Essential fatty acid deficiency in mice impairs lactose digestion


* both authors contributed equally to the study

Pediatric 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

Running head: EFA deficiency and intestinal function

Address correspondence and reprint requests to:
H.J. Verkade, MD, PhD
Pediatric Gastroenterology, Department of Pediatrics, Centre for Liver, Digestive and Metabolic Diseases, CMC IV, Room Y4.107a, University Medical Center Groningen
P.O. Box 30.001, 9700 RB Groningen, the Netherlands
Tel.: +31-50-3613157
Fax: +31-50- 5411031
E-mail: h.j.verkade@med.umcg.nl
Abstract

Essential fatty acid (EFA) deficiency in mice induces fat malabsorption. We previously reported indications that the underlying mechanism is located at the level of the intestinal mucosa. We have investigated the effects of EFA deficiency on small intestinal morphology and function. Mice were fed an EFA-deficient or control diet for 8 weeks. A 72 h fat balance, the EFA status, and small intestinal histology were determined. Carbohydrate absorptive and digestive capacities were assessed by stable isotope methodology after administration of U-\(^{13}\)C-glucose and 1-\(^{13}\)C-lactose. The mRNA expression and enzyme activity of lactase, and concentrations of the EFA linoleic acid (LA) were measured in small intestinal mucosa.

Mice fed the EFA-deficient diet were markedly EFA-deficient with a profound fat malabsorption. EFA deficiency did not affect the histology or proliferative capacity of the small intestine. Blood \(^{13}\)C\(_6\)-glucose appearance and disappearance were similar in both groups, indicating unaffected monosaccharide absorption. In contrast, blood appearance of \(^{13}\)C-glucose, originating from 1-\(^{13}\)C-lactose, was delayed in EFA-deficient mice. EFA deficiency profoundly reduced lactase activity (-58%, p<0.01) and mRNA expression (-55%, p<0.01) in mid small intestine. Both lactase activity and its mRNA expression strongly correlated with mucosal LA concentrations (r=0.77 and 0.79, resp., p<0.01).

EFA deficiency in mice inhibits the capacity to digest lactose, but does not affect small intestinal histology. These data underscore the observation that EFA deficiency functionally impairs the small intestine, which in part may be mediated by low LA levels in the enterocytes.

**Key words:** carbohydrate digestion, essential fatty acid deficiency, essential fatty acids, lactase, small intestine
Introduction

Essential fatty acid (EFA) deficiency can occur in cholestatic liver diseases as a consequence of fat malabsorption.(5; 34) However, EFA deficiency itself also induces fat malabsorption.(24; 45) The underlying mechanism of EFA-deficiency induced fat malabsorption remains unclear. Absorption of fat involves lipolysis, solubilization, intestinal translocation from the lumen into the mucosa, chylomicron assembly and transport into the lymph.(14; 28) Previous studies in EFA-deficient mice have indicated that impaired lipolysis or bile formation do not cause the fat malabsorption in EFA deficiency.(45) However, studies in rats show that EFA deficiency alters both the intraluminal and intracellular phases of fat absorption. (24) This implies that the effects of EFA deficiency on mucosal phases of fat absorption could be species-dependent.

Recently we reported data to suggest that EFA deficiency in mice affects fat absorption at the level of the small intestinal mucosa.(45) However, it has not been proven that EFA deficiency impairs the mucosal phase of fat absorption. Based on previous findings, we hypothesize that EFA deficiency functionally impairs the small intestine.

In contrast to fat absorption, the absorption of di- and monosaccharide carbohydrates exclusively depends on mucosal function.(36) The monosaccharide glucose is actively transported across the brush border membrane in the small intestine by the brush-border transporter SGLT1 (Sodium-dependent glucose transporter).(18; 36) The disaccharide lactose is first hydrolyzed by the mucosal membrane anchored lactase-phlorizin hydrolase (lactase, LPH) into glucose and galactose, prior to their active transport across the brush border by SGLT1.(17; 18) Besides being an important enzyme in lactose hydrolysis, lactase is a marker of enterocyte differentiation.(32) Throughout development total intestinal lactase activity remains similar to that found in newborns.(4) If EFA deficiency affects lactase expression and activity in the small intestine, even slight changes should easily be detectible in adult EFA-deficient mice. For this
reasons, lactase is a good marker for functional assessment of the small intestine in adult animal.

