In addition, there was focal expression of class I MHC molecules.

Figure 6 depicts representative photomicrographs of immunohistochemically stained paraffin sections of the biopsy. There was clear rhabdomyolysis shown by myofibre destruction and focal infiltration of leukocytes surrounding some of the damaged myofibres

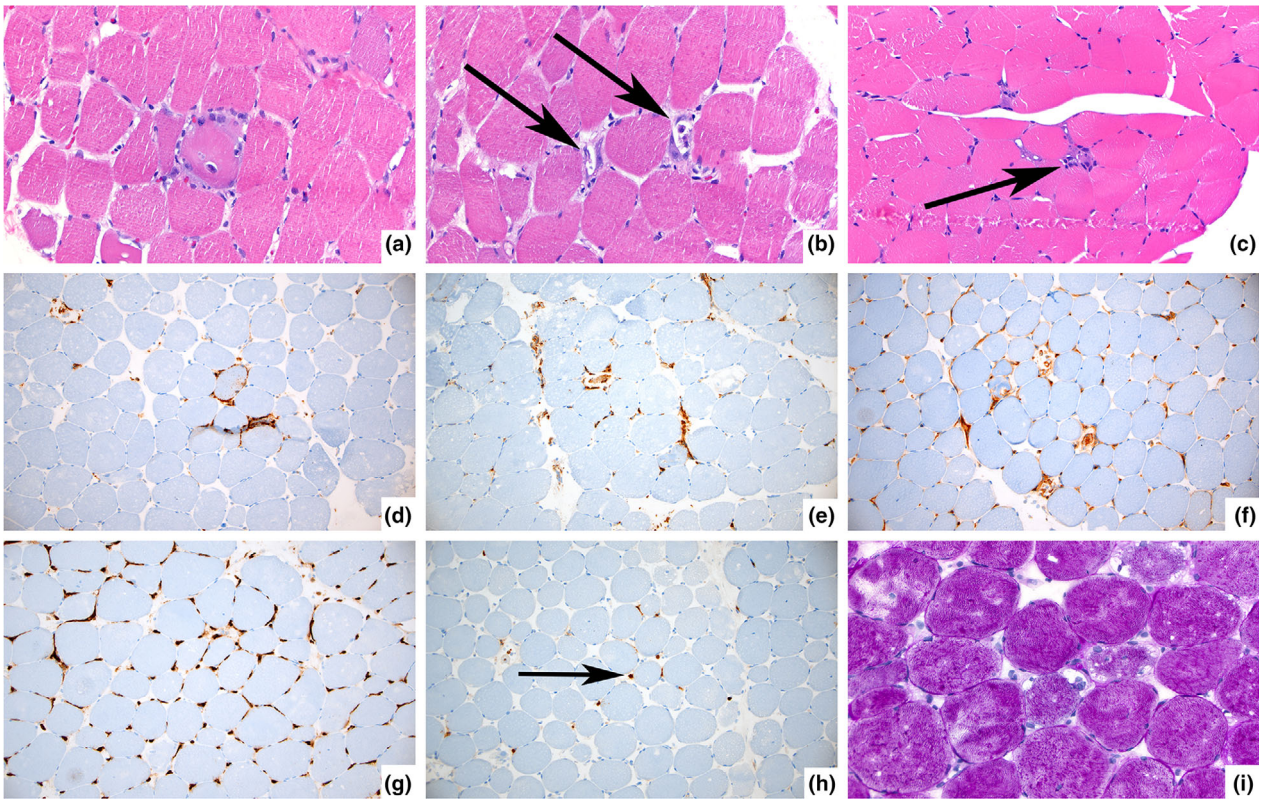

Figure 6. Focal inflammation in the muscle biopsy of a VLCADD patient with recurring rhabdomyolysis. Photomicrographs shown (all at 200× magnification) were raw images obtained from paraffin sections of a biopsy sample from Patient 3 (see Table 1); all sections counterstained with haematoxylin (blue-purple) and eosin (pink) (H&E). **(a–c)** Focal rhabdomyolysis with cell leukocyte infiltration, which are indicated by arrows. **(d, e)** Immunohistological staining of macrophages (brown peroxidase staining) around damaged muscles. **(f)** Perimysial expression of MHC I (brown peroxidase staining). **(g)** CD31 staining (brown peroxidase) for vascular endothelium. **(h)** CD3 staining (brown peroxidase and arrow) for infiltrating T cells. **(i)** Periodic acid–Schiff (PAS) intracellular staining of glycogen.

