The mouse as a model to understand peroxisomal disorders

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CHAPTER 7

ATAXIA WITH LOSS OF PURKINJE CELLS IN A MOUSE MODEL FOR REFSUM DISEASE

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Ataxia with loss of Purkinje cells in a mouse model for Refsum disease

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Refsum disease is characterized by cerebellar ataxia, polyneuropathy, and progressive retinitis pigmentosa, culminating in blindness, (1, 3). The age of onset of the symptoms can vary from early childhood to the third or fourth decade of life. No treatment is available for patients with Refsum disease, but they benefit from a low phytanic acid diet. Phytanic acid is derived from dietary sources only, specifically from the chlorophyll component phytol. When phytanic acid levels are kept low via dietary reduction, a halt in the progression of the symptoms has been reported (4–7).

Although the clinical symptoms of Refsum disease are well known, only limited postmortem data are available on Refsum patients and the pathogenesis of the disorder is poorly understood. Insight herein is of crucial importance for the development of potential therapeutic options. Phytanic acid has been claimed to be toxic, and it has been shown to be an activating ligand of the nuclear receptor peroxisome proliferator receptor α (PPARα). To study the pathological consequences of phytanic acid accumulation, we generated of a mouse model for Refsum disease by targeted disruption of the Phyh gene.

Results

Disruption of the Phyh Gene

To disrupt the mouse Phyh gene, part of the gene containing exons 4–7 was replaced with the hygromycin B-resistance gene by homologous recombination in ES cells as depicted in Fig. 1A. Correct targeting of Phyh was confirmed by Southern blot analysis and PCR on genomic DNA (Fig. 1B and C). The disruption of the Phyh gene resulted in the absence of detectable mRNA (Fig. 1D), PHYH protein, and a complete loss of PHYH enzyme activity (data not shown). The absence of the Phyh gene did not result in embryonic lethality nor did it affect postnatal viability. When kept under standard laboratory conditions, Phyh−/− mice developed normally and had no obvious developmental abnormalities. They were fertile, and interbreeding of Phyh−/− males and females led to viable progeny.

Biochemical Abnormalities in Phyh−/− Mice Fed a Phytol-Supplemented Diet. In contrast to the human diet, standard rodent chow contains only low amounts of branched-chain fatty acids and the


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Fig. 1. Generation of targeting vector and Phyh−/− mice. (A) Strategy of gene targeting. The WT allele, targeting vector and the targeted allele are depicted. After homologous recombination, exons 4–7 and parts of introns 3 and 7 are deleted. The location of the 5’ probe used for Southern blot analysis is shown. (B) Southern blot analysis of genomic DNA isolated from ES cells, digested with EcoRV and BamHI, and probed with the 5’ probe yielded a 12.3-kb fragment for the WT allele (+/+) and a 10-kb fragment for the targeted allele (+/−). (C) Genotyping by PCR on genomic DNA. Amplification from the WT allele (+/+) resulted in a 201-bp fragment and amplification from the targeted allele (+/−) a fragment of 260 bp. (D) Upper PCR analysis of liver cDNA from WT and Phyh−/− mice. No amplification of exons 1–8 could be detected in Phyh−/− mice. (Lower) A control for cDNA amplification was carried out with primers for β-actin.

Fig. 2. Phytanic acid levels in plasma and tissues of male WT and Phyh−/− mice. Phytanic levels were determined in liver (white bars), kidney (gray bars), cerebellum (black bars), testis (checked bars), and plasma (inset graph) from WT and Phyh−/− mice on a control diet (C), a 0.1% phytol diet, and a 0.25% phytol diet. Data represent mean ± SD.

Fig. 3. Phyh−/− mice on a phytol diet develop hepatic lipidosis. H&E staining of livers from WT (A–C) and Phyh−/− mice (D–F) on a control diet (A and D), on a 0.1% phytol diet (B and E), and on a 0.25% phytol diet (C and F). In Phyh−/− mice on a 0.25% phytol diet (F), steatosis is clearly detected by the large lipid vacuoles present throughout the liver parenchyma. On a 0.1% phytol diet (E), Phyh−/− mice showed signs of microsteatosis (small lipid vacuoles within hepatocytes). (Scale bar: 100 µm.)

