Original Research | *Highlight article*

Vitamin B6 deficiency in new born rats affects hepatic cardiolipin composition and oxidative phosphorylation

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Impact statement

Vitamin B6 (VitB6) is an active co-enzyme for more than 150 enzymes and is required for a great diversity of biosynthesis and metabolic reactions. There is an increased need for VitB6 during pregnancy and sufficient supply of VitB6 is crucial for the prevention of cleft palate and neural tube defects. We show that liver mitochondria from new-born rats with pre-term VitB6 deficiency respond with substantial alterations in cardiolipin (CL) composition and in the amount of oxidized CL species. These changes are associated with a decrease in the efficiency of oxidative phosphorylation. The results of this study support the significance of sufficient supply of VitB6 during pregnancy (and periconceptional) for diminishing the number of early abortions and minimizing malformation. The established link between VitB6 deficiency, CL composition, and mitochondrial respiration/energy production provides mechanistic insight as to how the VitB6 deficiency translates into the known pathophysiological and clinically relevant conditions.

Abstract

Vitamin B6 deficiency during pregnancy translates into a severe vitamin B6 deficiency (plasma levels decreased by 97%) in new-born rats. Further, hallmarks are increased (+89%) concentrations of homocysteine, gross changes in gene methylation and expression, and metabolic alterations including lipid metabolism. This study focuses on determining the effects of vitamin B6-deficiency on cardiolipin composition and oxidative phosphorylation in liver. For this purpose, hepatic cardiolipin composition was analyzed by means of LC/MS/MS, and mitochondrial oxygen consumption was determined by using a Clark-type electrode in a rat model of vitamin B6 deficiency. Liver mitochondria from new-born rats with pre-term vitamin B6 deficiency responded with substantial alterations in cardiolipin composition that include the following changes in the amounts of cardiolipin incorporated fatty acids: increase in C16, decrease in C18, decrease in saturated fatty acid, as well as increase in amount of oxidized cardiolipin species. These changes were accompanied by significantly decreased capacity of oxidative phosphorylation. In conclusion, vitamin B6 deficiency in new born rats induces massive alterations of cardiolipin composition and function of liver mitochondria. These findings support the importance of sufficient periconceptional supply of vitamin B6 to prevent vitamin B6 deficiency.

Keywords: Vitamin B6 deficiency, mitochondrial respiration, cardiolipin composition

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Introduction

Vitamin B6 (VitB6) is comprised of the pyridine derivatives, pyridoxol, pyridoxamine, and pyridoxal.[1] Pyridoxal phosphate functions as an active co-enzyme for more than 150 enzymes that catalyze several types of reactions such as transamination, decarboxylation, and dehydration. Thus, VitB6 is required for biosynthesis and metabolism of proteins, carbohydrates, lipids, and a great diversity of further relevant bioactive substances.[1–4] Plasma reference values for humans range from 23 to 223 nmol/l.[5]

Because of its anabolic activity, there is an increased need for VitB6 during pregnancy which amounts to 1.9 mg/d. It has been demonstrated that sufficient supply of VitB6 is crucial for the prevention of cleft palate and

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neural tube defects.[6–9] Moreover, since VitB6 facilitates the degradation of homocysteine (Hcy) from the methylation cycle to cystathionine by cystathionine synthase, deficiency in VitB6 leads to hyperhomocysteinemia (HHcy).[10] The latter has been shown to cause serious malformations.[11–18]

As comprehensively reviewed recently,[19] VitB6 is essential for mitochondrial and, therefore, the cellular energy metabolism. It is a cofactor of transaminases that catalyze the degradation of amino acids within the mitochondrial urea cycle. It supports the activity of the malate-aspartate shuttle that facilitates the oxidation of NADH via glycolysis. It further links amino acid metabolism to energy production by the formation of pyruvate from alanine or of α-ketoglutarate from glutamate as substrates for glycolysis or mitochondrial TCA and oxidative phosphorylation.

Furthermore, there is evidence that VitB6 deficiency is associated with alterations in lipid and phospholipid metabolism.[4,20–24] In particular, insufficient synthesis of phosphatidylcholine (PC) contributes to hepatic impairment by triacylglyceride (TAG) accumulation via decreased secretion of lipoproteins (VLDL).[25,26] VitB6-dependent restriction of liver function is of central impact in the early phase of development. Therefore, we placed the liver in the center of our interest.

Although the requirement of VitB6 for a proper intact cellular energy metabolism has been already demonstrated, there are no data available regarding the direct effects of VitB6 deficiency on oxidative phosphorylation. To elucidate this particular role of VitB6, mitochondria isolated from livers of VitB6 deficient and control animals were used in the experiments.

In the interrelationship between VitB6 deficiency and oxidative phosphorylation, the mitochondrial phospholipid, cardiolipin (CL), is of particular impact. The anionic phospholipid CL contains four fatty acyl chains organized in two phosphatidyl moieties that are bridged by glycerol.[27] Different assemblies of fatty acyl chain residues offer a high variety of molecular CL species. Thereby, the actual set of fatty acids present in CL has been found to be organism and tissue specific,[27] and is subject of change in response to physiological stimuli or under pathophysiological conditions.[28–30] It has been demonstrated that the fatty acid composition of CL can affect mitochondrial structure and biogenesis,[28,30,31] the activity and organization of mitochondrial membrane proteins and respiratory chain complexes,[32–34] and the efficiency of oxidative phosphorylation.[35–38]

In this study, we analyzed the composition of molecular CL species by LC/MS/MS technique and measured oxidative phosphorylation by quantifying oxygen consumption in isolated liver mitochondria of new born VitB6 deficient rats. We found substantial alterations in CL composition and impaired oxidative phosphorylation in liver mitochondria of VitB6 deficient new born rats.

Materials and methods

Animals

The animal experiments were approved by the LALLF Mecklenburg-Vorpommern (Az 7221.3–1.1–049/13). Twelve female primiparous inbred rats (LEW.1W) were used. Breeding rats had *ad libitum* access to pelleted food and acidulated drinking water. Each breeding pair was kept in a separate K3 cage. Ambient humidity was 50% to 60%. The animals were adapted to the light regimen (12-h light from 6:00 a.m. to 6:00 p.m.) for at least 14 days. During the experimental period, two females at a time were brought to a buck for copulation between 4:00 p.m. and 6:00 a.m., and were separated for treatment following a positive vaginal smear (proof of sperm). The day after successful mating was considered the start of the trial (day 1 post conceptionem (p.c.)). The gravid animals were divided into treatment groups: group C (control) and group B (VitB6 deficiency).

The control group (group C) received control food (ssniff, VE 100–400) during the whole experimental period, whereas the deficiency group B was fed control food from day 1 to day 17 of pregnancy. A VitB6-free food (ssniff, food number SO752-E010, Soest, Germany) was administered to pregnant female rats (group B) from day 18 p.c. until day 14 p.p. to induce functional VitB6 deficiency (hypovitaminosis). For analyses, each group consisted of six new-born, male rats.

On day 14 post-partum (p.p.) (group B1/C1) and day 28 p.p. (group B2/C2), new-born rats were euthanized and liver samples were taken, immediately frozen in liquid nitrogen, and stored at −196°C until preparation of mitochondria or CL analysis. The experimental design is illustrated in Figure 1. Mean body weight was 42.1 ± 1.7 (day 14) and 51.8 ± 2.3 (day 28); liver weights were 1.49 ± 0.08 (day 14) and 1.88 ± 0.07 (day 28). At these time points, groups C and B were not different.

Enzymatic activity assay

The enzymatic activity of aspartate-aminotransferase (ASAT) in plasma samples was determined using the Fluitest® AST/GOT (Analyticon-Biotechnologies AG, Lichtenfels, Germany) following the recommended protocol but without the addition of pyridoxal phosphate to the reaction mixture.