(Figure 6a–c; sections counterstained with haematoxylin [blue-purple] and eosin [pink] [H&E]). There was also high CD68 staining of tissue macrophages (brown peroxidase, Figure 6d and e) and perimysial staining of MHC I (brown peroxidase, Figure 6f). Staining for CD31 shows normal vascular endothelium (brown peroxidase, Figure 6g). There was notable CD3 staining for infiltrating T cells (brown peroxidase, Figure 6h and d–h counterstained with haematoxylin). As expected, the muscle fibres had high periodic acid–Schiff (PAS) staining of intracellular glycogen (Figure 6i).

Long-chain FFA-induced cytokine production in monocytes

Cytokines, such as IFNγ and the chemokines MCP1 and MIP-1β, are normally produced by activated monocyte/macrophage lineage cells.[39] Indeed, both cytokine and cytometric profiles of Patient 3 showed a dominant chronic activated state of monocytes. To further provide proof of principle that monocytes are sources of inflammatory cytokines in these patients, we examined the cytokine profiles of freshly isolated monocytes from healthy controls exposed to long-chain FFAs. Figure 7 shows that oleate (C18:1), palmitate (C16) and stearate (C18) induced statistically significant high levels of *in vitro* production by monocytes for 12 of 17 molecules examined ($P < 0.05$, ANOVA). IL-6, IL-8, MCP1 (CCL2) and MIP-1β (CCL4) showed highest levels of induction, between ~1.5 and ~8.0×10^4 pg mL^{-1}. IL-12p70, IL-17, IFNγ and TNFα were induced to ~600 pg mL^{-1}. There was negligible detection for IL-2, IL-4, IL-7, IL-13 and IL-15.

DISCUSSION

Very-long-chain acyl-CoA dehydrogenase deficiency patients share a common vulnerability to