Fig. 4. Loss of spermatogonia in phytol-fed Phyh−/− mice. Immunohistochemical detection of calreticulin in testis of WT mice (A–C) and Phyh−/− (D–F) mice on control diet (A and D), 0.1% phytol diet (B and E), and 0.25% phytol diet (C and F). Calreticulin is highly expressed in spermatogonia of WT mice on control diet (A). Loss of spermatogonia in Phyh−/− mice on the 0.1% phytol (E) and 0.25% phytol (F) diets is demonstrated by decreased numbers of calreticulin-positive spermatogonia leaving gaps (arrows in E and F) in the epithelium of the seminiferous tubules. The slide is hematoxylin QS counterstained. (Scale bar: 50 µm.)

Phytanic acid precursor phytol. For this reason, we supplemented standard chow with phytol. One group of animals was fed a control diet, 1 group received a diet supplemented with 0.1% (wt/wt) phytol for 6 weeks, and 1 group received a 0.25% (wt/wt) phytol diet for 3 weeks. The 0.25% phytol diet caused a decrease in body weight of 25 ± 5% in the Phyh−/− males and 16 ± 10% in the Phyh−/− females. This body weight loss is most probably caused by lipatrophy, because upon dissection, almost no white fat could be observed in the Phyh−/− mice after the 0.25% phytol diet.

Because of the phytol diets, phytanic acid accumulated in plasma and tissues (Fig. 2). After 3 weeks on the 0.25% phytol diet, plasma phytanic acid levels >1 mmol/L were reached. Such levels have also been reported in Refsum disease patients (8). Of the different tissues analyzed, liver showed the highest accumulation of phytanic acid, followed by kidney, testis, and cerebellum, and the extent of the accumulation was similar for males and females. These results show that the most important biochemical characteristic of Refsum disease, i.e., phytanic acid accumulation, is reproduced by disruption of the Phyh gene in the mouse.

Further biochemical analyses in plasma revealed increased amylase and alanine-aminotransferase (ALAT) activity in pooled plasma of Phyh−/− mice after 3 weeks on the 0.25% phytol diet, whereas albumin levels were unaltered [supporting information (SI) Table S1]. Cholesterol, triglycerides, free fatty acids, and total fatty acid levels were decreased in plasma from Phyh−/− mice on a phytol diet (Table S1). The levels of virtually
all of the fatty acids determined were decreased (C14:0, C16:0, C18:0, C20:0, C22:0, and C24:0 and 5, 6, 7, and 9 polyunsaturated fatty acids). These results confirm that high levels of phytanic acid cause increased fat degradation, resulting in the disappearance of the white fat, as described above.

Pathological Effects of Phytanic Acid Accumulation in Phyh+/− Mice.

The pathological effects of phytanic acid accumulation in the liver were studied by histological analysis. H&E staining of liver sections (Fig. 3) of Phyh−/− mice fed with the 0.25% phytol diet showed morphological changes that included steatosis, hepatocyte degeneration, and inflammatory infiltrates. The steatosis in livers from Phyh−/− mice was microvesicular when they were fed the 0.1% phytol diet (Fig. 3E) and macrovesicular when fed the 0.25% phytol diet (Fig. 3F). In contrast, no morphological changes were observed in WT mice when fed a phytol diet.

H&E staining of testis sections revealed that, despite having sperm at different stages of maturation, the seminiferous tubules of Phyh−/− mice on the phytol diet did not have the full complement of spermatogenic cells (Fig. 4). This loss of spermatogenic cells was clearly seen upon immunodetection of calreticulin, which is predominantly expressed in spermatogonia. After 6 weeks on the 0.1% phytol diet, loss of spermatogonia was clearly visible (Fig. 4E), but mild when compared with the loss observed after 3 weeks on the 0.25% phytol diet (Fig. 4F). No abnormalities were observed by histological analysis in heart, kidney, intestine, lung and eye.