Quantification of 5-hydroxytryptophan plasma concentrations

Quantification of 5-hydroxytryptophan in rat EDTA-blood plasma was performed by a liquid chromatography tandem mass spectrometry (LC-MS/MS)-based analysis modified from Fuertig *et al*.[39]

Sample preparation. Plasma (10 µL) was mixed with 10 µL of internal standard and with 10 µL of mobile phase (0.4% formic acid, 1% acetonitrile in water). Proteins were precipitated by adding 150 µL of ice-cold methanol followed by overnight incubation at −20°C. Then, samples were centrifuged at 0°C and 18,000×*g* for 15 min. Supernatants were removed, pellets evaporated to dryness at 30 °C in vacuum, and stored until use at −20°C. For analysis, precipitated material was reconstituted in 100 µL of acidified mobile phase (40°C, 1 h) and cleared by centrifugation at 4°C and 18,000×*g* for 5 min.

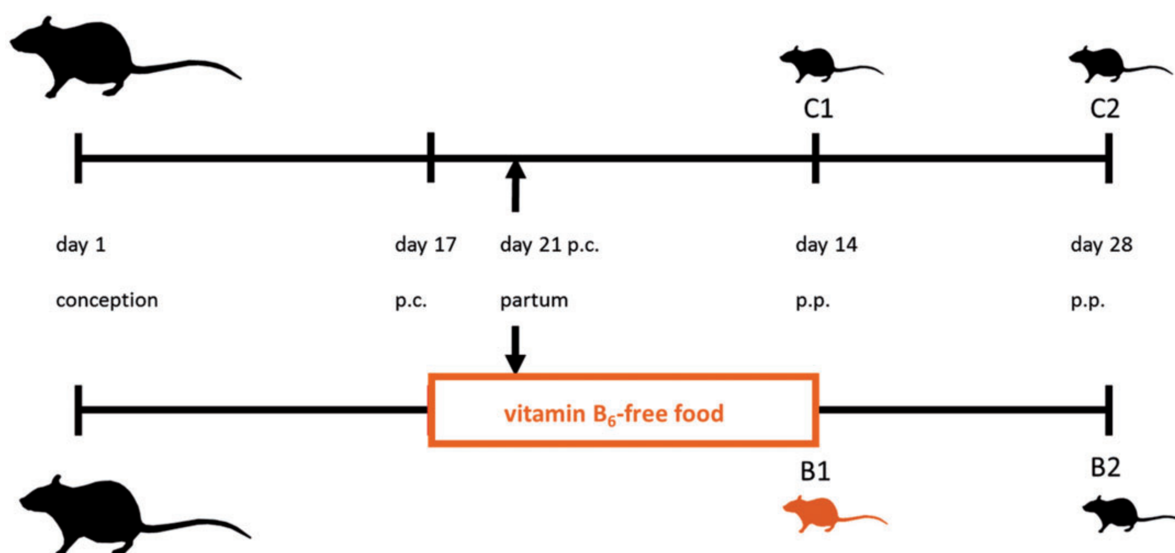

Figure 1. Experimental design. In the control group (C), gravid rats were fed normal diet throughout the experiment from day 1 (conception) until day 28. In the VitB6-deficient group (B), gravid rats were fed VitB6-free diet from day 17 post conception (p.c.) until day 14 post-partum (p.p), where after normal feeding was resumed for further 14 days. For read-out, neonatal rats from both groups were analyzed at days 14 p.c. (C1, B1) and 28 p.c. (C2, B2). (A color version of this figure is available in the online journal.)

LC-MS/MS

The measurements were performed on an AB Sciex 5500 QTrap^{TM} mass spectrometer (AB SCIEX, Darmstadt, Germany) with electrospray ionization in positive mode combined with a HPLC system (Agilent 1260 Infinity Binary LC, Santa Clara, USA). Twenty microliters of the samples were injected and separated by using a VisionHT C18 column (100 × 2.1 mm, particle size 3 µm; Grace, Maryland, USA). In order to prevent contamination of the analytical column, a pre-column (VisionHT C18, Guard 5 × 2 mm; Grace, Maryland, USA) containing the same column material was used. Separation was performed at 15°C with the flow rate of 0.4 mL/min and 11 min total run time using the following gradients of solution A (water + 0.1% FA + 0.01% TFA) and solution B (methanol + 0.1% FA + 0.01% TFA): 0–2.8 min, 97% A, 3% B; 2.8–3.3 min, 70% A, 30% B; 3.3–4.4, 40% A, 60% B; 4.5–5.0 min, 40% A, 60% B; 5.0–5.5 min, 5% A, 95% B; 5.5–6.9 min, 5% A, 95% B; 6.9–7.0 min, 97% A, 3% B; 7.0–11.0 min, 97% A, 3% B.

The eluate collected between 0.5 and 9 min was introduced into the mass spectrometer and analyzed in the MRM mode. The ion spray voltage was set at 4000 V, the curtain gas pressure was 40.0 psi, and the ion source temperature was set to 550°C.

For the quantification of the 5-hydroxytryptophan, a deuterated tryptophan, D5-tryptophan, was used as internal standard. The evaluation of the data, including peak integration and calculation of concentrations, was performed with Analyst Software (Version 1.5.1, AB Sciex, Darmstadt, Germany).

CL analysis

Extraction of lipids and CL analysis was performed as recently described.[40] Similar to our previous study, we analyzed (C18:2)3-CL as the representation of mono-lyso-CL (MLCL) and (C18:2)3-hydroxy-linolyl CL as indication for CL oxidation.

Determination of cellular fatty acid distribution

Liver fatty acid patterns were established by the analysis of fatty acids as their methyl esters, basically following the protocol of Nourooz-Zadeh *et al.*[41] with modifications described recently.[31]

Oxygen consumption

For the evaluation of mitochondrial oxygen consumption, the liver was cut into pieces using a sharp scissor with isolation buffer (250 mM D-Sucrose, 1 mM EGTA, 10 mM Tris, 0.1% BSA (fatty acid free), pH = 7.4). The liver mixture was homogenized using a Potter-Elvehjem homogenizer (500 r/min) after the addition of trypsin. The reaction was stopped by the addition of soybean trypsin inhibitor (Thermo Fisher Scientific, Waltham, USA). Subsequently, the following centrifugation steps were performed: 15 min at 600×*g*, supernatant 9000×*g* for 15 min, resuspension of the pellet in isolation buffer and repeated washing (9000×*g*, 15 min, three times). The final pellet was resuspended in incubation medium containing 10 mM KH_2PO_4, 0.5 mM EGTA, 60 mM KCl, 60 mM Tris, 110 mM mannitol, and 1 mM free Mg^{2+} (pH 7.4); 2 mL (0.3 mg/mL) of mitochondrial suspension were transferred into the incubation chamber for the analysis of oxygen consumption. Mitochondrial respiration was measured using a Mitocell MT200/oxygraph model 782 (Strathkelvin Instruments) at 30°C. The experimental approach was calibrated using the oxygen content of air-saturated medium of 435 ng atoms/mL at 30°C. Respiration was evaluated as follows: first, the substrate couple 5 mM glutamate/5 mM malate was added in order to adjust basal NADH-dependent respiration. Subsequently, active respiration was stimulated by adding 500 µM ADP

(glutamate/malate). Finally, maximal respiration was initiated by adding 10 mM succinate to the reaction mixture including FADH-dependent respiration (max). Each condition was recorded for at least 3 min to achieve stable rates of respiration before the addition of next substances. The respiration rates were related to protein content in each sample.

Immunoblot analysis

Tissue samples (0.1 g) were homogenized in 750 μL cold (4°C) lysis buffer (20 mM Tris-HCl pH 7.4, 50 μM ethylenediamine-tetraacetic acid [EDTA], 137 mM sodium chloride, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 1% n-dodecyl-β-D-maltoside) by using the mechanical homogenizer OMNI μH (Süd-Laborbedarf GmbH, Gauting, Germany). After incubating on ice for 30 min, the homogenates were centrifuged for 30 min at 10,000×g at 4°C to remove any particulate material. Protein content in the supernatant was determined according to Bradford[42] and were stored at −80°C until use.