Essential fatty acids (EFAs) are structural components of membrane phospholipids. Enterocyte membrane phospholipids are particularly rich in linoleic acid (LA, C18:2n-6) (22), which is necessary for modulations of a wide variety of biological functions and for physiochemical adaptations of the membrane lipid matrix to alterations in membrane fluidity.(20) The lipid matrix influences the conformation and function of proteins embedded in the inner and/or outer leaflet of the membrane.(41) Recently, an additional role of EFAs in alterations of bilayer elastic properties and lipid composition in lipid rafts have been reported.(3; 25) Through activation of peroxisome proliferator-activated receptors (PPARs), EFAs can regulate transcriptional activity of several genes, including of those involved in fatty acid transport and metabolism.(12; 33)

In the present study we characterized the effects of EFA deficiency on small intestinal morphology and function in mice. Korotkova et al. have shown that EFA deficiency affects the fatty acid composition in the phospholipids of the rat small intestinal mucosa by decreasing jejunal concentrations of linoleic acid.(22) However, no studies have been performed on the effect of EFA deficiency on the small intestinal function concerning carbohydrate absorption. We assessed the absorption of glucose, a major source of metabolic energy for mammalian cells, (1) and the expression and activity of the lactase enzyme, as appropriate functional markers of the small intestine, in a previously developed and characterized murine model of EFA deficiency.(45) We applied stable isotope methodology (35), since this approach allows extension to similar studies in patients with EFA deficiency, cholestasis, or other forms of malabsorption.(11; 21; 42) U-13C-labeled glucose and 1-13C-labeled lactose were administered to EFA-deficient and control mice. Blood appearance of labels derived from administered glucose (13C6-glucose) and lactose (13C-glucose) into the blood glucose fraction was subsequently quantified. We also determined the activity and expression of lactase, as well as the concentration of LA, in the mucosa along the proximal-to-distal axis of the small intestine. To
determine whether EFA deficiency specifically affects lactase activity or disaccharide activity in general, we in addition measured the activity of another disaccharide, sucrase.

Our data show that EFA deficiency is associated with impaired lactose digestion in mice. This functional observation is specific for lactase and corresponds with lower lactase mRNA expression and enzyme activity in the mid small intestine of EFA-deficient mice. All together, these findings support the idea that EFA deficiency functionally impairs the small intestine.
Materials and methods

Mice and housing
Wild type mice with a free virus breed background were obtained from Harlan (Horst, the Netherlands). Male mice (25-35 g) were housed in a light-controlled (lights on 6 AM-6 PM) and temperature-controlled facility and were allowed tap water and chow (AB diets, Woerden, the Netherlands) ad libitum. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, the Netherlands.

Materials
U-13C-glucose and 1-13C-lactose were obtained from Isotec Inc. (Miamisburg, Ohio, USA) with isotopic enrichments of 99%. Unlabeled lactose was obtained from Fluka (Buchs, Switzerland).

Experimental diets
Similar to previous studies, we used high-fat EFA-deficient and EFA-sufficient (control) diets (16 wt% and 34 energy% fat), in order to mimic more closely the human diet composition.(45) The diets were custom synthesized by Arie Bloks BV (Woerden, the Netherlands, diet codes EFA-deficient #4141.08 and EFA-sufficient #4141.07). The EFA-deficient diet contained 64 mol% palmitic acid (C16:0), 18 mol% stearic acid (C18:0), 13 mol% oleic acid (C18:1n-9) and 5 mol% linoleic acid (C18:2n-6). The isocaloric EFA-sufficient diet contained 36 mol% C16:0, 5 mol% C18:0, 31 mol% C18:1n-9 and 29 mol% C18:2n-6. Fatty acid contents of the diets were analyzed by extracting, hydrolyzing and methylating total dietary fatty acids as described by Muskiet at al. and subsequent separation and quantification of fatty acid methyl esters was performed by gas chromatography as described previously.(29; 45)
**Experimental procedures**