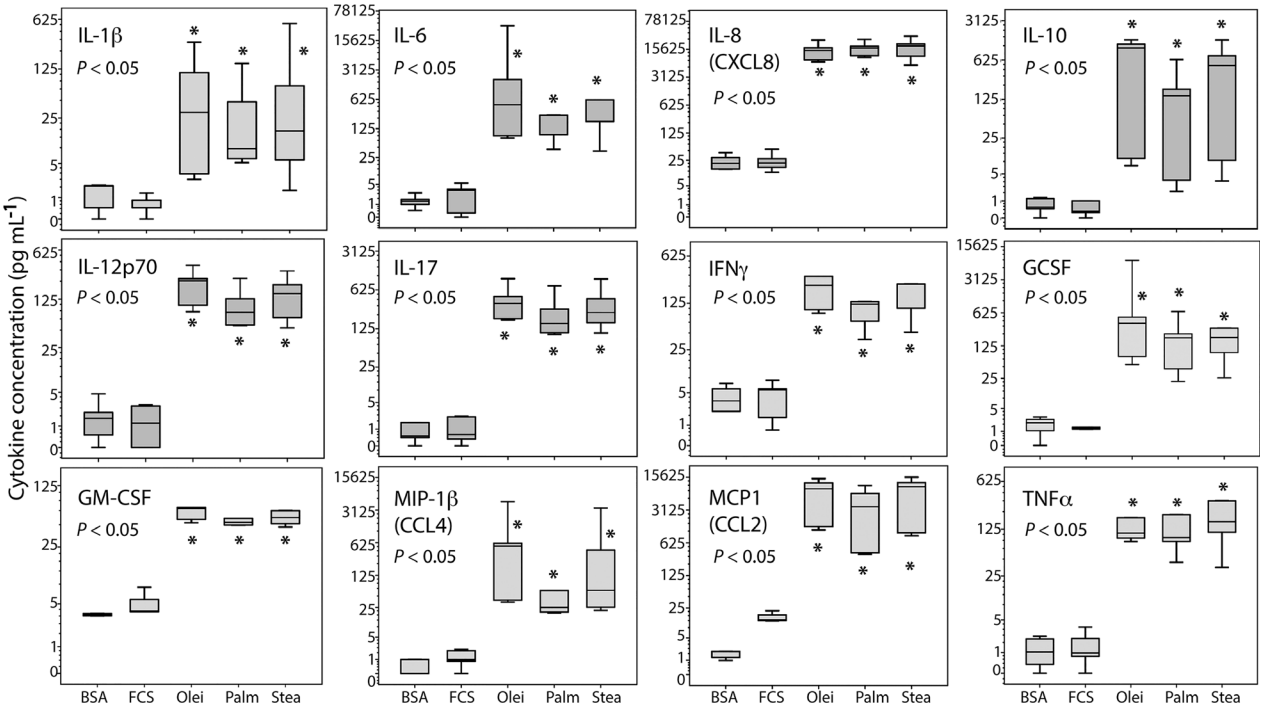

Figure 7. Production of inflammatory cytokines by exposed to long-chain FA. Freshly isolated monocytes from PBMC from healthy donors ($n = 5$ per group) were incubated with media containing either foetal calf serum [FCS] or bovine serum albumin [BSA], or 30 μM each of BSA-bound palmitate [Palm], oleate [Olei] or stearate [Stea]. After 24 h, the culture supernatant was examined for cytokine content by Luminex. Data shown are box/whisker/median plots constructed as in Figure 1. *P*-values indicated were determined by Kruskal–Wallis ANOVA. ** indicates *post hoc* comparison (Tukey, $P < 0.005$) of the FA-treated cultures and either of the media controls.

rhabdomyolysis in spite of varied *ACADVL* mutations.[1] While rhabdomyolysis in this disorder has been assumed to largely be the cause of abnormal muscle energetics, it occurs in patients treated with both standard of care and an anaplerotic agent (triheptanoin) currently in clinical trial[12] and in the absence of hypoglycaemia.[3] Additionally, rhabdomyolysis occurs with or without accompanying mutations of genes of other metabolic pathways and muscle structure/function.[15,40] Regardless of aetiology or precipitating event, rhabdomyolysis involves ongoing muscle degeneration/regeneration cascade(s), a physiologic stress that can elicit inflammatory activation.[40,41] Hence, we sought to examine whether or not VLCADD is a chronic inflammatory state.

Our data show that inflammation in VLCADD is pervasive. It involves both the upregulation of systemic cytokines and chemokines, and the dominance of pre-activated phenotypes of immune cells in blood. The cytokine/chemokine profiles of patients show upregulations of several molecules even at asymptomatic outpatient visits. Among these is IL-12p70, the quintessential inflammatory cytokine that is a master regulator of many downstream inflammatory cascades including those mediated through IFNγ, MCP1 (CCL2), MIP-1β (CCL4) and TNFα.[42–45] Hence, detection of these five molecules among VLCADD patients is biologically relevant. We also found IL-17, another inflammatory factor that is elaborated independent of IL-12p70/IFNγ, although it has been reported that functionality of IL-17-producing cells themselves could be influenced by IL-12p70.[34] In addition, we detected IL-6, IL-8 and GM-CSF, which are known to cross-regulate TNFα,[46–48] with GM-CSF also cross-regulating IL-17.[49,50] Such crosstalk between cytokines explains the preponderance of T cells, B cells and monocytes with high intracellular stores of IL-6, MIP-1β (CCL4), IFNγ and TNFα. Further, the high levels of expression of the active phosphorylated forms of the transcription factors p65-NFκB and STAT1 by the same immune cells provide experimental confidence for the idea of pervasive inflammation. STAT1 is a direct

regulator of IFNγ.[38] p65-NFκB is central to many inflammatory processes including its direct regulation of the expressions of IL-6, MIP-1β (CCL4), IFNγ and TNFα.[51,52] The expression of IL-17, MCP1 (CCL2), IL-8 (CXCL8) and IL-12p70, the other cytokines found in plasma, also requires occupancy of their respective gene promoters by p65-NFκB.[51–53] It should be noted, however, that in B cells the intracellular levels of the cytokines and the transcription factors in immune cells of patients were notable increased but they were not statistically significant. Nevertheless, detection of intracellular cytokines in B could still be additive to the overall inflammatory environment in VLCADD with the pre-activated T cells, NK cells and monocytes that are major sources of cytokines.