Amounts of cytochrome C (CYC) protein were determined by immunoblot analysis as described previously.[43] Twenty micrograms of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. Membranes were incubated with anti-CYC rabbit polyclonal antibody (#4272, Cell Signaling Technology, Frankfurt a. M., Germany) followed by incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Cell Signaling Technology). Thermo Scientific™ SuperSignal™ West Dura Chemilumineszenz-Substrat (Thermo Fisher Scientific) was used for detection and the AlphaEaseFC software (AlphaImager System, Alpha Innotech, CA, USA) was used for quantification.

Statistical analysis

The data were statistically evaluated using the software GraphPad Prism, Version 7 (GraphPad Inc., La Jolla, USA). For all four groups (C1, C2, B1, B2) VitB6 markers (Figure 2) and individual CL-species were compared with ANOVA for repeated measures followed by post hoc pairwise comparisons (Tukey's multiple comparisons test). P values of the post hoc test lower than 0.05 were considered to indicate significant differences.

In the graphs, quantitative data are shown as informative boxplots with medians, quartiles, and an interquartile range (IQR) $\pm 1.5 \times$ IQR with outliers indicated (Tukey method). In Table 1, data are summarized as mean ± SD.

Results

VitB6 deficiency in the experimental groups

VitB6 deficiency in new-born rats has been confirmed by three different methods. First, the enzymatic activity of the VitB6-dependent plasmatic ASAT was measured. As expected, feeding gravid rats a VitB6-freed diet over a period of 18 days (from day 17 p.c. to day 14 p.p.) led to substantially reduced ASAT enzymatic activity in the plasma of offspring (B1: 423.8 nkat/l vs. C1:592.2 nkat/l; $P < 0.01$) (Figure 2(a)). The subsequent feeding with normal diet over a period of 14 days caused a complete recovery of ASAT enzymatic activity with no obvious differences between the groups C2 and B2 (Figure 2(a)).

Second, analysis of plasma 5-hydroxytryptophan levels by mass spectrometry revealed significantly reduced plasma concentrations in the B1 group (1023 nM vs. C1: 2190 nM, $P < 0.05$), further confirming a present VitB6 deficiency (Figure 2(b)). Third, quantification of hepatic CYC by immunoblot analysis demonstrated significantly lower amounts of this protein in the B1 group (Figure 2(c)). As observed with ASAT, 5-HT and CYC returned to normal upon resumption of normal diet (groups C2 and B2).

VitB6 deficiency alters CL-composition

VitB6-deficiency, although not altering the total amount of hepatic CL (Figure 4(j)), provoked highly significant changes in the CL composition of liver mitochondria (C1 vs. B1; Table 1). Of the 19 different CL species investigated, 13 were significantly altered in response to VitB6 deficiency, and two more species showed a tendency towards increased levels ($P = 0.08$ or $P = 0.06$), respectively. Specifically, a >1.5-fold increase in the amounts of the CL-species $(C18{:}1)_2/(C16{:}0)_2$ ($P < 0.001$), $(C18{:}1)_2/C18{:}2/C16{:}0$ ($P < 0.0001$), $(C18{:}1)_3/C16{:}0$ ($P < 0.0001$), $(C18{:}1)_2/(C18{:}3)_2$ ($P < 0.0001$), and $C22{:}6/(C18{:}2)_3$ ($P < 0.01$) was observed in the livers of B1 rats when compared to the livers of C1 control animals. In contrast, amounts of $(C18{:}2)_4$ were lower in the livers of B1 rats compared to C1 (B1: 25.2 ± 1.1 vs. C1: 31.7 ± 0.6; $P < 0.0001$) (Table 1, Figure 3(a) to (f)).

In addition, VitB6 deficiency caused an increase in the amounts of oxidized CL (C1: 1.11% ± 0.07% vs. B1: 1.29% ± 0.12%; $P = 0.01$) (Table 1, Figure 4(l)).

When normal feeding was resumed, CL composition approached that of controls. Only a few differences in CL-species remained after feeding food with normal VitB6 content (C2 vs. B2). Consequently, the profound differences that exist between the CL-compositions of the VitB6-deficient B1 group and controls (C1) became evident between B1 and the VitB6-restituted B2 group (Table 1).

The analyses performed in this study not only identified VitB6-dependent alterations of hepatic CL composition, but in addition revealed the existence of substantial age-dependent effects on the abundance of several CL-species (C1 vs. C2) (Table 1). Elder control animals (C2) showed less oxidized CL (C2: 0.77 ± 0.07% vs. C1: 1.11 ± 0.07%; $P < 0.005$) (Figure 4(l)).

The abundance of individual fatty acids present in the CL was different between the VitB6-deficient rats (B1) and controls (C1). As shown in Figure 4(a) to (f), VitB6-deficiency led to increased amounts of C18:1 (1.15fold; $P < 0.0001$), C18:3 (2.5fold; $P < 0.0001$), C16:0, (1.5fold; $P < 0.0001$), and C20:4 (1.1fold; $P = 0.026$) FAs, whereas that of C18:2 appeared to be slightly, but significantly decreased (−5.3% $P < 0.0001$). Overall, these changes in CL-composition are reflected in an increase in the total amount of C16 (1.5fold; $P < 0.0001$) and a slight decrease in the total amount of C18-FAs ($P < 0.001$) (Figure 4(g) and (h)). Furthermore, the degree of saturation of the FAs