Fig. 5. Automated gait analysis using CatWalk revealed ataxia in Phyh−/− mice on a phytol diet. (A) Paw prints of the fore- and the hindpaws for representative runs by male WT and Phyh−/− mice on different diets are shown. The paw print area of both the fore- and hindpaw of the Phyh−/− mice on 0.25% phytol were markedly decreased. (B) Successive frames of the paw-floor contact area for fore- and hindpaws revealed a decreased contact area for male Phyh−/− mice compared with WT mice on a 0.25% phytol diet. C, control diet; P, 0.25% Phytol diet. (C) Base of support for the hindpaws (white bars) was significantly decreased (*, P < 0.05, t test) in the Phyh−/− mice on the phytol diets compared with the base of support of the WT mice on the same diets. The decrease was also significant for the Phyh−/− mice before and after the 0.25% phytol diet (data not shown). There were no differences for base of support of the forepaws (black bars).
phenotype revealed that the mice developed an abnormal gait because of phytanic acid accumulation. The plasma phytanic acid levels were the highest expression after the 0.25% phytol diet (Fig. S3). The MNCV in Phyh−/− mice was normal regardless of the dietary regimen (data not shown). How-ever, glial fibrillary acidic protein (GFAP) immunohistochemistry (Fig. S1) revealed reactive astrocytosis in the brain of Phyh−/− mice fed with the 0.25% phytol diet. Astrocytosis was prominent in the inferior colliculus (Fig. S1F) and thalamus (data not shown). Occasionally, reactive astrocytes were identified in the granular layer of the cerebellum (Fig. S1J), but normal appearing astrocytes were present in the hippocampus (Fig. S1A) and spinal cord (Fig. S1L). Calbindin-D28K immunohistochemistry (Fig. 6) and quantification of Purkinje cells revealed a decrease in Purkinje cell numbers in Phyh−/− mice fed phytol (Fig. S2). When Phyh−/− mice were fed with the 0.1% phytol diet, we observed focal loss of Purkinje cells within the cerebellar Purkinje cell layer (Fig. 6E). A severe loss of Purkinje cells with only some remaining foci of surviving Purkinje cells (Fig. 6F), that showed an abnormal truncated arborization, was observed after the 0.25% phytol diet. Within the cerebrum, an increase in the number of calbindin-D28K-positive neurons was observed in the cortex, thalamus, and medulla of Phyh−/− mice fed with the 0.25% phytol diet (data not shown). This observation led us to investigate the expression of calcium-binding proteins in the CNS. Parvalbumin expression was clearly increased in the thalamus and cerebral cortex of Phyh−/− mice with the highest expression after the 0.25% phytol diet (Fig. S3). The number of cortical neurons expressing parvalbumin was also increased in Phyh−/− mice fed the phytol diets (Fig. S3). Expression of calreticulin in the CNS was similar between WT and Phyh−/− mice regardless of the dietary regimen (data not shown). In conclusion, our immunohistochemical analyses have shown that phytanic acid accumulation causes pathological and adaptive changes in the CNS of Phyh−/− mice.

Peripheral Neuropathy in Phyh−/− mice. To determine whether a peripheral neuropathy is present in Phyh−/− mice, motor nerve conduction velocities (MNCV) were measured. The measurements revealed a significant decrease in MNCV in Phyh−/− mice fed with a 0.1% phytol diet for 8 weeks (Fig. S4). The MNCV in Phyh−/− mice after the phytol diet was 22.7 ± 3.2 m/s compared with 29.6 ± 0.6 m/s in WT mice. This decrease in MNCV was due to an increased latency in the compound muscle action potentials after stimulation (Fig. S4). The action potentials were also extended in Phyh−/− mice. On the control diet, Phyh−/− mice had normal MNCV with normal compound muscle action potentials. These results show that the phytol diet causes a peripheral neuropathy in Phyh−/− mice. Histological analyses and MBP immunohistochemistry of sciatic nerves from WT and Phyh−/− mice did not reveal gross abnormalities in myelination (Fig. S4).

Discussion

Only limited postmortem data are available on Refsum patients, and the pathophysiology of Refsum disease is poorly understood. In this article, we report studies on the pathological consequences of phytic acid accumulation in a Phyh−/− mouse, which we generated as model for the human disorder. Because phytic acid is present in numerous dietary sources, Refsum patients are life-long exposed to elevated phytic acid levels. During metabolic stress due to fasting or a viral infection, stored phytic acid is mobilized from adipose tissue, causing a rapid rise of plasma phytic acid levels, thus accelerating the progression of clinical symptoms (1, 3). The Phyh−/− mice in this study received a phytol-supplemented diet for a relatively short period (up to 6 weeks), allowing us to study the short-term effect of phytic acid accumulation. The plasma phytic acid levels that were reached in the Phyh−/− mice were similar to those found in plasma from Refsum patients (8). We observed several detrimental effects caused by the high phytic acid levels. First of all, Phyh−/− mice displayed a severe weight loss on the diet...
with the highest phytol concentration. This was accompanied by lipotoxopathy and hepatic lipodosis. At the same time, plasma levels of triglycerides, cholesterol, and fatty acids were decreased. These changes are most likely due to activation of peroxisome proliferator receptor α (PPARα) by phytanic acid. Phytanic acid has been shown to be an activating ligand of this nuclear receptor (11–13), which regulates the expression of genes involved in lipid and lipoprotein metabolism and fatty acid oxidation both in humans and rodents (14–16). The expression of peroxisomal straight-chain acyl-CoA oxidase, a target gene of PPARα in humans and rodents (14–16), was up-regulated in Phyh−/− mice on a phytol diet (data not shown) pointing to PPARα activation in these mice. Indeed, Phyh−/− mice did not lose any weight, confirming the involvement of PPARα in the phytanic acid induced weight loss in Phyh−/− mice (data not shown).

