Essential fatty acid deficiency and the small intestine
Lukovac, Sabina

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
ESSENTIAL FATTY ACID DEFICIENCY IN MICE IS ASSOCIATED WITH CHOLESTEROL MALABSORPTION AND INCREASED JEJUNAL LIPID SYNTHESIS

S. Lukovac¹, M.Y.M. van der Wulp¹, V.W. Bloks¹, M.V. Boekschoten²,³, J. Dekker³, A.K. Groen¹, E.H.H.M. Rings¹, H.J. Verkade¹

¹Pediatric Gastroenterology, Department of Pediatrics, Beatrix Children’s Hospital, Groningen University Institute for Drug Exploration (GUIDE), Center for Liver, Digestive and Metabolic Diseases, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands.

²Nutrigenomics Consortium, Top Institute Food and Nutrition, Wageningen, The Netherlands.

³Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands.

Manuscript in preparation
ABSTRACT

Essential fatty acid (EFA) deficiency in mice is associated with reduced small intestinal function, as demonstrated by fat malabsorption, impaired lactose digestion and altered small intestinal feedback regulation of bile salt synthesis. The effects of EFA deficiency on cholesterol metabolism in the small intestine remained unknown. Here, we studied the effects of EFA deficiency on jejunal cholesterol and fatty acid metabolism in a mouse model of EFA deficiency.

EFA deficiency was induced in mice by feeding an EFA-deficient diet for 8 weeks. EFA-deficient mice excreted 57% more cholesterol via the feces compared with control mice (p<0.05). A well-known surrogate marker for cholesterol absorption (plasma plant sterols/cholesterol ratio) was significantly reduced in EFA-deficient mice (-60%, p<0.05), accompanied by reduced jejunal mRNA expression of the apical sterol uptake transporter Npc1l1 (-70%, p<0.05). Plasma concentrations of major plant sterols derived from dietary sources were significantly lower in EFA-deficient mice (p<0.05). EFA deficiency did not affect total cholesterol concentrations in jejunal mucosa. Triglyceride and oleic acid concentrations were significantly increased in jejunum of EFA-deficient mice and lipid staining revealed lipid droplet accumulation in EFA-deficient jejunal epithelium. Transcriptional analysis of jejunum of EFA-deficient mice revealed significant induction of Srebpl and Srebp2 and its target genes involved in fatty acid and cholesterol synthesis. An unexpected observation from the transcriptional analysis of jejunal segments of EFA-deficient mice was induced gene expression of genes of the proteasome complex, suggestive for proteasome inhibition.

Our data show that EFA deficiency is associated with cholesterol malabsorption and subsequent increased jejunal lipid synthesis, which might serve as a compensatory mechanism for cholesterol and fatty acid malabsorption. Our observations suggest that during EFA deficiency in mice, jejunal proteasome degradation pathway is shut down in order to maintain the lipid homeostatic response in jejunal epithelium.
INTRODUCTION

Essential fatty acid (EFA) deficiency is associated with several functional abnormalities in broad range of tissues and organs in both humans and experimental animals.\textsuperscript{1,2,3,4} Studies in EFA-deficient mice and rats demonstrated several morphological and functional abnormalities in the small intestine, although some effects were species specific.\textsuperscript{5,6,7,8,9} In mice, we have shown that EFA deficiency is associated with reduced fat absorption, impaired lactose digestion and altered regulation of the enterohepatic circulation of bile salts.\textsuperscript{6,7,10} All together, these data strongly suggest that EFA deficiency negatively affects small intestinal function, and more specifically at the intracellular level.\textsuperscript{6} Very little is known about the effects of EFA deficiency on the cholesterol absorption, and small intestinal lipid metabolism in general. Christon et al. demonstrated that there was no effect of low linoleic diet on cholesterol content in enterocyte brush border membrane in rats.\textsuperscript{9} However, in the epidermal tissue, increased cholesterol synthesis in EFA-deficient rats was demonstrated by Proksch et al.\textsuperscript{2} In both rats and mice, EFA deficiency leads to development of hepatic steatosis, as indicated by elevated hepatic and reduced plasma triglyceride concentrations.\textsuperscript{4,11} Werner et al. previously excluded reduced VLDL-TG secretion from the liver in EFA-deficient mice as the cause of hepatic steatosis.\textsuperscript{4} Moreover, \textit{Ppara} and \textit{Ppara}-target genes (\textit{Acc1}, \textit{Cpt1a}) were upregulated in livers of EFA-deficient mice, probably due to induced \textit{de novo} synthesis of non-essential fatty acids.\textsuperscript{4} Therefore, hepatic triglyceride accumulation in EFA-deficient mice was most likely due to the increased lipogenic activity, increased uptake of circulating lipids, or a combination of both.

The aim of our study was to determine the effects of EFA deficiency on small intestinal function regarding cholesterol absorption. We hypothesized that impaired small intestinal function during EFA deficiency might lead to reduced absorption of cholesterol. Theoretically, increased lipid synthesis as shown in the liver and epidermis during EFA deficiency might exist in the small intestinal epithelium. Although the whole small intestine is capable of cholesterol absorption, the main site of absorption is the jejunum.\textsuperscript{12} Furthermore, regarding absorption of other nutrients, studies in EFA-deficient rats and mice revealed that delay in transport of fatty acids and lactose was most severe in the jejunal part of the small intestine.\textsuperscript{7,13} Therefore, in the present study we focused our attention on the effects of EFA deficiency on jejunum.