*Induction of EFA deficiency*

Mice were fed standard laboratory chow containing 6 weight% fat from weaning, and were switched to EFA-deficient or control diet at eight weeks of age. After eight weeks of EFA-deficient or control diet, fat absorption was assessed by measuring food intake and collecting feces for 72 h. Mice underwent a glucose/lactose absorption test with U-\(^{13}\)C-glucose and 1-\(^{13}\)C-lactose (details see below).(39; 46) After the test the mice were anesthetized and sacrificed by obtaining a large blood sample through cardiac puncture for determination of erythrocyte EFA-status by the triene/tetraene (C20:3n-9/C20:4n-6) ratio. (45) The small intestine was excised, flushed with ice-cold PBS and divided into a proximal, mid and distal segment of similar size. Smaller parts from the middle of each small intestinal segment were harvested for histology and gene expression. Another part of the proximal, mid and distal small intestine was opened lengthwise and the mucosa was removed by scraping the luminal surface with a glass coverslip. Mucosa was homogenized in buffer (see below for details) and used for the determination of enzyme activity, proteins and LA concentrations in mucosal phospholipids.

*Glucose/lactose absorption*

Glucose absorption and lactose digestion were determined by a combined U-\(^{13}\)C-glucose/1-\(^{13}\)C-lactose absorption test. After an overnight fast, mice received 0.5 mg U-\(^{13}\)C-glucose, 5 mg 1-\(^{13}\)C-lactose and 5 mg naturally enriched lactose in 300 \(\mu\)L PBS via gastric gavage. Before and at time points 7.5, 15, 30, 45, 60, 90, 120 and 180 min. after administration, blood samples were obtained by blood spot technique from the tail for determination of blood concentrations of (total) glucose, \(^{13}\)C\(_6\)-glucose (glucose originating from U-\(^{13}\)C-glucose) and \(^{13}\)C-glucose (originating from 1-\(^{13}\)C-lactose).(39) For reasons of clarity, we will address “blood” \(^{13}\)C\(_6\)-glucose and \(^{13}\)C-glucose as “plasma” in the Results and Discussion sections.
Analytical methods

Lipid absorption, triene/tetraene ratio, erythrocyte fatty acid concentrations, blood glucose and serum insulin concentrations

Lipid absorption, erythrocyte fatty acid concentrations, and triene/tetraene ratio were determined as described previously.(27; 45) Blood glucose levels were measured with a Lifescan EuroFlash glucose meter (Lifescan Benelux, Beerse, Belgium). Insulin was measured in a solid phase two-site enzyme immunoassay in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule (Ultrasensitive Mouse Insulin kit; Mercodia, Uppsala, Sweden).

Histology and villus length along the small intestinal axis

Morphology of proximal, mid and distal small intestine was assessed by hematoxylin/eosin staining of formalin-fixated material. Proliferating cells were detected by staining of nuclear Ki-67 antigen. Morphometrical analysis of small intestinal samples was performed as described by evaluation of approximately 5 vertically oriented villi per intestinal segment of 4 to 6 animals per group. The digitized images were evaluated at 10x magnification using the calibrated image analysis system (Leica Quantimet 570 C; Leica Qwin Pro V 2.8). The epithelial surface lining was demarcated and measured as a parametrical length, whereby 1 pixel was equal to 0.544 µm.

Glucose/lactose absorption

The analysis of $^{13}$C$_6$-glucose and $^{13}$C-glucose concentrations from blood spots was performed according to Van Dijk et al. by gas chromatography-mass spectrometry (SSQ700, ThermoFischer B.V., Breda, The Netherlands).(39)
*Disaccharidase activity assay in mucosal homogenates*

A portion of small intestinal mucosa (proximal, mid and distal) was homogenized with PBS buffer containing protease inhibitors (Roche, Indianapolis, USA) in order to make 4% homogenates for use in enzyme activity assay. Enzyme activity level of lactase and sucrase were measured in freshly scraped intestinal mucosa as described previously by Dahlqvist.\(^{(9)}\) Activity was normalized to protein levels, measured by the BCA method as described by the manufacturer (Pierce, Rockford, IL).