The cytokine/chemokine profiles of VLCADD patients were atypical and do not follow the TH paradigms.[54] Th1 (IFNγ) and Th2 cytokines (IL-5, IL-6, IL-13) were not inversely correlated as might be expected, but they were in fact directly correlated. There was no distinctive cytokine/chemokine correlation pattern(s). There was, however, high correlation of IFNγ with the rest of the cytokines, which is perhaps unsurprising since IFNγ is a general indicator of an ensuing inflammatory activation as documented for many chronic diseases including inflammatory myopathies.[35,55] Blood cytokine/chemokine levels were not correlated with phenomenally high levels of CPK, a classic clinical marker for VLCADD, rhabdomyolysis and various muscle pathologies.[56–58] Whereas CPK is central to the energy system of normal physiologic processes including in muscle and the immune system,[59–61] high serum CPK levels in the patients may just reflect ongoing muscle destruction[62] and metabolic hypercompensation because of energy deficit in VLCADD.[30,63] Whether or not CPK is regulated by or has cross-communication with inflammatory cytokines will have to be examined.

The case analysis of one VLCADD patient demonstrates the ontogeny of pervasive inflammation. Consistent with the cross-sectional analysis, the data show that there was already systemic cytokine upregulation in a sample taken at the age of 4 years when the CPK value was within normal range (average of ~97.5 μg L^{-1}) and prior to developing episodes of recurrent rhabdomyolysis. Such cytokine upregulations evolved into sustained high levels of cytokine that increased with rhabdomyolysis events. Unexpectedly, CPK in this case was correlated with IL-12p70 and IL-17, two cytokines that are classically linked to inflammatory activation.[34,64] Whether IL-12p70 and/or IL-17 regulates, or cross-regulates CPK is not yet clear, but it was remarkable that these two cytokines were highly correlated underscoring the notion of persistent inflammatory condition of this patient. IL-12p70 was also independently correlated with the macrophage chemokines MCP1 (CCL2) and MIP-1β (CCL4). The latter two, however, did not correlate with each other and that correlations among the other cytokines were as sporadic as in the cross section of VLCADD patients.

Histopathological evaluation of muscle biopsy of the patient with recurrent hospitalisations clearly show muscle destruction. There was also strong evidence of focal infiltration of $CD3^+$ T cells and $CD68^+$ macrophages surrounding damaged muscle. The observed expression of MHC I molecules in muscle, a tissue that is normally MHC I negative, is a strong bioindicator of immune and inflammatory activation explaining the T-cell/macrophage infiltration.[65] Whether or not muscle MHC I expression in VLCADD could be a form of a T-cell/macrophage-mediated autoimmune event analogous to recurrent myalgias in inflammatory myositis[66,67] will have to be examined. Plausibility of this suggestion might be inferred from the data showing identifiable and quantifiable activated phenotypes of T cells, NK cells and monocytes in blood of this patient. Most notable is the apparent dominance of CD14 and CD16 subsets of monocytes expressing high levels of IL-6, TNFα, MIP-1β (CCL4) and IFNγ, along with the transcriptional regulators p65-NFκB and STAT1.

In summary, VLCADD, as an inborn error of fatty acid oxidation, illustrates the biological theme about the adverse effects of alteration of metabolic pathways in health and disease.[68,69] In addition to direct mitochondrial dysfunction, the negative effects of VLCADD on cell/organ function including its direct link to the clinical manifestation of rhabdomyolysis are well characterised.[70–72] The present data support the idea that inflammation, both humoral and cellular, is a novel physiologic dimension of VLCADD. Despite the varied *ACADVL* mutations and the intrinsically small number of patients in this genetic disorder, the data show that inflammation occurs in both symptomatic and asymptomatic periods. Further studies are needed

to examine the exact driver(s) of quiescent and/or active hospitalisation-causing inflammation. Considering that immune homeostasis depends on a functional energetic system,[73,74] it is possible that inflammatory activation could be mediated by a maladaptive 'inside-out' signalling from the dysfunctional VLCAD-deficient mitochondria. Such signalling could be amplified by the accumulation of very-long-chain FFA (acylcarnitines) because of *ACADVL* mutation(s).[10] Indeed, the current data show direct activation of immune cells by such FFA consistent with well-documented effects of lipids on normal and pathologic immune and inflammatory cascades.[74,75] These cascades could be further fuelled by rhabdomyolysis, also a known tissue destructive process that ramps up cellular activation and production of inflammatory mediators. Whether and how alarmins and other biological sensors may be involved will have to be examined. Irrespective of cause, however, clinical diagnosis of rhabdomyolysis in the setting of many conditions including strenuous physical activity has indeed been associated with systemic cytokine upregulation.[40,76,77] Of particular interest is whether inflammation in VLCADD would be an autoinflammatory or autoimmune condition that might be a result of an innate and/or adaptive immunologic trigger.