First, we demonstrated the physiological consequences of EFA deficiency on cholesterol metabolism by determining the fecal cholesterol excretion and sterol absorption in EFA-deficient mice. In addition to cholesterol metabolism, we also analyzed triglyceride and fatty acid levels in jejunum of EFA-deficient mice. Cholesterol and fatty acid metabolism are closely associated metabolic pathways and are known to have a cross talk mediated mainly by the sterol-regulatory element-binding proteins (SREBPs).\textsuperscript{14,15,16} In order to characterize the effects of the EFA deficiency on the metabolic pathways in jejunum, we analyzed the transcriptional response to the EFA deficiency in mice by microarray analysis.

Our data clearly show that EFA deficiency in mice leads to malabsorption of cholesterol, accompanied by an induction of the transcriptional cascade involved in cholesterol and fatty acid synthesis. We suggest that the reduced proteasome activity during EFA
deficiency is responsible for the induction of lipogenic gene expression via activation of the transcription factor SREBPs. The increased cholesterol and fatty acid synthesis suggest a compensatory mechanism for reduced cholesterol and fatty acid absorption in EFA-deficient mice.

**MATERIAL AND METHODS**

**Mice and diet**
FVB (free virus breed) male mice of eight weeks old were purchased from Harlan (Horst, the Netherlands) and were housed in a light- and temperature-controlled facility. Tap water and food were allowed *ad libitum*. At the age of eight weeks, two groups of mice were switched to either control (#4141.07) or EFA-deficient (#4141.08) diet for eight weeks, which were both custom synthesized by Arie Blok BV (Woerden, the Netherlands). Fatty acid composition of the diets is indicated in Table 1. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, Netherlands.

**Table 1** Composition of the major fatty acids in the experimental diets. Concentrations are indicated in mol% of total fatty acid concentrations determined by gas chromatography analysis.

<table>
<thead>
<tr>
<th>Fatty Acid (Chain Length:ω)</th>
<th>Control diet (#4141.07)</th>
<th>EFA-deficient diet (#4141.08)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid (16:0)</td>
<td>36</td>
<td>64</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Oleic acid (18:1ω-9)</td>
<td>31</td>
<td>13</td>
</tr>
<tr>
<td>Linoleic acid (18:2ω-6)</td>
<td>29</td>
<td>6</td>
</tr>
</tbody>
</table>

**Induction of EFA deficiency, bile cannulations and sample preparations**
EFA deficiency was induced in mice by means of an EFA-deficient diet for eight weeks, as described earlier. Eight weeks of diet were sufficient to induce EFA deficiency as determined by the (biochemical) marker for EFA deficiency, triene/tetraene ratio, in erythrocytes and plasma. At the end of the dietary period, food intake was measured and feces was collected during a 72h period. Subsequently, bile was cannulated for 30 minutes as previously described. At the end of the experiment, mice were anesthetized and sacrificed through cardiac puncture. The small intestine was rinsed with phosphate-buffered saline (PBS) and mid part, corresponding to jejunum, was sliced and immediately frozen in liquid nitrogen. Subsequently, small intestinal and hepatic tissue was stored at -80°C for further analysis.

**Cholesterol balance**
Cholesterol balance was determined by calculating the dietary intake and hepatobiliary secretion of cholesterol, along with fecal output of neutral sterols per day per 100 g of body weight, as previously described by van der Velde et al. Dietary cholesterol intake
and fecal neutral sterol excretion were analyzed during the 72 hours of feces collection and weighing of the food pellets in this period.

**Analytical methods**

**Analysis of biliary, dietary, fecal and plasma sterols**

Hepatobiliary secretion of cholesterol was analyzed by lipid extraction from bile, according to the method of Bligh and Dyer, and subsequent cholesterol measurement according to Gamble et al.\(^1\)\(^8\),\(^19\) Food pellets and fecal samples were grind and 50 mg was prepared for neutral sterol analysis by gas chromatography as described previously.\(^2\)\(^0\) Neutral sterol profile and concentrations in plasma were analyzed by gas chromatography mass spectrometry as described previously.\(^2\)\(^1\)

**Determination of lipids in total jejunal mucosa homogenate**

Thirty milligram of intestinal mucosa was homogenized in 200 μl of 0.9% NaCl, and lipids were extracted according to Bligh and Dyer.\(^1\)\(^8\) Triglyceride and cholesterol concentrations were determined by means of commercially available kits (Roche Diagnostics, Mannheim, Germany; DiaSys Diagnostic Systems, Holzheim, Germany).

**Determination of fatty acids in total jejunal mucosa**

Thirty milligram of intestinal mucosa was homogenized in 200 μl of 0.9% NaCl. Subsequently, ten microliter of the homogenate was used for fatty acid determination according to Muskiet et al., after the addition of the internal standard (C17:0) and antioxidant butylated hydroxytoluene.\(^2\)\(^2\) Total homogenate was used for fatty acid profile and quantification by gas chromatography.

**Histology**

Jejunal lipids were examined on frozen jejunal sections after Oil Red O staining for neutral lipids by standard procedures.