*Measurement of mRNA expression by real-time PCR (Taqman)*

mRNA expression of lactase and sucrase isomaltase was measured in proximal, mid and distal small intestine by real-time PCR as described previously.\(^{(15)}\) In addition, mRNA expression levels of intestine-specific transcription factors (Cdx-2, Gata-4 and Hnf-1α) were measured by real-time PCR in the mid part of the small intestine. PCR results were normalized to β-actin mRNA levels. The sequences of the primers and probes are listed in Table 1.
Table 1 The PCR Primers and TaqMan Probes

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank</th>
<th>Forward Primer</th>
<th>Reversed Primer</th>
<th>TaqMan® probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>NM_0073</td>
<td>AGCCATGTACGTAGCCATCC</td>
<td>TCTCCGGAGTCCATCAC</td>
<td>TGTCCTGTG ATG</td>
</tr>
<tr>
<td></td>
<td>93</td>
<td>A</td>
<td>A ATG</td>
<td>CCTCTGGTGCTGATCA C</td>
</tr>
<tr>
<td>Lactase</td>
<td>XM_1294</td>
<td>CGTCTGCTTCTATCAGGTT</td>
<td>GTGGGAAAATGTGTCCCC</td>
<td>TCTTTTGCCATCTGC</td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>GAA</td>
<td>A GATACT</td>
<td>TCTTACGC</td>
</tr>
<tr>
<td>Sucrase-</td>
<td>NM_0130</td>
<td>TGTTTGGAATGACTGAG</td>
<td>CCCACCCTCGATGTTG</td>
<td>ACTGTTAACCTGGC</td>
</tr>
<tr>
<td>isomaltase</td>
<td>61</td>
<td>A TG</td>
<td>TG</td>
<td>CATACTCTCAATA</td>
</tr>
<tr>
<td>Cdx-2</td>
<td>NM_0076</td>
<td>GTCCCTAGGAAGCCAGTGA</td>
<td>CTCCTGATGTTGATGTA</td>
<td>CCT TCTCACAGTCT</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td>AA</td>
<td>TC GACTA</td>
<td>CCA GCGCTG</td>
</tr>
<tr>
<td>Gata-4</td>
<td>NM_0080</td>
<td>GAGATGCGCCCC ATCAAG</td>
<td>GAC ACA GTA CTG AAT</td>
<td>CTGTCATCTCAGTAT</td>
</tr>
<tr>
<td></td>
<td>92.2</td>
<td></td>
<td>GTC TGG GAC AT</td>
<td>GGGACACAGCAGCTC</td>
</tr>
<tr>
<td>Hnf-1α</td>
<td>NM_0093</td>
<td>CTCCAGCAGCCTGTTGTTG</td>
<td>GAGGCCATCTGGTG</td>
<td>CACAGCCACCTGCTG</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td></td>
<td>GAGAT</td>
<td>CCATCCAAC</td>
</tr>
</tbody>
</table>

LA determination in phospholipids of intestinal mucosa

Thirty mg of intestinal mucosa was homogenized in 200 µl of 0.9% NaCl and lipids were extracted according to Bligh and Dyer, after the addition of the fatty acid internal standard (C17:0) and anti-oxidant (BHT).(2) Lipid extracts were fractionated into phospholipids and other lipids using TLC (20 x 20 cm, Silica gel 60 F254; Merck) with hexane/diethyl ether/acetic acid (80:20:1, v:v:v) as running solvent. Phospholipid spots were scraped and phospholipids were extracted by methanol/chloroform. Phospholipid LA ratio was determined according to Muskiet et al. as described previously.(29; 43)

Statistical analysis
Values represent means ± SD for the indicated number of mice per group. Using SPSS version 12.0.2 statistical software (Chicago, IL, USA), we calculated significance of differences with the Mann-Whitney U-test. P-values below 0.05 were considered statistically significant.

Correlations between the linoleic concentrations in the mucosa of the mid small intestine, and mRNA expression and enzyme activity of lactase and sucrase were determined by means of linear regression and are expressed as non-parametric Spearman correlation coefficient (SPSS version 14.0, Chicago, IL, USA). Differences between means were considered significant at the level of p<0.01.
Results

Body weight and food ingestion were assessed every two weeks and there were no significant differences in basal or final body weight, nor in food intake between EFA-deficient and control mice (data not shown).