A broader prospective study will be also needed to further examine whether there is a shared inflammatory fingerprint of VLCADD disease progression. Of biological interest is how innate and adaptive immune, or perhaps autoimmune or autoinflammatory, cascades operate in the setting of VLCADD. The data suggest that these overlapping arms of immune and inflammatory responses are operational. Dissection of these processes will be key to understanding whether favorable and less favorable outcomes of dietary treatment regimens in VLCADD[63] may be linked to distinct inflammatory or autoinflammatory, or autoimmune fingerprint(s). Our case analysis of a VLCADD patient with recurrent rhabdomyolysis showing the ontogeny of inflammation illustrates an individualised assessment of progression of inflammation. We suggest that an individualised approach relative to inflammation will provide the opportunity for rational adoption of already existing anti-inflammatory interventions for VLCADD including anti-cytokine therapy, for example IL-6/TNF-blockers,[78,79] and/or small molecule inhibitors of inflammatory processes, for example NFκB inhibitors.[80]

METHODS

Human subjects and collection of biospecimens

Human research protocols were in accordance with the principles of the Declaration of Helsinki and were approved by institutional review boards of the University of Pittsburgh Human Research Protection Office. VLCADD patients (n = 18) were identified from the Medical Genetics Clinic of Children's Hospital of Pittsburgh of the University of Pittsburgh Medical Center (UPMC) and underwent written informed consent/assent process. Healthy controls also provided written consent/assent from our ongoing community recruitment. For research purposes, blood samples collected from patients were separate from those designated for clinical diagnoses. In accordance with the approved research protocol, such research samples were collected during outpatient and/or at hospital admission when phlebotomy was a necessary part of the delivery of care during the visit and without impeding medical procedures especially during admission. With this procedural guideline, we collected more outpatient samples than for inpatient cases. For healthy controls (n = 9), samples were chosen such that they were within the age range of VLCADD patients. They had no diagnoses of inflammatory and/or chronic diseases and had no reported acute symptoms at the time of blood sampling. For healthy young controls aged $\leq$ 10 years, we collected excess de-identified samples from Children's Hospital of Pittsburgh Clinical Laboratory Services that processes blood samples from routine paediatric visits. Clinical information, including results of genetic screening, CPK values and biopsy reports, was abstracted from the medical record.

Humoral profiling and flow cytometry

We employed validated procedures for the separation of plasma and peripheral blood mononuclear cells (PBMC) from whole blood and their cryostorage.[81,82] Analysis of plasma humoral factors was performed according to our standardised multiplex protocols.[81–85] Cytokine/chemokine profiling utilised a multiplex kit for 17 traditional inflammatory mediators (Bio-Plex Pro™ kit, Bio-Rad). Using previously described procedures,[81] each multiplex experiment included an establishment of standard curves for each assay plate that were then used in integral equations to determine the final standard curve upon which concentrations of molecules were calculated.

Cell surface and intracellular immunostaining, controls for all multicolour flow cytometry experiments, live/dead cell discrimination, instrument calibration and offline signal compensation and analyses of cell populations were carried out according to our validated protocols.[81,82,86] Raw cytometry data were collected by a Fortessa cytometer (BD), and off-line cell subset analyses were performed with FlowJo software (FlowJo LLC). Analytical discrimination and quantification of each cell surface and intracellular molecule followed the Overton subtraction method.[87] We examined phenotypes of T cells, B cells, NK cells and monocytes. From the forward (FSC) and side scatter (SSC) profiles, the smaller low FSC/SSC lymphoid cluster of cells