Toxicity of high phytanic acid levels could be observed in different tissues of the Phyh−/− mice. In the liver of Phyh−/− mice, the phytol in phytol diet causes not only steatosis but also hepatocyte degeneration and infiltration. Phytol treatment also resulted in increased plasma levels of ALAT and amylase, indicative of liver and pancreas damage. In addition, a loss of spermatogonia was observed in the testes of phytol-fed Phyh−/− mice. Longer exposure to these high levels of phytanic acid may lead to a block in spermatogenesis, which has been described for Refsum patients (17).

Phenotype assessment with the SHIRPA protocol and subsequent automated gait analysis using the CatWalk system revealed an abnormal gait for Phyh−/− mice on the phytol diet. The toe spread, paw print area, and base of support of the hindpaws showed an increase of cytosolic calcium due to activation of calcium-binding proteins could be a neuroprotective mechanism to protect against excitotoxicity caused by phytanic acid.

In conclusion, our studies have provided important insights in the pathophysiology of Refsum disease. As a consequence of phytanic acid accumulation, Phyh−/− mice, just like human patients, developed a peripheral neuropathy and cerebellar ataxia that was accompanied by a loss of Purkinje cells. In addition, the increased phytanic acid levels caused reactive astrocytosis, and up-regulation of the calcium-binding proteins in the CNS, which is most likely a neuroprotective mechanism against the excitotoxic effects of phytanic acid. Our studies showed that Phyh−/− mice are a good model for this human disorder. Future studies will focus on the effects of long-term exposure to high levels of phytanic acid. Studies in Phyh−/− mice will be valuable for the development and evaluation of potential new treatment options.

Materials and Methods

Construction of the Targeting Vector and Generation of Phyh−/− Mice. The Phyh gene (GenBank accession no. NP_034856.1) was cloned from a 129 SVJ mouse genomic library (Stratagene). The targeting vector was constructed by removing an EcoRI-ClaI fragment including exons 4–7 and replacing it with the hygromycin B resistance gene in the opposite transcriptional orientation to 0.1% phythol diet. The final construct containing 6.3 kb of 5′ and 3′ homology, respectively. The targeting vector (30 μg) was linearized with HindIII and introduced into IB10 ES cells (The Netherlands Cancer Institute, 6 × 10⁵ cells) by electroporation (0.8 kV and 3 μF) in a Bio-Rad gene pulsor. Targeted ES cells were screened by PCR for homologous recombination. The primers annealed to the hygromycin B resistance marker and the 3′ genomic flank outside the construct, respectively. PCR positive clones were also analyzed by Southern blot for correct 5′ homologous recombination. Genomic DNA was digested overnight with EcoRV and BamHI and hybridization of the Southern blot was carried out with an external 5′ probe (EcoRI-HindII fragment, containing exon 1). Targeted ES cells were injected into C57BL/6 blastocysts, resulting in several chimeric mice that showed germ-line transmission. Phyh−/− mice (50% Swiss/25% 129SvJ/25% FVB) were bred to obtain Phyh−/− animals. F₂ offspring were used for the experiments.