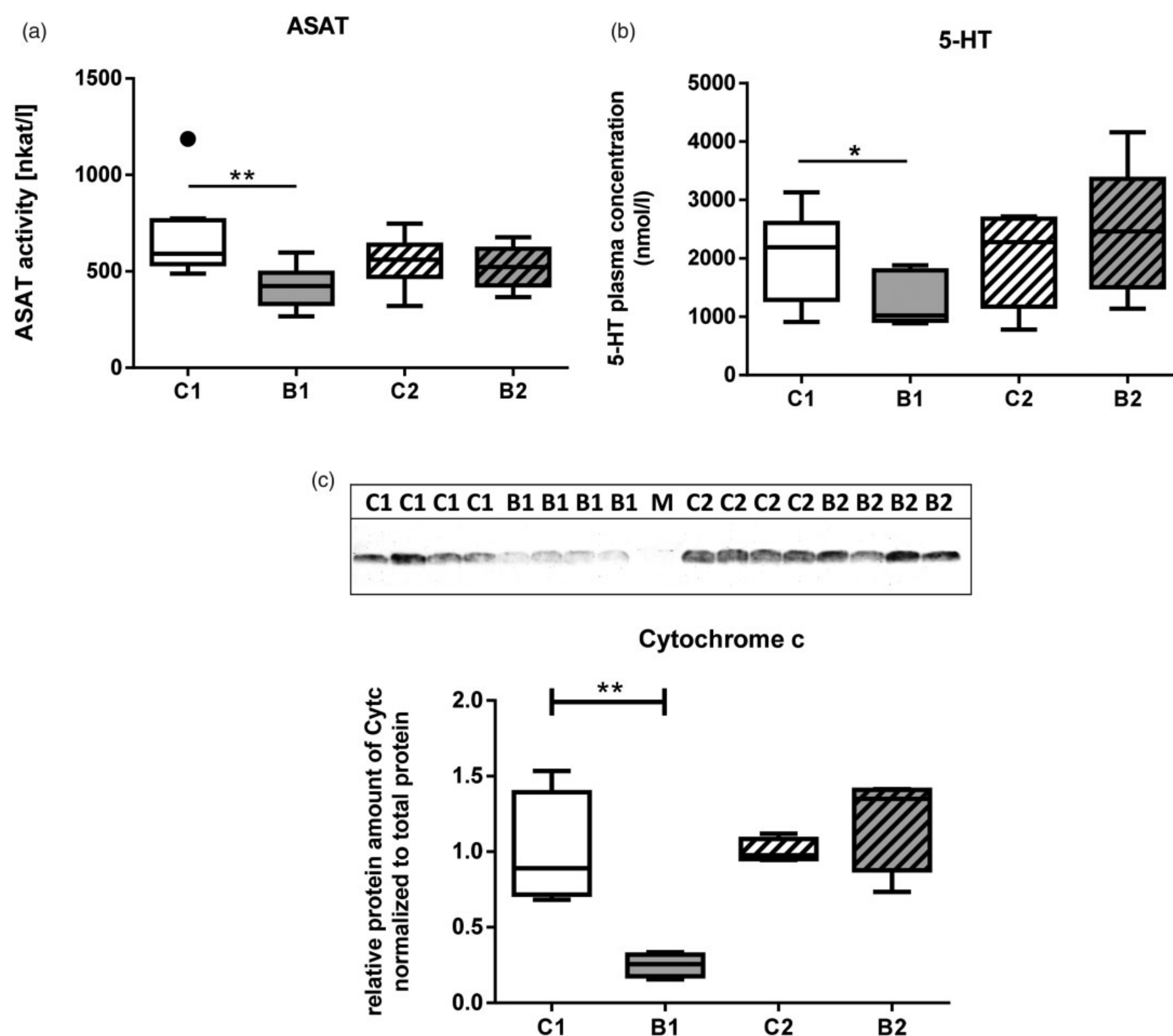

Figure 2. Verification of VitB6 deficiency. The presence of a functional VitB6-deficiency in neonatal rats of the experimental group B1 could be verified by (a) decreased plasmatic activity of aspartate-aminotransferase (ASAT), (b) lower plasma levels of 5-hydroxytryptophan (5-HT), and (c) decreased hepatic amounts of cytochrome C (CYC) as determined by immunoblot analysis. Representative immunoblot is shown on top of the quantitative data. Data are given as boxplots with medians, quartiles, and an interquartile range (IQR) $\pm$ 1.5 $\times$ IQR with outliers as indicated (Tukey method) ($n = 6$; *$P < 0.05$, **$P < 0.01$).

present in CL is significantly ($P < 0.0001$) reduced in the VitB6-deficient group, B1 (Figure 4(k)).

In contrast to the effects of VitB6-deficiency on CL-composition, the effects on the abundance of cellular fatty acids were much less pronounced, with only the cellular content of alpha-C18:3, C20:3, and C22:6 found to be slightly increased in the B1 group when compared to C1 (Table 2).

VitB6 deficiency affects mitochondrial respiration

In parallel to the observed changes in CL-composition, VitB6 deficiency provoked significant changes in the mitochondrial respiration. Liver mitochondria from VitB6-deficient rats showed decreased respiratory control ratios (RCR) (C1: 4.7 vs. B1: 3.2; $P < 0.01$) (Figure 5(a)). This change in RCR might be due to increased state IV respiration (C1: 15.33 vs. B1: 18.32 nmol O_2/min/mg mitochondrial protein), whereas state III respiration was not affected by VitB6-deficiency (not shown). Maximum (uncoupled) respiration was also not different between the experimental groups (Figure 5(b)). This finding demonstrates that ADP-dependent respiration was reduced in mitochondria of VitB6-deficient animals indicating decreased capacity of oxidative phosphorylation.

Discussion

It has been shown previously that induction of vitamin deficiencies/hypovitaminosis (VitB6 and folic acid) in rats due to restricted feeding decreased the VitB6 blood plasma levels by 97%, while that of Hcy increased by 89% after a period of 14 days.[18]

The development of a functional VitB6-deficiency under the conditions applied in this study in the B1 group could be verified by three different techniques.

The activity of transaminases like ASAT is substantially decreased under conditions of VitB6 deficiency.[44] Therefore, reduction in ASAT activity observed in the B1 group confirmed an existing functional deficiency of VitB6 in this group. The reduced hepatic levels of CYC reflect the dependency of heme synthesis on VitB6[44] and, thus, further

Table 1 Cardiolipin composition in livers of new-born rats.

	Cardiolipin species	C1 mean ± SD (% of total CL)	B1 mean ± SD (% of total CL)	C2 mean ± SD (% of total CL)	B2 mean ± SD (% of total CL)	ANOVA *P*-value C1 vs. B1	ANOVA *P*-value C2 vs. B2	ANOVA *P*-value C1 vs. C2	ANOVA *P*-value B1 vs. B2
1	(C18:2)$_4$	31.66 ± 0.58	25.24 ± 1.07	41.89 ± 1.97	40.71 ± 1.88	**<0.0001**	n.s.	**<0.0001**	**<0.0001**
2	(C18:2)$_3$/C18:1	28.54 ± 0.28	28.73 ± 0.60	26.23 ± 0.48	26.89 ± 0.35	n.s.	**0.02**	**<0.0001**	**<0.0001**
3	(C18:2)$_2$/(C18:1)$_2$	10.84 ± 0.22	13.27 ± 0.49	8.28 ± 0.53	8.71 ± 0.60	**<0.0001**	n.s.	**<0.0001**	**<0.0001**
4	C18:2/C18:1/(C16:0)$_2$	0.62 ± 0.14	0.77 ± 0.13	0.49 ± 0.09	0.46 ± 0.07	0.08	n.s.	n.s.	**<0.0001**
5	(C18:1)$_2$/(C16:0)$_2$	1.17 ± 0.25	1.89 ± 0.17	1.17 ± 0.19	1.21 ± 0.35	**<0.0001**	n.s.	n.s.	**<0.001**
6	(C18:2)$_3$/C16:1	1.16 ± 0.12	1.36 ± 0.20	1.64 ± 0.43	1.63 ± 0.34	0.06	n.s.	**0.02**	n.s.
7	(C18:2)$_2$/C18:1/C16:1 or (C18:2)$_3$/C16:0	1.28 ± 0.07	1.74 ± 0.25	1.26 ± 0.22	1.32 ± 0.18	**<0.001**	n.s.	n.s.	**0.01**
8	(C18:1)$_2$/C18:2/C16:1 or (C18:2)$_2$/C18:1/C16:0	1.21 ± 0.07	1.77 ± 0.19	0.97 ± 0.11	0.98 ± 0.13	**<0.0001**	n.s.	**<0.001**	**<0.0001**
9	(C18:1)$_2$/C18:2/C16:0	0.69 ± 0.04	1.07 ± 0.08	0.59 ± 0.06	0.58 ± 0.12	**<0.0001**	n.s.	**<0.01**	**<0.0001**
10	(C18:1)$_3$/C16:0	0.43 ± 0.04	0.68 ± 0.09	0.33 ± 0.04	0.31 ± 0.06	**<0.0001**	n.s.	**<0.001**	**<0.0001**
11	(C18:1)$_2$/(C18:3)$_2$	0.11 ± 0.01	0.20 ± 0.02	0.14 ± 0.02	0.14 ± 0.01	**<0.0001**	n.s.	**<0.01**	**<0.0001**
12	(C18:1)$_3$/C18:2	2.02 ± 0.11	2.73 ± 0.11	1.41 ± 0.15	1.47 ± 0.19	**<0.0001**	n.s.	**<0.0001**	**<0.0001**
13	(C18:2)$_3$/C20:4	2.06 ± 0.19	2.13 ± 0.21	1.85 ± 0.20	1.82 ± 0.05	n.s.	n.s.	n.s.	**<0.01**
14	(C18:2)$_3$/C20:3 or (C18:2)$_2$/C18:1/C20:4	3.64 ± 0.20	4.43 ± 0.28	3.05 ± 0.22	3.26 ± 0.28	**<0.0001**	n.s.	**<0.0001**	**<0.0001**
15	(C18:2)$_3$/C20:2 o. (C18:2)$_2$/C18:1/C20:3	8.22 ± 0.13	7.71 ± 0.48	6.69 ± 0.49	6.60 ± 0.48	**0.03**	n.s.	**<0.0001**	**<0.0001**
16	(C18:2)$_2$/C18:1/C20:2	5.07 ± 0.19	4.93 ± 0.31	3.28 ± 0.33	3.20 ± 0.26	n.s.	n.s.	**<0.0001**	**<0.0001**
17	(C18:2)$_3$/C20:0 o. (C18:1)$_2$/C18:2/C20:2	1.22 ± 0.10	1.27 ± 0.11	0.71 ± 0.10	0.68 ± 0.06	n.s.	n.s.	**<0.0001**	**<0.0001**
18	C22:6/(C18:2)$_3$	0.01 ± 0.00	0.03 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	**0.01**	n.s.	**<0.001**	**<0.0001**
19	C22:6/(C18:2)$_2$/C18:1	0.04 ± 0.01	0.05 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	**0.04**	**0.05**	**<0.0001**	**<0.0001**
	CL total (μg/mg protein)	14.58 ± 3.56	11.70 ± 1.82	13.26 ± 2.62	13.32 ± 1.88	n.s.	n.s.	n.s.	n.s.
	CL oxidized	1.11 ± 0.07	1.29 ± 0.12	0.77 ± 0.07	0.71 ± 0.07	**0.01**	n.s.	**<0.01**	**<0.0001**