**RNA isolation and measurement of RNA expression levels by microarray analysis**

For the microarray analysis, the Affymetrix microarray platform was used. After RNA isolation from jejunal tissue with TRIzol reagent, RNA was used individually and further purified using RNeasy MinElute micro columns (Qiagen, Venlo, the Netherlands). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) using 6000 Nano Chips according to the manufacturer’s instructions. RNA was judged as suitable for array hybridization only if samples exhibited intact bands corresponding to the 18 and 28S ribosomal RNA subunits, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number 7.0-8.5). Five hundred nanograms of RNA were used for one cycle cRNA synthesis (Affymetrix, Santa Clara, CA). Control and EFA-deficient samples (n=6) were hybridized, washed and scanned on the Affymetrix Gene chip mouse 1.0 ST arrays, according to standard Affymetrix protocols. Scans of the Affymetrix arrays were processed using packages from the Bioconductor project.\(^2\)\(^3\) Quality control of microarray data (using simpleaffy and affyplm packages), normalization, differential expression analysis and Gene Set Enrichment Analysis were performed through the Management and Analysis Database.
for MicroArray eXperiments (MADMAX) analysis pipeline, (Wageningen, the Netherlands). Expression levels of probe sets were calculated using regular normalization strategies: VSN in jejunum followed by identification of differentially expressed probe sets using Limma.\textsuperscript{24} Comparison was made between treated (EFA-deficient mice) and untreated (control mice) groups (Limma package, applying linear models and moderated t-statistics that implement empirical Bayes regularization of standard errors.\textsuperscript{25} False discovery rate (FDR) of 1 % (p <0.01) was used as a threshold for significance of differential expression. The limma t-test values of differential expression between groups where used as the input for the PreRanked scoring method within the Gene Set Enrichment Analysis (GSEA).\textsuperscript{26} Normalized enrichment scores (NES) of significantly enriched pathways and the corresponding FDR p-values are available upon request. Identification of overrepresented functional categories among responsive genes and their grouping into functionally related clusters (Biological Processes:BP-4) was performed using DAVID Functional Annotation Clustering tool.\textsuperscript{27} All microarray data reported in the manuscript is described in accordance with MIAME guidelines.\textsuperscript{28}

Quantitative PCR
Total RNA was prepared from mouse intestinal (jejunum) or hepatic tissue using TRIzol reagent (Invitrogen, Breda, The Netherlands). Subsequently, cDNA synthesis and quantitative PCR analysis were performed as described by Greffhorst et al.\textsuperscript{29} PCR results were normalized to RNA expression of the housekeeping gene \textit{18S}. Primer and probe sequences for the Q-PCR analysis have been published (www.LabPediatricsRug.nl: Realtime PCR Primers & Probes Database), except for \textit{Ehhadh} (GeneID 74147, NM_023737; forward primer: GCCCTTTCTGTGCACCAATACC; reverse primer: GAAGAAGTGGGTGCCAATCAC; probe: CATTGCTTCTTCCACAGATCGCCC).

Statistical analysis
Using SPSS version 16 statistical software (Chicago, IL, USA), we calculated significance of differences between EFA-deficient and control (FVB) mice with the Mann-Whitney \textit{U}-test and p-values below 0.05 were considered statistically significant. All data represent mean values ± SD. Statistical analysis for the microarray analysis is described in the section above (RNA isolation and measurement of RNA expression levels by microarray analysis).

RESULTS
EFA-deficient mice showed increased biomarker of EFA deficiency (triene:tetraene ratio) in erythrocytes and jejunal tissue, which exceeded the threshold value for EFA deficiency (>0.2; data not shown). In agreement with previous observations, EFA-deficient mice showed reduced fat absorption without significant changes in body weight or food intake (data not shown) after eight weeks of EFA-deficient diet.\textsuperscript{6}

\textbf{EFA deficiency in mice is associated with cholesterol malabsorption}
In order to determine the physiological relevance of EFA deficiency on jejunal cholesterol
absorption, we measured cholesterol excretion in feces of EFA-deficient mice. Neutral sterol analysis in fecal samples of EFA-deficient mice after 72h of collection revealed significantly higher concentrations of cholesterol and dihydroxycholesterol compared with control mice (Figure 1a, 1b). In addition, we analyzed the plasma sterol concentrations to determine whether EFA deficiency is associated with reduced concentrations of plant sterols (phytosterols) in plasma, which can only be derived from the dietary sources, thus from the intestinal uptake. Cholesterol measured in plasma, on the other hand, can, in addition to intestinal uptake, be derived from the periphery by means of the reversed cholesterol transport. Our plant sterol analysis in plasma show significantly lower concentrations of two major plant sterols campesterol and β-sitosterol, and of cholestanol (Figure 1c).31

**Figure 1** Cholesterol concentrations in feces and plasma of EFA-deficient (white bars) and control (black bars) mice. (a) Fecal cholesterol and (b) dihydroxycholesterol concentrations were determined by gas chromatography subsequent to 72h feces collection. (c) Plasma sterol profile was determined by gas chromatography-mass spectrometry; from different sterol concentrations in plasma, (d) marker for cholesterol absorption (as indicated by the plant sterol/cholesterol ratio) and (e) markers for cholesterol synthesis (as indicated by the lathosterol/cholesterol and desmosterol/cholesterol ratio) were calculated. (f) Total cholesterol concentrations were determined in jejunal mucosa. Values are means ±SD for n=5-6. *p<0.05 is the significant difference between the two groups.

Concentrations of lanosterol and desmosterol in plasma were significantly increased in EFA-deficient mice compared with control mice. Furthermore, the marker of cholesterol absorption, the ratio of plant sterols (campesterol+β-sitosterol) to cholesterol was significantly decreased in EFA-deficient mice (Figure 1d).32 Most commonly used marker for cholesterol synthesis, lathosterol/cholesterol ratio, was not different between EFA-deficient and control mice (Figure 1e). However, another marker for cholesterol synthesis (desmosterol/cholesterol ratio) was significantly higher in EFA-deficient mice (Figure 1e). Total cholesterol concentration in jejunal mucosa was not affected by EFA deficiency in mice (Figure 1f).
**Chapter 3**

**Cholesterol balance in EFA-deficient mice**

In order to determine the cholesterol intake and excretion, we measured the food intake and collected feces during 72h. In parallel, bile was collected by means of bile cannulation to determine the hepatobiliary secretion of cholesterol. Total cholesterol balance shows that there is no significant difference in cholesterol intake between EFA-deficient and control mice (Table 2).