Animal Experiments. Seven-week-old WT and Phyh−/− mice were fed pellet mouse chow (AB Diets) without supplements (control) or supplemented with 0.1% (wt/wt) phythol or 0.25% (wt/wt) phythol diet (Sigma-Aldrich). Each group consisted of 6 animals, including 3 males and 3 females that were 7 weeks old at the start of the experiment. The mice received the diet for 3 weeks in the case of the 0.25% phythol diet and for 6 weeks in the case of the control and 0.1% phythol diet. At the start and at the end of the diet period, the SHIRPA protocol was performed with all animals, and the gait was analyzed by using CatWalk. At the end of the experiment, mice were anesthetized by using isoflurane, blood was collected by cardiac puncture, and tissues were harvested. The animals were killed the day after the final SHIRPA and CatWalk analysis, and they had free access to water and food until that moment. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. For the MNCV measurements, another set of animals was used. The mice were killed the day after the final SHIRPA and CatWalk analysis, and they had free access to water and food until that moment. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. For the MNCV measurements, another set of animals was used. The mice were killed the day after the final SHIRPA and CatWalk analysis, and they had free access to water and food until that moment. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. For the MNCV measurements, another set of animals was used. The mice were killed the day after the final SHIRPA and CatWalk analysis, and they had free access to water and food until that moment. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. For the MNCV measurements, another set of animals was used. The mice were killed the day after the final SHIRPA and CatWalk analysis, and they had free access to water and food until that moment. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. For the MNCV measurements, another set of animals was used. The mice were killed the day after the final SHIRPA and CatWalk analysis, and they had free access to water and food until that moment. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. For the MNCV measurements, another set of animals was used. The mice were killed the day after the final SHIRPA and CatWalk analysis, and they had free access to water and food until that moment. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. For the MNCV measurements, another set of animals was used. The mice were killed the day after the final SHIRPA and CatWalk analysis, and they had free access to water and food until that moment. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. For the MNCV measurements, another set of animals was used. The mice were killed the day after the final SHIRPA and CatWalk analysis, and they had free access to water and food until that moment. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis.
animals were allowed to traverse the walkway as many times as needed to obtain at least 3 fluent crossings (without stopping or hesitations). Both qualitative and quantitative data can be assessed with the CatWalk program. For this study, we focused on the paw print, the paw print area, and the base of support (average distance between the mass-midpoints of the prints at maximum contact).

Historical and Immunohistochemical Analyses. Harvested tissues were fixed by immersion in formalin at 4 °C for 48 h and processed for paraffin embedding. For immunohistochemistry, sections were cut on a Leica RM2255 microtome and stained with H&E for microscopic pathologic analyses. For immunohistochemistry, sections were cleared in Histoclear II (National Diagnostics). Sections were counterstained with hematoxylin Q5 (Vector Laboratories), dehydrated in graded alcohol series, and processed for DAB-immunostaining following a standard protocol using Vectorstain Elite ABC kit (Vector Laboratories) for polyclonal antibodies and the Vector M.O.M. kit (Vector Laboratories) for mouse monoclonals and visualized by using DAB (Sigma). Sections were mounted with DPX (Fluka). Sections were analyzed in a Zeiss Axiophot microscope.

MNCV Measurements. Mice were anesthetized with ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively). Recording needle electrodes were placed in the foot pad, and supramaximal stimulation of sciatic nerves was performed distally at the ankle and proximally at the sciatic notch. Recordings were obtained on a PowerLab 4/25ST (AD Instruments) using Chart5 software. Conduction velocities were calculated as (proximal distance – distal distance)/ (proximal latency – distal latency), with latencies corresponding to the time lapse between the stimulus and the onset of the compound muscle action potential and expressed in meters per second.