n.s.: not significant.

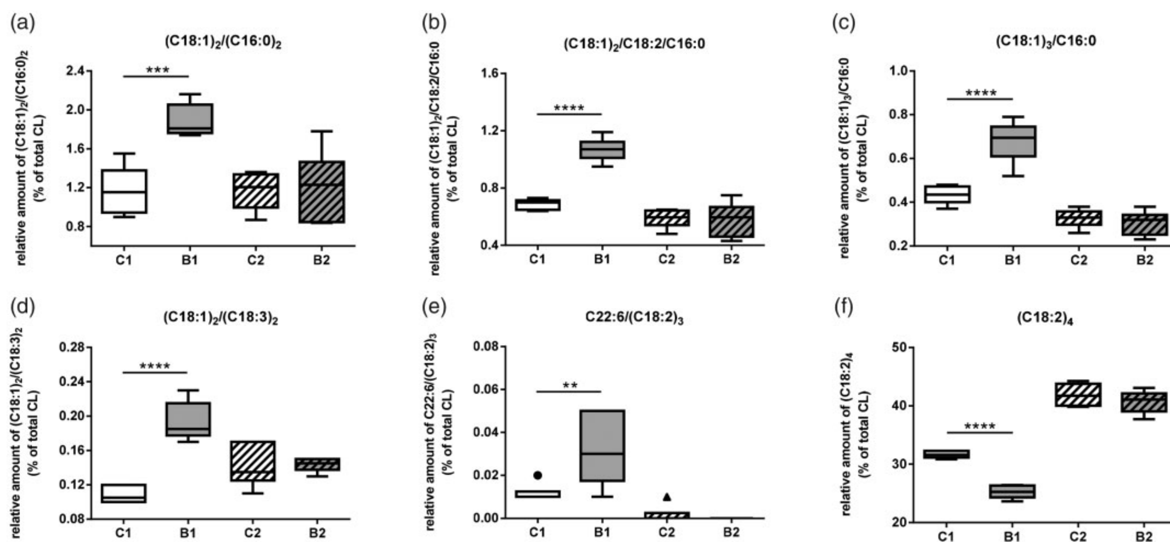

Figure 3. Effects of VitB6-deficiency on hepatic amounts of CL-species. Shown are molecular CL species which are subject to >1.5-fold change in response to VitB6-depletion (C1 vs. B1). The content of molecular CL species in all groups is presented in per cent of the sum of analyzed CL species. Data are given as boxplots with medians, quartiles, and an interquartile range (IQR) ± 1.5 × IQR with outliers as indicated (Tukey method) ($n = 6$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

confirmed the VitB6 deficiency that developed in the B1 group. VitB6-deficieny could be also verified by the detection of reduced serotonin (5-HT) plasma levels in the B1 group.[45] In summary, these findings demonstrate that feeding a VitB6-free diet to gravid rats translates into functional VitB6 deficiency (hypovitaminosis) in new-born rats.

VitB6 deficiency has been associated previously with substantial changes in lipid metabolism and hepatic histology.[21,23,46,47] Notably, in new-born rats of VitB6, deficient mothers substantial alterations of liver metabolism could be detected.[48] However, the impact of VitB6-deficiency on hepatic mitochondrial CL composition and mitochondrial respiration has not been studied yet.