**Table 2** Cholesterol balance (dietary ingestion, hepatobiliary secretion, fecal output; μmol/day/100g body weight). Data represent means ± SD of 4-6 mice per group. *p<0.05 is the significant difference between EFA-deficient and control mice after eight weeks of EFA-deficient or control diet, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EFA-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary cholesterol intake</td>
<td>2.1 ± 0.3</td>
<td>2.2 ± 0.8</td>
</tr>
<tr>
<td>Biliary cholesterol secretion</td>
<td>1.6 ± 0.4</td>
<td>3.1 ± 1.3 *</td>
</tr>
<tr>
<td>Fecal neutral sterol excretion</td>
<td>3.2 ± 0.3</td>
<td>8.8 ± 3.0 *</td>
</tr>
<tr>
<td>TICE</td>
<td>range=(-0.8)-(0.2)</td>
<td>range=(-1.3)-(7.4)</td>
</tr>
</tbody>
</table>

Biliary secretion of cholesterol was significantly higher in EFA-deficient mice, in agreement with previous studies in EFA-deficient mice (Table 2). However, fecal excretion of cholesterol is extremely increased in EFA-deficient mice to such an extent that it is even higher than the sum of cholesterol derived from the intake and the cholesterol of hepatobiliary origin (Table 2). This suggests that the alternative route of cholesterol excretion from the intestine, transintestinal cholesterol efflux (TICE)-route, might be responsible for the missing cholesterol excreted in EFA-deficient mice (Table 2). However, our indirect measurement of TICE did not reach the significant difference between EFA-deficient and control mice due to the large variation within the groups (p=0.07) (Table 2).

**EFA deficiency in mice leads to increased oleic acid and triglyceride concentrations in jejunal mucosa**

Molar concentrations of polyunsaturated fatty acids (PUFA) were significantly reduced, while molar concentrations of monounsaturated fatty acids (MUFA) were increased in jejunal mucosa of EFA-deficient mice (Figure 2a). EFA deficiency had no effect on the molar concentrations of the saturated acids (SAFA) in mouse jejunum (Figure 2a). Decreased molar concentrations of PUFA in jejunum of EFA-deficient mice were mainly due to lower molar concentrations of linoleic acid (18:2ω-6) and its metabolite arachidonic acid (20:4ω-6) (Figure 2b). Fatty acid profile revealed significantly increased molar concentrations of 18:1ω9 and 18:1ω7, while the molar concentration of the precursor of the synthesis of these two fatty acids, stearic acid (18:0) was decreased in EFA-deficient mice (Figure 2b). Decrease in molar concentrations of palmitate (18:0) were accompanied by increased molar concentration of oleic acid (18:1ω-9), leading to...
Figure 2 Fatty acids and triglycerides in jejunal mucosa of EFA-deficient (white bars) and control (black bars) mice. (a) Saturated, monounsaturated and polyunsaturated fatty acid concentrations, as well as (b) the concentrations of the individual fatty acids are indicated as molar percentages of total fatty acid concentrations. (c) Desaturation index is determined by the oleic acid/stearic acid ratio. (d) Total triglyceride concentrations were determined in the freshly scraped jejunal mucosa. (e) Elevated fat accumulation in EFA-deficient jejunum is indicated by the representative picture of the Oil Red O staining (40x magnification). Values are means ±SD for n=4-6. *p<0.05 is the significant difference between the two groups.
significantly increased desaturation index (18:1ω-9/18:0) in both plasma and jejunum (Figure 2c) of EFA-deficient mice. Triglyceride concentrations in jejunal mucosa were increased by more than six fold (Figure 2d), consistent with accumulation of lipid droplets in jejunal tissue of EFA-deficient mice, as demonstrated by the Oil Red O staining (Figure 2e).

**EFA deficiency in mice is associated with inhibition of the proteasomal activity**

Overall microarray analysis revealed in total 1388 of the jejunal genes which were differentially regulated during EFA deficiency in mice, when the false discovery rate (FDR) of <1% was used as a threshold for significance of differential expression. From these genes, 857 were upregulated and 531 were down regulated in jejunal tissue during EFA deficiency. DAVID annotation of the microarray analysis pointed the attention on the proteasome (Table 3, bold fond). The importance of the proteasomal pathway in this model was confirmed by GSEA (NES=2.66), Metacore GeneGo (P<1e-7) and ErmineJ (raw score 3.38, P=1.67e-11) (data not shown, available upon request). Over 20 members of the genes of the proteasome complex were significantly upregulated in jejunum of EFA-deficient mice (Supplementary Table; p<0.05). Within the top of most affected pathways in EFA-deficient mice were also biosynthesis of steroids and polyunsaturated fatty acids, as well as the fatty acid biosynthesis in general (Table 3).

**Table 3** Cellular pathways enriched during EFA deficiency in mouse jejunum – microarray analysis (KEGG-Pathway). False discovery rate (FDR) <1%. Proteasome pathway, with the largest number of genes affected, is indicated in bold font.

<table>
<thead>
<tr>
<th>KEGG-pathways</th>
<th>Number of genes</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteasome</td>
<td>22</td>
<td>5.8E-15</td>
</tr>
<tr>
<td>Biosynthesis of steroids</td>
<td>14</td>
<td>2.6E-8</td>
</tr>
<tr>
<td>Fatty acid metabolism</td>
<td>16</td>
<td>5.8E-6</td>
</tr>
<tr>
<td>Polyunsaturated fatty acid biosynthesis</td>
<td>10</td>
<td>8.4E-6</td>
</tr>
</tbody>
</table>

**EFA deficiency in mice increased transcriptional activity of jejunal genes involved in lipid metabolism**

Based on the Gene Ontology categorization, EFA deficiency mainly affects metabolic processes (Table 4). In top 10 of the processes most significantly enriched during EFA deficiency in mice, lipid biosynthethic processes and processes involved in steroid and fatty acid metabolism were listed (Table 4, indicated in bold font). This was in agreement with the enrichment data of the most affected pathways (Table 3).