contained T cells identified by fluorochrome-conjugated antibodies to TCRαβ (T10B9, BD) and CD4 (OKT4, BD) or CD8 (RPA-T8, BioLegend), as well as B cells identified by CD19 (SJ25-C1, BD). Monocytes were identified from the higher FSC/SSC and by staining for CD14 (M5E2, BD) and/or CD16 (3G8, BD). In addition, NK cells were identified from the low FCS/SSC lymphoid cluster of cells as $TCR\alpha\beta^{-}CD19^{-}CD14^{-}$ cells that co-stained for CD16 and CD56 (B149, BD). For each cell subset, intracellular immunostaining was also performed for IFNγ (4S.B3, BD), IL-6 (AS12, BD), MCP1 (5D3-F7, BD), MIP-1β (D21-1351, BD) and TNFα (Mab11, BD), as well as for the phosphorylated forms of STAT1 (phospho-Y701; 4a, BD) and p65 subunit of NFκB (phospho-S529; K10-895.12.50, BD).

Cytokine production bioassays

Bioassays focused on monocytes, the first line of immune and inflammatory activation in muscle injury,[88] known for their high sensitivity to FFA.[26,89] We used healthy blood samples in order to provide proof of concept for the stimulatory activity of long-chain fatty acids even in an asymptomatic state. Monocytes were isolated by standard plastic adherence procedure,[90] and purity (> 95%) was verified by cytometry. Cultures in the presence or absence of 30 μM oleate, stearate or palmitate (albumin-bound) in lipid-free culture media (Sigma-Aldrich) were performed using our previously validated procedure.[91] Supernatants were collected after 24 h, and cytokine content was measured by multiplex Luminex assay.

Histological evaluation of muscle biopsy

Histological evaluation of muscle biopsy for a hard-to-treat patient (referred to as Patient 3, see Table 1) was performed by conventional light microscopy. Specimens were processed according to established clinical diagnostic histopathological procedures performed by CLIA (Clinical Laboratory Improvement Amendments)-accredited Pathology Laboratory of UPMC Clinical Laboratory Services. Frozen sections of biopsy samples were subjected to routine H&E and immunoperoxidase techniques employing antibodies specific for tissue macrophages (CD68, clone PGM1, Roche), endothelial cells (CD31, clone JC70A, Dako Agilent) and MHC I (anti-HLA-A, clone EP1395Y, Abcam). Immunostaining included isotype controls. PAS staining was also carried out. All immunohistochemically stained slides were counterstained with haematoxylin. Slide preparations utilised the Benchmark ULTRA System (Roche Diagnostics). Histological evaluation was performed by a paediatric pathologist (author MRM) using a bright-field Olympus® BX51 microscope, and images were obtained with a Jenoptik® Gryphax camera. Raw images were used without manipulation.

Statistical analyses

Cytokine data were analysed by Kruskal–Wallis ANOVA using SPSS Ver 24 software (IBM Corporation). *Post hoc* comparisons between any two groups employed the Tukey statistic. Correlation matrices were constructed from Spearman correlation coefficients, determined using Prism 8 (GraphPad Software Inc.), in order to examine whether CPK values were associated with systemic cytokine/chemokine levels. For cytometry data analysis, one-way ANOVA using Brown–Forsythe and Welch tests were employed since equal variance cannot be assumed because different cell types have varying mechanisms that regulate expression of the molecules of interest. Cytometry data analyses were also performed using Prism 8. In all statistical analyses, *P*-values < 0.05 were considered significant.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Abbe N Vallejo: Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Project administration; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing. **Henry J Mroczkowski:** Formal analysis; Investigation; Writing-original draft; Writing-review & editing. **Joshua J Michel:** Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Writing-original draft; Writing-review & editing. **Michael Woolford:** Data curation; Formal analysis; Investigation; Software; Writing-review & editing. **Harry C Blair:** Methodology; Resources; Supervision; Writing-review & editing. **Patricia Griffin:** Investigation; Methodology; Resources; Visualization; Writing-review & editing. **Elizabeth McCracken:** Data curation; Investigation; Resources; Validation; Writing-review & editing. **Stephanie J Mihalik:** Data curation; Formal analysis; Investigation; Methodology; Validation; Writing-review & editing. **Miguel Reyes-Mugica:** Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Visualization; Writing-review & editing. **Jerry Vockley:** Conceptualization; Data curation; Funding acquisition; Methodology; Project administration; Resources; Supervision; Validation; Writing-review & editing.

ETHICAL APPROVAL

Human research protocols were in accordance with the principles of the Declaration of Helsinki and were approved

by institutional review boards of the University of Pittsburgh Human Research Protection Office.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.