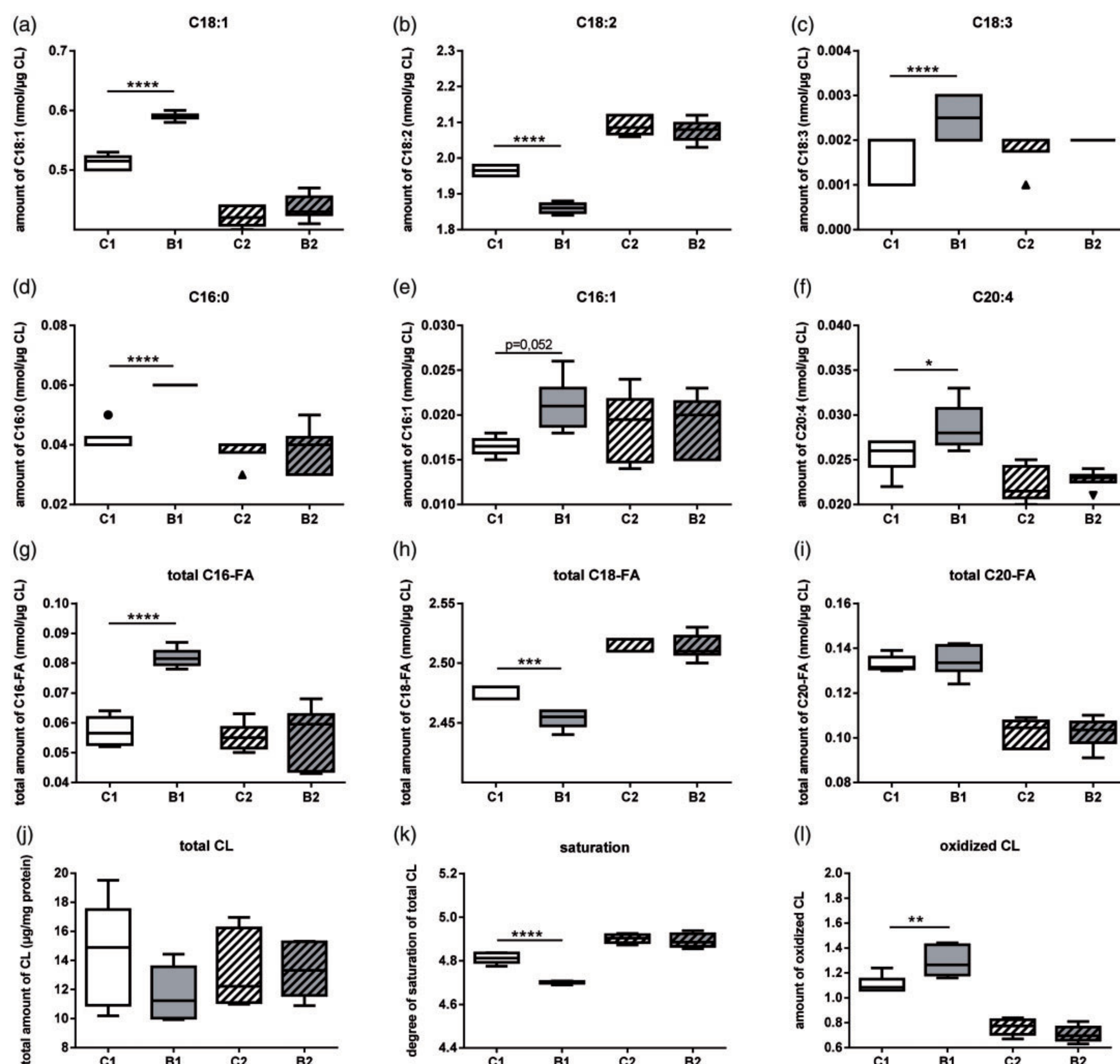

Figure 4. Effects of VitB6-deficiency on the abundance of fatty acids incorporated into hepatic CL. In the B1 group, amounts of (a) C18:1, (c) C18:3, (d) C16:0, (e) C16:1, and (f) C20:4 FAs incorporated into Cl were found to be increased, whereas (b) C18:2 was less abundant. Total amounts of (g) C16 FAs in CL were increased, the opposite effect was observed for (h) C18 FAs. VitB6 deficiency led to a (k) lower degree of saturated FAs in Cl, but (l) increased the amounts of oxidized CL. Total CL content (j) was not changed. Data are given as boxplots with medians, quartiles, and an interquartile range (IQR) $\pm$ 1.5 $\times$ IQR with outliers as indicated (Tukey method) ($n = 6$; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

Table 2 Abundance of cellular fatty acids in livers of new-born rats.

Cardiolipin species		C1 mean ± SD (µg/mg protein)	B1 mean ± SD (µg/mg protein)	C2 mean ± SD (µg/mg protein)	B2 mean ± SD (µg/mg protein)	ANOVA *P*-value C1 vs. B1	ANOVA *P*-value C2 vs. B2	ANOVA *P*-value C1 vs. C2	ANOVA *P*-value B1 vs. B2
1	C16:0	37.16 ± 5.03	38.75 ± 5.38	25.48 ± 2.63	24.73 ± 3.41	n.s.	n.s.	**<0.001**	**<0.0001**
2	C16:1	0.59 ± 0.09	0.76 ± 0.12	1.00 ± 0.24	1.24 ± 0.71	n.s.	n.s.	n.s.	n.s.
3	C18:0	24.18 ± 4.05	23.21 ± 3.01	22.02 ± 1.68	22.09 ± 2.09	n.s.	n.s.	n.s.	n.s.
4	C18:1	13.68 ± 2.64	17.97 ± 4.39	11.98 ± 0.91	12.74 ± 3.37	n.s.	n.s.	n.s.	**<0.05**
5	C18:2	23.50 ± 4.73	23.68 ± 5.15	15.91 ± 1.95	15.10 ± 1.80	n.s.	n.s.	**<0.05**	**<0.01**
6	γC18:3	0.37 ± 0.08	0.45 ± 0.11	0.38 ± 0.05	0.37 ± 0.13	n.s.	n.s.	n.s.	n.s.
7	αC18:3	0.46 ± 0.10	0.76 ± 0.29	0.31 ± 0.06	0.27 ± 0.07	**<0.05**	n.s.	n.s.	**<0.001**
8	C20:0	0.07 ± 0.08	0,03 ± 0.06	0.00 ± 0.00	0.04 ± 0.09	n.s.	n.s.	n.s.	n.s.
9	C20:2	0.93 ± 0.22	0,92 ± 0.15	0.48 ± 0.09	0.46 ± 0.07	n.s.	n.s.	**<0.001**	**<0.001**
10	C20:3	1.01 ± 0.15	1.27 ± 0.22	0.47 ± 0.07	0.54 ± 0.10	**<0.05**	n.s.	**<0.0001**	**<0.0001**
11	C20:4	39.05 ± 6.97	36.27 ± 4.58	28.77 ± 3.43	28.48 ± 2.75	n.s.	n.s.	**<0.01**	**<0.05**
12	C22:6	39.13 ± 6.69	52.05 ± 7.85	14.36 ± 3.09	18.57 ± 2.36	**<0.01**	n.s.	**<0.0001**	**<0.0001**

n.s.: not significant.

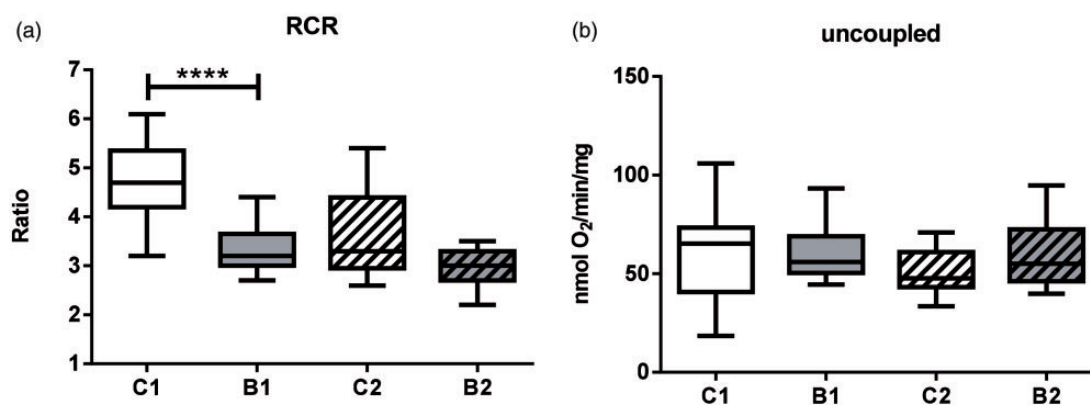

Figure 5. Effects of VitB6-deficiency on mitochondrial respiration in liver tissue. Mitochondria from the VitB6-deficient group, B1, exhibited a substantially reduced respiration control ratio (RCR) when compared to controls of equal age, C1. However, VitB6 deficiency did not affect maximum (uncoupled) respiration. (Data are given as boxplots with medians, quartiles and an interquartile range (IQR) $\pm$ 1.5 $\times$ IQR with outliers as indicated (Tukey method) ($n = 6$; ****$P < 0.0001$).

Results of this study demonstrate profound alterations in the CL-composition in response to VitB6 deficiency. These changes affect almost all CL-species investigated. In general, these changes can be described by an increase in the levels of unsaturated fatty acids and oxidized CL as well as increase in the amounts of C16 FAs, whereas C18 FAs appeared to be less abundant in the CL. With respect to the chain length, similar changes in CL have been observed in fast proliferating (tumor) cells previously.[49,50] Despite of the profound effects of VitB6-deficiency on CL-composition, we could not detect similar changes in the amounts of cellular FAs. This finding supports the hypothesis that VitB6 deficiency rather affects remodeling but not *de novo* synthesis of CL, since the mitochondrial CL content did not change. Additionally, the independency of the distribution of cellular free fatty acids with respect to VitB6 indicates that the substrate supply for CL *de novo* synthesis was unaffected by VitB6 deficiency. At present there is no information available concerning a role of VitB6 in tafazzin-dependent remodeling of CL. This question should be addressed by future studies of CL metabolism.

Even marginally decreased VitB6 levels have been shown to decrease plasma concentrations of polyunsaturated fatty acids (PUFAs) in humans.[51] Both insufficient availability of VitB6 as a co-factor for Δ6 desaturase and the reduced expression of fatty acid desaturase (FADS) due to hypermethylation of, e.g., the FADS2 promoter, have been proposed to contribute to these effects.[52,53] Along this line, VitB6-depletion has been shown to increase the hepatic amounts of saturated FAs and decrease that of unsaturated FAs.[22] Such changes could not be observed in our study. A possible explanation for this apparent discrepancy could be the relatively short period of VitB6-deficiency rats were exposed to in our study. Alternatively, effects on desaturase expression might be less prominent here due to incomplete hepatic maturation and low FADS expression levels. Postnatal hepatic remodeling has been shown to be mediated by large-scale transcriptional and post-transcriptional alterations[48,54,55] with *fatty acid metabolism* identified among the top enriched pathways.[55] In full accordance with these alterations of lipid metabolism, “age-dependent” changes in both CL-composition and the amounts of cellular FAs could be observed in our study (C1 vs. C2).