**EFA deficiency in mice is associated with enhanced transcription of genes involved in jejunal cholesterol synthesis**

Microarray analysis of jejunal tissue revealed that many genes involved in sterol metabolic processes were enriched in EFA-deficient mice (Table 4). More detailed analysis revealed that the expression of several genes important for cholesterol
absorption and efflux was affected by EFA deficiency (Table 5). The mRNA expression of Niemen-Pick C1-like 1 protein (Npc1l1), critical player in the absorption of intestinal sterol expressed at the apical surface of enterocytes, was decreased in jejunum of EFA-deficient mice (Table 5).\textsuperscript{12} The mRNA expression of the basolateral ATP-binding cassette (ABC) protein Abca1, which mediates HDL secretion from enterocytes, was decreased in jejunal tissue of EFA-deficient mice (Table 5).\textsuperscript{35} The mRNA expression of two other cholesterol transporters scavenger receptor class B, member 1 (Sr-B1; Scarb1) and low density lipoprotein receptor (Ldlr), both implied in basolateral cholesterol uptake into the enterocytes, was increased upon EFA deficiency in mouse jejunum (Table 5).\textsuperscript{35,36}

\textbf{Table 4 Biological processes enriched during EFA deficiency in mouse jejunum – microarray analysis} (GOTERM_BP_4). False discovery rate (FDR) <1%. Processes involved in steroid and fatty acid metabolism are indicated in bold font.

<table>
<thead>
<tr>
<th>GOTERM_BP_4: Biological processes</th>
<th>Number of genes</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular lipid metabolic process</td>
<td>104</td>
<td>3,7E-25</td>
</tr>
<tr>
<td>Carboxylic acid metabolic process</td>
<td>88</td>
<td>8,1E-20</td>
</tr>
<tr>
<td>Lipid biosynthetic process</td>
<td>52</td>
<td>1,5E-14</td>
</tr>
<tr>
<td>Fatty acid metabolic process</td>
<td>41</td>
<td>3,0E-14</td>
</tr>
<tr>
<td>Sterol biosynthetic process</td>
<td>24</td>
<td>6,0E-11</td>
</tr>
<tr>
<td>Steroid metabolic process</td>
<td>34</td>
<td>1,1E-10</td>
</tr>
<tr>
<td>Steroid biosynthetic process</td>
<td>22</td>
<td>8,8E-10</td>
</tr>
<tr>
<td>Isoprenoid biosynthetic process</td>
<td>11</td>
<td>3,1E-8</td>
</tr>
<tr>
<td>Isoprenoid metabolic process</td>
<td>13</td>
<td>6,3E-7</td>
</tr>
<tr>
<td>Coenzyme metabolic process</td>
<td>31</td>
<td>7,5E-7</td>
</tr>
</tbody>
</table>

The differences in mRNA expression of the jejunal cholesterol transporters between EFA-deficient and control mice detected by the microarray analysis were validated by the quantitative PCR (Q-PCR) analysis. Q-PCR analysis demonstrates decreased mRNA of Npc1l1 and Ldlr, while no significant difference in the mRNA expression of the Abca1 between EFA-deficient and control mice was detected (Figure 3a). There was no significant difference in mRNA expression of Abcg5/8 in jejunum of EFA-deficient mice compared with control mice, as demonstrated by both the microarray (Table 5) and Q-PCR analysis (data not shown).

Microarray analysis revealed that 34 jejunal genes involved in steroid metabolic process were enriched in EFA-deficient mice (Table 4). Detailed analysis of the microarray data shows that the mRNA expression of the rate controlling enzyme in the cholesterol synthesis, 3-hydroxy-3-methyl-glutaryl-CoA reductase (Hmgcr), was significantly increased in EFA-deficient mice (Table 5). In agreement, the mRNA expression of other relevant genes in the cholesterol biosynthesis pathway, soluble 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (Hmgcs1, soluble) and 3-hydroxy-3-
methylglutaryl-Coenzyme A synthase 2 (Hmgcs2, mitochondrial), were increased in EFA-deficient mouse jejunum (Table 5). When cellular cholesterol concentrations decrease, SREBP is released from the ER or nuclear membrane and transported to the nucleus where it binds to the sterol regulatory element on the HMGCR-gene. As the result, transcription of the HMGCR gene is initiated. Microarray analysis demonstrated induction of the Srebp2 mRNA expression in jejunal tissue of EFA-deficient mice (Table 5). Quantitative PCR analysis confirmed the increased mRNA expression of Hmgcr, Hmgcs1, Hmgcs2 and Srebp2 (Figure 3b). Moreover, Q-PCR analysis of the hepatic Hmgcr revealed significantly higher expression in EFA-deficient mice compared with control mice (Figure 3c). The mRNA expression of the liver X receptors Lxrα (Nr1h3) and Lxrβ (Nr1h2), other important transcriptional regulators of cholesterol metabolism, were also significantly higher in jejunum of EFA-deficient mice (Table 5).