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Supporting Information

Fig. S1. Astrocytosis in Phyh−/− mice due to phytanic acid accumulation. Immunohistochemical detection of glial fibrillary acidic protein (GFAP) in WT (A–C and G–I) and Phyh−/− (D–F and J–L) mice in the inferior colliculus (A–F), cerebellum (G and J), hippocampus (H and K), and spinal cord (I and L) of mice fed a control diet (A and D), 0.1% phytol diet (B and E), and 0.25% phytol diet (C, F, and G–L). Astrocytosis is clearly detected in the inferior colliculus of Phyh−/− mice on the 0.25% phytol diet (F) but absent in Phyh−/− mice fed the 0.1% phytol diet (E). Within the cerebellum, hippocampus, and spinal cord, the number and appearance of astrocytes were similar in WT and Phyh−/− mice. The slide was hematoxylin QS counterstained. (Scale bar: 100 μm.)
Fig. S2. Quantification of Purkinje cells in WT and Phyh⁻/⁻ mice fed control and phytol diets. Cerebellar sections were immunostained with an antibody against calbindin-D28K to visualize Purkinje cells. Phyh⁻/⁻ mice fed a phytol diet showed significant decreases in Purkinje cell numbers, with the lowest number of Purkinje cells in Phyh⁻/⁻ mice fed the 0.25% phytol diet. *, P < 0.01.
Fig. S3. Expression of parvalbumin by immunohistochemical detection in the cortex (A–F) and anterodorsal thalamic nuclei (ATN) (G–L) of WT (A–C and G–I) and Phyh<sup>−/−</sup> mice (D–F and J–L) on a control diet (A, D, G, and J), a 0.1% phytol diet (B, E, H, and K), and a 0.25% phytol diet (C, F, I, and L). The cerebral cortex of Phyh<sup>−/−</sup> mice fed the 0.25% phytol diet showed increased numbers of parvalbumin-positive neurons (M) as well as increased levels of parvalbumin (F). In the ATN, increased parvalbumin-reactivity was detected in Phyh<sup>−/−</sup> mice from all dietary groups (J–L), with the highest expression in Phyh<sup>−/−</sup> mice fed the 0.25% phytol diet (L). The slide was hematoxylin QS counterstained. (Scale bars: 30 μm in A–F and 100 μm in G–L. *, * < 0.05.)
Fig. S4. Peripheral neuropathy in Phyh<sup>+/−</sup> mice fed a phytol diet. (A) Motor nerve conductance velocity (MNCV) measurements revealed a decrease in Phyh<sup>+/−</sup> mice fed a 0.1% phytol diet for 8 weeks. * P < 0.05. (B) Compound muscle action potentials (CMAPs) recorded after stimulation at the sciatic notch showed a delay in onset and a longer duration in Phyh<sup>+/−</sup> mice fed the phytol diet. (C) Immunofluorescent detection of MBP in cross-sections of sciatic nerves from Phyh<sup>+/+</sup> and Phyh<sup>−/−</sup> mice fed the 0.1% and 0.25% phytol diets. (Scale bar: 10 μm.) (D) Immunohistochemical detection of MBP in longitudinal sections of sciatic nerves from Phyh<sup>+/+</sup> and Phyh<sup>−/−</sup> mice fed the 0.1% and 0.25% phytol diets. (Scale bar: 30 μm.)
Table S1. Clinical chemical parameters in pooled plasma from WT and Phyh\(^{+/−}\) male mice on a control or phytol-supplemented diet

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Albumin, g/L</th>
<th>ALAT, units/L</th>
<th>Amylase, units/L</th>
<th>Cholesterol, mmol/L</th>
<th>Triglycerides, mmol/L</th>
<th>Free FAs, mmol/L</th>
<th>Total FAs, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phyh(^{+/−})</td>
<td>Control</td>
<td>21</td>
<td>45</td>
<td>477</td>
<td>2.61</td>
<td>1.40</td>
<td>0.66</td>
<td>8,131 ± 681</td>
</tr>
<tr>
<td>Phyh(^{+/−})</td>
<td>0.1% Phytol</td>
<td>21</td>
<td>28</td>
<td>450</td>
<td>2.08</td>
<td>0.78</td>
<td>0.30</td>
<td>5,738 ± 539</td>
</tr>
<tr>
<td>Phyh(^{+/−})</td>
<td>0.25% Phytol</td>
<td>18</td>
<td>28</td>
<td>396</td>
<td>1.62</td>
<td>0.66</td>
<td>0.28</td>
<td>4,601 ± 840</td>
</tr>
<tr>
<td>Phyh(^{+/−})</td>
<td>Control</td>
<td>21</td>
<td>47</td>
<td>451</td>
<td>2.12</td>
<td>1.14</td>
<td>0.71</td>
<td>6,841 ± 1,423</td>
</tr>
<tr>
<td>Phyh(^{+/−})</td>
<td>0.1% Phytol</td>
<td>21</td>
<td>331</td>
<td>406</td>
<td>2.06</td>
<td>0.57</td>
<td>0.36</td>
<td>4,771 ± 1,056</td>
</tr>
<tr>
<td>Phyh(^{+/−})</td>
<td>0.25% Phytol</td>
<td>23</td>
<td>NA</td>
<td>571</td>
<td>1.83</td>
<td>0.32</td>
<td>0.24</td>
<td>3,267 ± 1,238</td>
</tr>
</tbody>
</table>

FA, fatty acids; NA, not analyzed.