In line with results of previous studies, changes in CL composition, as caused here by VitB6-deficiency, were associated with altered mitochondrial function. In this study, we found a decreased capacity of oxidative phosphorylation (maximal ADP-dependent oxygen consumption) in liver mitochondria from VitB6 deficient new born rats. This we conclude although uncoupled respiration as well as active respiration in the presence of both NADH- and FADH-dependent respiration remained unaffected by VitB6 deficiency. However, increased state IV oxygen consumption values in isolated liver mitochondria from VitB6 deficient new born rats indicate (i) alteration of the permeability of the mitochondrial membrane system (proton leak) and (ii) restriction of the capacity for ATP synthesis since ADP-dependent respiration (maximal respiration minus state IV oxygen consumption) decreased. It has been shown that properties of the mitochondrial membrane system depend on the content and composition of CL.[56,57] Electrophysical properties, membrane curvature (cristae structure), and the assembling of membrane proteins depend on CL.[31,33,58] Thus, the observed VitB6-dependent alterations in CL could be responsible for the changes in membrane permeability and subsequently for the restriction in the capacity of oxidative phosphorylation in situations of VitB6 deficiency.

Conclusion

VitB6 deficiency during pregnancy and lactation induces massive alterations of CL composition in the liver mitochondria of new-born rats. This is associated with a decrease in the efficiency of oxidative phosphorylation.

These findings indicate that a sufficient supply of VitB6 during pregnancy (and periconceptional) contributes to an appropriate mitochondrial CL-composition and, thus, supports mitochondrial respiration/energy production.

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experiments: JW, CW, DP, GD, LS, SG, UL. Analyzed the data: CW, GD, SG, LS, UL, JW. Wrote the manuscript: UL, CW, LS, GD.

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The author(s) declared no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

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REFERENCES

1. Parra M, Stahl S and Hellmann H. Vitamin B_6 and its role in cell metabolism and physiology. *Cells* 2018;**7**: pii:E84
2. Ueland PM, Ulvik A, Rios-Avila L, Midttun Ø, Gregory JF. Direct and functional biomarkers of vitamin B6 status. *Annu Rev Nutr* 2015;**35**:33–70
3. Bender DA. Non-nutritional uses of vitamin B6. *Br J Nutr* 1999;**81**:7–20
4. Bordoni A, Cabrini L, Marchetti M, Danesi F, Bochicchio D, Biagi PL, Maranesi M. Vitamin B6 deficiency and dietary fats: effects on lipid composition and glutathione peroxidase activity in rat liver. *Ann Nutr Metab* 2006;**50**:305–12
5. Panton KK, Farup PG, Sagen E, Sirum UF, Asberg A. Vitamin B6 in plasma – sample stability and the reference limits. *Scand J Clin Lab Invest* 2013;**73**:476–9
6. Cortes F, Mellado C, Pardo RA, Villarroel LA, Hertrampf E. Wheat flour fortification with folic acid: changes in neural tube defects rates in Chile. *Am J Med Genet* 2012;**158A**:1885–90
7. Elmazar MM, Thiel R, Nau H. Effect of supplementation with folinic acid, vitamin B6, and vitamin B12 on valproic acid-induced teratogenesis in mice. *Fundam Appl Toxicol* 1992;**18**:389–94
8. Jacobsson C, Granström G. Clinical appearance of spontaneous and induced first and second branchial arch syndromes. *Scand J Plast Reconstr Surg Hand Surg* 1997;**31**:125–36
9. Krapels IP, van Rooij IA, Ocke MC, van Cleef BA, Kuijpers-Jagtman AM, Steegers-Theunissen RP. Maternal dietary B vitamin intake, other than folate, and the association with orofacial cleft in the offspring. *Eur J Nutr* 2004;**43**:7–14
10. Smolin LA, Benevenga NJ. Factors affecting the accumulation of homocyst(e)ine in rats deficient in vitamin B-6. *J Nutr* 1984;**114**:103–11
11. Copp AJ. Prevention of neural tube defects: vitamins, enzymes and genes. *Curr Opin Neurol* 1998;**11**:97–102
12. Eskes TK. Folates and the fetus. *Eur J Obstet Gynecol Reprod Biol* 1997;**71**:105–11
13. Hol FA, van der Put NM, Geurds MP, Heil SG, Trijbels FJ, Hamel BC, Mariman EC, Blom HJ. Molecular genetic analysis of the gene encoding the trifunctional enzyme MTHFD (methylenetetrahydrofolate-dehydrogenase, methenyltetrahydrofolate-cyclohydrolase, formyltetrahydrofolate synthetase) in patients with neural tube defects. *Clin Genet* 1998;**53**:119–25
14. Pietrzik K, Prinz-Langenohl R, Thorand B. Mikronährstoffe in der schwangerschaft. *Z Geburtshilfe Neonatol* 1997;**201**:21–4
15. Steegers-Theunissen RP, Smithells RW, Eskes TK. Update of new risk factors and prevention of neural-tube defects. *Obstet Gynecol Surv* 1993;**48**:287–93
16. Eskes TK. Open or closed? a world of difference: a history of homocysteine research. *Nutrit Revs* 1998;**56**:236–44
17. Goddijn-Wessel TA, Wouters MG, van de Molen EF, Spuijbroek MD, Steegers-Theunissen RP, Blom HJ, Boers GH, Eskes TK. Hyperhomocysteinemia: a risk factor for placental abruption or infarction. *Eur J Obstet Gynecol Reprod Biol* 1996;**66**:23–9
18. Weingärtner J, Fanghänel J, Bienengräber V, Gundlach K. Initial findings on teratological and developmental relationships and differences between neural tube defects and facial clefting. First experimental results. *J Craniomaxillofacial Surg* 2005;**33**:297–300
19. Depeint F, Bruce WR, Shangari N, Mehta R, O'Brien PJ. Mitochondrial function and toxicity: role of the B vitamin family on mitochondrial energy metabolism. *Chem Biol Interact* 2006;**163**:94–112
20. Dasarathy J, Gruca LL, Bennett C, Parimi PS, Duenas C, Marczewski S, Fierro JL, Kalhan SC. Methionine metabolism in human pregnancy. *Am J Clin Nutr* 2010;**91**:357–65
21. Cabrini L, Bochicchio D, Bordoni A, Sassi S, Marchetti M, Maranesi M. Correlation between dietary polyunsaturated fatty acids and plasma homocysteine concentration in vitamin B6-deficient rats. *Nutr Metab Cardiovasc Dis* 2005;**15**:94–9
22. McNeil CJ, Beattie JH, Gordon MJ, Pirie LP, Duthie SJ. Nutritional B vitamin deficiency disrupts lipid metabolism causing accumulation of proatherogenic lipoproteins in the aorta adventitia of ApoE null mice. *Mol Nutr Food Res* 2012;**56**:1122–30
23. Zhao M, Ralat MA, da Silva V, Garrett TJ, Melnyk S, James SJ, Gregory JF. 3rd. Vitamin B-6 restriction impairs fatty acid synthesis in cultured human hepatoma (HepG2) cells. *Am J Physiol Endocrinol Metab* 2013;**304**:E342–51
24. Kaimoto T, Shibuya M, Nishikawa K, Maeda H. High incidence of lipid deposition in the liver of rats fed a diet supplemented with branched-chain amino acids under vitamin B6 deficiency. *J Nutr Sci Vitaminol* 2013;**59**:73–8
25. Noga AA, Vance DE. Insights into the requirement of phosphatidylcholine synthesis for liver function in mice. *J Lipid Res* 2003;**44**:1998–2005
26. Vance DE. Role of phosphatidylcholine biosynthesis in the regulation of lipoprotein homeostasis. *Curr Opin Lipidol* 2008;**19**:229–34
27. Schlame M, Greenberg ML. Biosynthesis, remodeling and turnover of mitochondrial cardiolipin. *Biochim Biophys Acta* 2017;**1862**:3–7
28. Neustein HB, Lurie PR, Dahms B, Takahashi M. An X-linked recessive cardiomyopathy with abnormal mitochondria. *Pediatrics* 1979;**64**:24–9
29. Acehan D, Xu Y, Stokes DL, Schlame M. Comparison of lymphoblast mitochondria from normal subjects and patients with Barth syndrome using electron microscopic tomography. *Lab Invest* 2007;**87**:40–8
30. Frohman MA. Role of mitochondrial lipids in guiding fission and fusion. *J Mol Med* 2015;**3**:263–9
31. Gürtler S, Otto O, Heise N, Scholz F, Laporte A, Elsner M, Jörns A, Weinert S, Wolke C, Döring M, Jansing S, Gardemann A, Lendeckel U, Schild L. Tafazzin-dependent cardiolipin composition in C6 glioma cells correlates with changes in mitochondrial and cellular functions, and cellular proliferation. *Biochim Biophys Acta Mol Cell Biol Lipids* 2019;**1864**:452–65
32. Paradies G, Paradies V, De Benedictis V, Ruggiero FM, Petrosillo G. Functional role of cardiolipin in mitochondrial bioenergetics. *Biochim Biophys Acta* 2014;**1837**:408–17
33. Acehan D, Malhotra A, Xu Y, Ren M, Stokes DL, Schlame M. Cardiolipin affects the supramolecular organization of ATP synthase in mitochondria. *Biophys J* 2011;**100**:2184–92
34. Pfeiffer K, Gohil V, Stuart RA, Hunte C, Brandt U, Greenberg ML, Schägger H. Cardiolipin stabilizes respiratory chain supercomplexes. *J Biol Chem* 2003;**278**:52873–80
35. Kiebish MA, Han X, Cheng H, Chuang JH, Seyfried TN. Cardiolipin and electron transport chain abnormalities in mouse brain tumor