Table 5 Fold changes of genes involved in intestinal cholesterol transport and synthesis – microarray analysis. Fold changes are indicated by the arrows in the direction of up- (↑) or downregulation (↓) of gene expression. False discovery rate (FDR) <1%. mRNA expression of genes indicated with an asterix (*) has been confirmed by the Q-PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description and protein ID</th>
<th>Fold change EFA-deficient versus control mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol transport</td>
<td>Abcg5/6, Srebl (Scarb1), Abca1, Npc1l1, Uldr</td>
<td>No change*</td>
</tr>
<tr>
<td></td>
<td>ATP-binding cassette sub-family G member 5/6, Scavenger receptor class B, member 1, ATP-binding cassette, sub-family A (ABC1), member 1, Low density lipoprotein receptor</td>
<td>1.2, 1.6* (NS in Q-PCR), 1.8*, 2.0*</td>
</tr>
<tr>
<td>Cholesterol synthesis</td>
<td>Lxra (Nr1h3), Lxrb (Nr1h2), Srebp2 (Srebf2), Hmgcr, Hmgcs1, Hmgcs2</td>
<td>1.2, 1.4, 1.3*, 1.5*, 1.9*, 3.0*</td>
</tr>
</tbody>
</table>

**EFA deficiency in mice is associated with enhanced transcription of genes involved in jejunal lipogenesis and beta oxidation**

Previous studies in mouse model of EFA deficiency revealed hepatic steatosis characterized by triglyceride accumulation in hepatic tissue. In parallel to these physiological changes, EFA deficiency in mice was accompanied by increased hepatic mRNA expression of genes involved in lipogenesis and beta oxidation. Microarray analysis in present study demonstrated that 41 genes involved in fatty acid metabolic processes were enriched in jejunum of EFA deficient mice. In EFA-deficient mice, the mRNA expression of almost all lipogenic genes was increased in jejunum (Table 6). More specifically, the mRNA expression of Scd1, Acc2 and Fasn was significantly increased in jejunum of EFA-deficient mice, as determined by both the microarray and Q-PCR analysis. (Table 6; Figure 4a). Scd1 gene encodes the enzyme responsible for the conversion of stearic acid (18:0) into its metabolite oleic acid (18:1ω9). Thus, the
increased mRNA expression of Scd1 in EFA-deficient mouse jejunum was in agreement with the increased desaturation index (marker for the activity of SCD1) in plasma of EFA-deficient mice (Figure 2c). In addition, the jejunal mRNA expression of Srebpl, transcription factor regulating fatty acid synthesis, was also induced by EFA deficiency (Table 6). The mRNA expression of Gpam, involved in triglyceride synthesis, was more than three times higher in EFA-deficient mice compared with control mice (Table 6, Figure 4a). This is in agreement with increased triglyceride concentrations in mouse jejunal mucosa (Figure 2d). Moreover, jejunal mRNA expression of most genes involved in fatty acid oxidation was significantly increased in EFA-deficient mice, as demonstrated by the microarray analysis (Table 6). Significantly increased mRNA expression of two relevant beta oxidation genes, Ehhadh and Cpt1a, was confirmed by means of Q-PCR analysis (Figure 4b).

**Figure 3** Jejunal expression of genes involved in (a) cholesterol transport and (b) synthesis in EFA-deficient (white bars) versus control (black bars) mice. (c) Hepatic mRNA expression of Hmgcr in EFA-deficient (white bars) and control (black bars) mice. Data represent mRNA expression relative to the RNA expression of the housekeeping gene 18S. Values are means ±SD for n=6. *p<0.05 is the significant difference between the two groups.

**DISCUSSION**

In the present study, we addressed the effects of EFA deficiency on jejunal lipid metabolism in a mouse model of EFA deficiency. Previous studies in this model suggested impaired small intestinal function during EFA deficiency. However, the effects of EFA deficiency on jejunal cholesterol metabolism remained unclear. Our data indicate
that EFA deficiency in mice leads to reduced \textit{Npc1l1} mRNA expression in jejunal epithelium and subsequent cholesterol malabsorption. Transcriptional analysis of jejunal tissue of EFA-deficient mice revealed increased transcription of proteasomal genes, accompanied by increased \textit{Srebp2} mRNA expression. Increased jejunal \textit{Srebp2} expression in EFA-deficient mice was accompanied by increased transcription of its target genes involved in cholesterol synthesis. In addition to alterations in cholesterol metabolism, EFA deficiency in mice was associated with increased triglyceride and oleic acid concentrations in jejunal mucosa, supported by increased transcription of lipogenic genes. We propose that during EFA deficiency jejunal tissue induces compensatory mechanisms to correct for reduced lipid absorption. By shutting down the proteasomal complex, \textit{Srebp2} activation is increased leading to induction of target genes involved in cholesterol and fatty acid metabolism. These data underscore previous findings suggesting that EFA deficiency leads to altered small intestinal function. Previous studies in mouse model of EFA deficiency revealed negative effects on the small intestine,

\begin{table}[h]
\begin{center}
\begin{tabular}{|l|l|l|}
\hline
\textbf{Gene} & \textbf{Description and protein ID} & \textbf{Fold change EFA-deficient versus control mice} \\
\hline
\hline
\textit{Lipogenesis} &  &  \\
\textit{Srebp1 (Sreb1)} & Sterol regulatory element binding transcription factor 1 & ↑1.3 \\
\textit{Pparg1b} & PPAR gamma, coactivator 1 beta & ↑1.4 \\
\textit{Elov5} & ELOVL family member 5 & ↑1.4 \\
\textit{Elov6} & ELOVL family member 6 & ↑1.5 \\
\textit{Fads1} & Fatty acid desaturase 1 & ↑1.6 \\
\textit{Acc1 (Acac)} & Acetyl-Coenzyme A carboxylase alpha & ↑1.7 \\
\textit{Fasn} & Fatty acid synthase & ↑1.9* \\
\textit{Fads2} & Fatty acid desaturase 2 & ↑2.7 \\
\textit{Aoc2 (Acacb)} & Acetyl-Coenzyme A carboxylase beta & ↑2.7* \\
\textit{Gpm1} & Glycerol-3-phosphate acyltransferase (mitochondrial) & ↑3.5* \\
\textit{Scd1} & Stearoyl-Coenzyme A desaturase 1 & ↑4.8* \\
\hline
\textbf{β-oxidation} &  &  \\
\textit{Decr1} & 2.4 Dienoyl CoA reductase & ↑1.2 \\
\textit{Ech1} & Enoyl CoA hydratase (peroxisomal) & ↑1.2 \\
\textit{Echs1} & Enoyl CoA hydratase (mitochondrial) & ↑1.2 \\
\textit{Gpr8} & Carnitine palmitoyltransferase 2 & ↑1.3 \\
\textit{Acaa1} & Acyl-CoA:Dehydrogenase, long chain & ↑1.4 \\
\textit{Dci} & Enoyl CoA isomerase & ↑1.5 \\
\textit{Cpt1a} & Carnitine palmitoyltransferase 1 & ↑1.6* \\
\textit{Ehhadh} & L-β-hydroxyacyl CoA dehydrogenase & ↑1.9* \\
\hline
\end{tabular}
\end{center}
\caption{Fold changes of genes involved in intestinal lipogenesis and β-oxidation – microarray analysis. Fold changes are indicated by the arrows in the direction of up- (↑) or downregulation (↓) of gene expression. False discovery rate (FDR) <1%. mRNA expression of genes indicated with an asterix (*) has been confirmed by the Q-PCR analysis.}
\end{table}

including reduced fat absorption and impaired lactose digestion.\textsuperscript{6,7,10} Therefore, we hypothesized that EFA deficiency additionally might lead to reduced cholesterol absorption. In order to test this hypothesis we measured fecal cholesterol excretion in EFA-deficient mice, which appeared to be 50% higher in EFA-deficient mice compared with control mice. In agreement, the marker for cholesterol absorption was decreased by 59\% in EFA-deficient mice. Besides the reduced absorption, increased dietary intake or increased biliary secretion of cholesterol to the intestinal lumen might be the cause of the
increased fecal excretion of cholesterol. Therefore, we determined these parameters by measuring both the dietary intake and the hepatobiliary secretion of cholesterol. Although the dietary intake was similar between EFA-deficient and control mice, EFA deficiency significantly increased hepatobiliary secretion of cholesterol, in agreement with previous data in EFA-deficient mice.\(^6\) However, the increased hepatobiliary secretion of cholesterol was not sufficient to compensate for the difference in fecal excretion of cholesterol between EFA-deficient and control mice. This suggested the presence of an alternative route for cholesterol secretion in EFA-deficient mice, besides the hepatobiliary route. Theoretically, increased cholesterol concentration in feces of EFA-deficient mice could be due to the increased shedding of the small intestinal enterocytes. However, this is rather unlikely since previous studies in EFA-deficient mice showed by means of Ki67 staining in jejunum that there were no significant differences in proliferative capacity of jejunal enterocytes between EFA-deficient and control mice.\(^7\)

Recently, several studies in mice described that small intestine plays a significant role in removal of cholesterol from the body and that the capacity of the transintestinal cholesterol efflux (TICE) is sufficient to account for the missing cholesterol in the balance studies.\(^{40,41}\) It is possible that increased TICE in EFA-deficient mice is responsible for increased cholesterol excretion. Interestingly, previous study in mice demonstrated that increased mRNA expression of Sr-BI (Scarb1) correlated with TICE; in EFA-deficient mice we show that Sr-BI expression is increased, along with increased TICE.\(^{42}\) However, direct measurement of TICE by perfusion of isolated jejunal segments of the small intestine in Sr-BI deficient mice revealed a significant, twofold increase in TICE compared with wild-type mice.\(^{43}\) The underlying mechanism of this discrepancy remained unclear. Future studies with direct TICE measurements by perfusion of the intestinal segments should reveal whether EFA deficiency increases TICE and in which part of the small intestine. Jejunal Abca1 mRNA expression was reduced in EFA-deficient mice, probably as the result of reduced cholesterol absorption and to maintain

---

**Figure 4** Jejunal expression of genes involved in (a) fatty acid synthesis and (b) beta oxidation in EFA deficient (white bars) versus control (black bars) mice. Data represent mRNA expression relative to the RNA expression of 18S. Values are means ±SD for n=6. *p<0.05 is the significant difference between the two groups.
sufficient cholesterol levels within the enterocytes. However, negative regulation of Abca1 by unsaturated fatty acids has previously been described.\textsuperscript{44} Therefore, reduced expression of Abca1 which might also be the result of increased jejunal oleic acid concentration. In addition to cholesterol malabsorption in EFA-deficient mice, our data clearly demonstrate that EFA deficiency in parallel leads to the induction of mRNA expression of majority of genes involved in cholesterol and fatty acid metabolism. In addition, microarray analysis demonstrated increased gene expression of the proteasomal pathway in jejunum during EFA deficiency. Increased gene expression of proteasomal complex leads to negative feedback regulation of the proteasomal activity, thus inhibition of the proteasome degradation. Theoretically, this could lead to prolonged expression and activity of certain cellular proteins and transcription factors. Previous studies revealed increased Srebp activity upon inhibition of the ubiquitin-proteasome pathway.\textsuperscript{45} Furthermore, SREBPs are shown to be possible substrates for the ubiquitin-proteasome system, which in turn controls the expression of SREBP-responsive genes.\textsuperscript{46} Surprisingly, mRNA expression of Srebp was demonstrated to be increased in jejunal tissue of EFA-deficient mice, leading to increased mRNA expression of its target genes involved in both cholesterol and fatty acid metabolism. Although we did not measure direct intestinal cholesterol synthesis, it has been previously shown that mRNA levels of HMGCR strongly correlate with changes in cholesterol synthesis in several tissues.\textsuperscript{47} We additionally demonstrated increased expression of HMGCR in the liver of EFA-deficient mice, suggesting that increased cholesterol synthesis also takes place in the hepatic tissue during EFA deficiency. Moreover, Proksch et al. demonstrated increased cholesterol synthesis in epidermal tissue of EFA-deficient mice. Compensatory increase of cholesterol synthesis in jejunum could explain maintained jejunal cholesterol concentrations in EFA-deficient mice, despite the malabsorption of cholesterol. One of the markers of the cholesterol synthesis, namely plasma ratio of lathosterol to cholesterol, was not affected by EFA deficiency. However, the ratio of two other cholesterol precursors, desmosterol and lanosterol, to cholesterol was increased in plasma of EFA-deficient mice. Plasma markers determined do not discriminate between the intestinal cholesterol synthesis and that in other (i.e. hepatic) tissues, and are therefore not suitable as specific jejunal markers of cholesterol synthesis.