mitochondria: lipidomic evidence supporting the Warburg theory of cancer. *J Lipid Res* 2008;**49**:2545–56
36. Raja V, Greenberg ML. The functions of cardiolipin in cellular metabolism-potential modifiers of the Barth syndrome phenotype. *Chem Phys Lipids* 2014;**179**:49–56
37. Schlame M. Cardiolipin remodeling and the function of tafazzin. *Biochim Biophys Acta* 2013;**1831**:582–8
38. Dudek J, Cheng IF, Balleininger M, Vaz FM, Streckfuss-Bömeke K, Hübscher D, Vukotic M, Wanders RJ, Rehling P, Guan K. Cardiolipin deficiency affects respiratory chain function and organization in an induced pluripotent stem cell model of Barth syndrome. *Stem Cell Res* 2013;**11**:806–19
39. Fuertig R, Ceci A, Camus SM, Bezard E, Luippold AH, Hengerer B. LC-MS/MS-based quantification of kynurenine metabolites, tryptophan, monoamines and neopterin in plasma, cerebrospinal fluid and brain. *Bioanalysis* 2016;**8**:1903–17
40. Ohlig T, Le DV, Gardemann A, Wolke C, Gürtler S, Peter D, Schild L, Lendeckel U. Effects of siRNA-dependent knock-down of cardiolipin synthase and tafazzin on mitochondria and proliferation of glioma cells. *Biochim Biophys Acta* 2018;**1863**:379–87
41. Nourooz-Zadeh J, Liu EH, Yhlen B, Angard EE, Halliwell B. F4-Isoprostanes as specific marker of docosahexaenoic acid peroxidation in Alzheimer's disease. *J Neurochem* 1999;**72**:734–40
42. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt Biochem* 1976;**72**:248–54
43. Chilukoti RK, Lendeckel J, Darm K, Bukowska A, Goette A, Sühling M, Utpatel K, Peters B, Homuth G, Völker U, Wolke C, Scharf C, Lendeckel U. Integration of "omics" techniques: dronedarone affects cardiac remodeling in the infarction border zone. *Exp Biol Med* 2018;**243**:895–910
44. Vanderlinde RE. Review of pyridoxal phosphate and the transaminases in liver disease. *Ann Clin Lab Sci* 1986;**16**:79–93
45. Schulman MP, Richert DA. Heme synthesis in vitamin B6 and pantothenic acid deficiencies. *J Biol Chem* 1957;**226**:181–9
46. Weissbach H, Bogdanski DF, Redfield BG, Udenfriend S. Studies on the effect of vitamin B6 on 5-hydroxytryptamine (serotonin) formation. *J Biol Chem* 1957;**227**:617–24
47. Ferro Y, Carè I, Mazza E, Provenzano F, Colica C, Torti C, Romeo S, Pujia A, Montalcini T. Protein and vitamin B6 intake are associated with liver steatosis assessed by transient elastography, especially in obese individuals. *Clin Mol Hepatol* 2017;**23**:249–59
48. Bhate A, Parker DJ, Bebee TW, Ahn J, Arif W, Rashan EH, Chorghade S, Chau A, Lee JH, Anakk S, Carstens RP, Xiao X, Kalsotra A. ESRP2 controls an adult splicing programme in hepatocytes to support post-natal liver maturation. *Nat Commun* 2015;**6**:8768
49. Liu Z, Li P, Zhao ZH, Zhang Y, Ma ZM, Wang SX. Vitamin B6 prevents endothelial dysfunction, insulin resistance, and hepatic lipid accumulation in apoe (-/-) mice fed with High-Fat diet. *J Diabetes Res* 2016;**2016**:1748065
50. Schild L, Lendeckel U, Gardemann A, Wiswedel I, Schmidt CA, Wolke C, Walther R, Grabarczyk P, Busemann C. Composition of molecular cardiolipin species correlates with proliferation of lymphocytes. *Exp Biol Med* 2012;**237**:372–9
51. Hartz JW, Morton RE, Waite MM, Morris HP. Correlation of fatty acyl composition of mitochondrial and microsomal phospholipid with growth rate of rat hepatomas. *Lab Invest* 1982;**46**:73–8
52. Devlin AM, Singh R, Wade RE, Innis SM, Bottiglieri T, Lentz SR. Hypermethylation of Fads2 and altered hepatic fatty acid and phospholipid metabolism in mice with hyperhomocysteinemia. *J Biol Chem* 2007;**282**:37082–90
53. Zhao M, Lamers Y, Ralat MA, Coats BS, Chi YY, Muller KE, Bain JR, Shankar MN, Newgard CB, Stacpoole PW, Gregory JF 3rd. Marginal vitamin B-6 deficiency decreases plasma (n-3) and (n-6) PUFA concentrations in healthy men and women. *J Nutr* 2012;**142**:1791–7
54. Costa RH, Kalinichenko VV, Holterman AX, Wang X. Transcription factors in liver development, differentiation, and regeneration. *Hepatology* 2003;**38**:1331–47
55. Kyrmizi I, Hatzis P, Katrakili N, Tronche F, Gonzalez FJ, Talianidis I. Plasticity and expanding complexity of the hepatic transcription factor network during liver development. *Genes Dev* 2006;**20**:2293–305
56. Ikon N, Ryan RO. Cardiolipin and mitochondrial cristae organization. *Biochim Biophys Acta Biomembr* 2017;**1859**:1156–63
57. Schlame M, Ren M. The role of cardiolipin in the structural organization of mitochondrial membranes. *Biochim Biophys Acta* 2009;**1788**:2080–3
58. Claypool SM, Boontheung P, McCaffery JM, Loo JA, Koehler CM. The cardiolipin transacylase, tafazzin, associates with two distinct respiratory components providing insight into Barth syndrome. *MBoC* 2008;**19**:5143–55

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