In agreement with induced jejunal expression of target genes of Srebp involved in fatty acid metabolism, we demonstrate that physiological consequence of increased lipogenic genes during EFA deficiency leads to increased oleic acid and triglyceride concentrations. Lack of EFA in the tissues is known to be demarcated by an increased synthesis of the non-essential fatty acid oleate. Increased oleic acid synthesis in jejunal mucosa of EFA-deficient mice is most likely originating from its precursor palmitic acid. Preliminary study of Hamel suggested that oleic acid itself might inhibit the proteasomal pathway.\textsuperscript{48} Whether EFA deficiency directly inhibits the proteasomal complex gene expression, or indirectly via increased oleic acid synthesis, remains to be elucidated. Although jejunal tissue seems to compensate for the reduced absorption of lipids, lipids accumulation occurs within the jejunum. Previous studies in EFA-deficient mice revealed smaller chylomicrons in these mice, which could explain reduced jejunal capacity to secrete the synthesized lipids.\textsuperscript{49}
Increased fatty acid synthesis and oxidation during EFA deficiency are not specific for the jejunum. Werner et al. demonstrated earlier that similar pathways and genes are induced by EFA deficiency in mouse hepatic tissue. In both liver and jejunum triglyceride concentrations are severely increased during EFA deficiency in parallel to increased mRNA expression of *Gpam*, essential for triglyceride synthesis. Previous studies in mice fed high fat diet revealed increased triglyceride accumulation in the liver which was attributed to the high dietary content of the saturated fatty acids. EFA-deficient diet contains more saturated fatty acids palmitate and stearate, thus the effects seen in jejunal lipid metabolism could be, at least in part, due to increased dietary intake of saturated fat. However, since fat balance studies revealed overall malabsorption of both saturated and unsaturated fatty acids, triglyceride accumulation cannot exclusively be due to the increased intake of saturated fatty acids.

Altogether, our data show that EFA deficiency leads to cholesterol malabsorption. Metabolic pathways involved in jejunal lipid synthesis are activated in order to compensate for the malabsorption of both cholesterol and fatty acids. However, we suggest that elevated synthesis is not effective in improving the absorption of fatty acids and cholesterol, since most of the lipids accumulate within the enterocytes. In order to improve the nutritional status of patients with EFA deficiency, future studies should focus on the mechanism underlying lipid accumulation during EFA deficiency and improvement of the intestinal function.

**ACKNOWLEDGEMENTS**

The authors thank Ingrid Martini, Renze Boverhof, Juul Baller and Gertrud Kortman for their excellent technical assistance. Mechted Grootte-Bromhaar is acknowledged for her excellent technical assistance with microarray analyses. Part of this study was supported by the Dutch Digestive Foundation and Top Institute Food and Nutrition (TIFN; Wageningen, the Netherlands).

**GRANTS**

This study was supported by the Dutch Digestive Foundation (MLDS).

**REFERENCES**

22 Muskiet FA, van Doormaal JJ, Martini IA, Wolthers BG and van der Silk W. *J Chromatogr* 1983; 278(2):231-244.


Kuhn DJ, Burns AC, Kazi A and Ping Dou Q. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 2004; 1682(1-3):1-10.


**Supplementary table** DAVID software analysis of the KEGG-pathways enriched in EFA-deficient mouse jejunum reveal proteasome complex genes as the most significantly enriched cellular process upon EFA deficiency. Fold change of the expression of these genes is indicated for false discovery rate (FDR) <1%.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change EFA-deficient versus control mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psmd7</td>
<td>1.5</td>
</tr>
<tr>
<td>Psmd4</td>
<td>1.5</td>
</tr>
<tr>
<td>Psmd12</td>
<td>1.4</td>
</tr>
<tr>
<td>Psmd6</td>
<td>1.4</td>
</tr>
<tr>
<td>Psmd3</td>
<td>1.4</td>
</tr>
<tr>
<td>Psmb7</td>
<td>1.4</td>
</tr>
<tr>
<td>Psmb4</td>
<td>1.3</td>
</tr>
<tr>
<td>Psmd2</td>
<td>1.3</td>
</tr>
<tr>
<td>Psmb2</td>
<td>1.3</td>
</tr>
<tr>
<td>Psma7</td>
<td>1.3</td>
</tr>
<tr>
<td>Psma6</td>
<td>1.3</td>
</tr>
<tr>
<td>Psma1</td>
<td>1.3</td>
</tr>
<tr>
<td>Psmc2</td>
<td>1.3</td>
</tr>
<tr>
<td>Psmd14</td>
<td>1.3</td>
</tr>
<tr>
<td>Psmb5</td>
<td>1.3</td>
</tr>
<tr>
<td>Psmc3</td>
<td>1.2</td>
</tr>
<tr>
<td>Psmb6</td>
<td>1.2</td>
</tr>
<tr>
<td>Psmc1</td>
<td>1.2</td>
</tr>
</tbody>
</table>