**Pronounced EFA deficiency of EFA-deficient mice**

After eight weeks of treatment, in mice fed the EFA-deficient diet, the triene/tetraene ratio in red blood cell membranes was strongly increased compared with the control group (0.23 ± 0.06 vs. 0.01 ± 0.00; respectively, p<0.01). Fatty acids profile in erythrocyte lipids is very similar to those obtained in our previous studies in mice. (44) They revealed a severe decrease in n-6 family of essential fatty acids, accompanied by an increase in concentrations of non-essential fatty acids of the n-7 and n-9 families (fig 1B). Fat balance during 72 hours revealed a decreased total fat absorption in EFA-deficient compared to control mice (81% vs. 99%, respectively, p<0.01; fig 1A). The absorption of saturated fatty acids, palmitic (C16:0) and stearic (C18:0) acids, was affected to a greater extent than that of the unsaturated fatty acids oleic (C18:1n-9) and linoleic acid (C18:2n-6). Together, these observations indicated that the mice fed the EFA-deficient diet had profound EFA deficiency after 8 weeks on the experimental diet.(45)

**EFA deficiency in mice is not associated with alterations in intestinal morphology**

Hematoxylin/eosin (data not shown) and Ki67 staining of the three segments of the small intestine revealed no clear differences in morphology or proliferative capacity between EFA-deficient and control mice (fig 2A). The villus lengths were similar in EFA-deficient and control mice, as determined by morphometrical measurements (fig 2B).

**EFA deficiency is associated with delayed glucose clearance**
Basal blood glucose concentrations were similar in EFA-deficient and control mice. After intragastric administration of the glucose/lactose bolus, glucose concentrations rapidly increased in control mice, with a maximum concentration at 30 min. after administration (fig 3A). In EFA-deficient mice, the increase in blood glucose levels was similar to that in control mice up to 30 min., but then continued to increase, reaching a maximum concentration at 60 min. The glucose concentrations between 60 and 180 min. were slightly, but significantly higher in EFA-deficient mice compared to controls (+10-15%, p<0.05). Accordingly, the area under the curve was higher for the EFA-deficient mice compared with controls (+15%, p<0.05, data not shown). Based on the apparently delayed glucose clearance, we determined insulin concentrations at the end of the experiment (at ~180 min.). In EFA-deficient mice insulin concentrations were significantly higher than in control mice (0.55±0.10 µg/ml vs. 0.35±0.02 µg/ml, respectively, p<0.01).

Similar glucose absorption but delayed lactose digestion in EFA-deficient mice

To assess the competence of monosaccharide absorption in EFA deficiency, we determined plasma appearance of $^{13}$C$_6$-glucose (fig 3B). After the administration of the bolus, plasma $^{13}$C$_6$-glucose concentration rapidly increased with a maximum at 45 min. for both groups. After 45 min., $^{13}$C$_6$-glucose rapidly disappeared until 120 min., after which the rate of disappearance decreased in both EFA-deficient and control mice. Thus, the plasma $^{13}$C$_6$-glucose appearance and disappearance was similar in EFA-deficient and control mice, supporting unaffected monosaccharide absorption in the former.

In order to measure the competence of disaccharide digestion and absorption, we determined plasma appearance of $^{13}$C-glucose, originating from the administered 1-$^{13}$C-lactose (fig 3C). $^{13}$C-glucose reached a maximum concentration in control mice at 45 min. after bolus administration. The $^{13}$C-glucose disappeared from the blood within the next 2 hours, with the slowest disappearance during the last hour after the bolus administration. Plasma appearance of $^{13}$C-glucose in EFA-deficient mice, however, increased to a slower extent and reached its maximal
concentration at approximately 60 min. after the bolus administration. In addition, the peak of $^{13}$C-glucose absorption in EFA-deficient mice was lower compared to control mice. Thus, the lactose uptake was delayed in EFA-deficient compared to control mice.

**Specific decrease in mRNA expression and activity of lactase in mid small intestine of EFA-deficient mice**

Measurement of the enzyme activity of lactase along the proximal-to-distal axis of the small intestine revealed a lower activity in the mucosa of the mid part of the small intestine of EFA-deficient mice (fig 4A). The decreased lactase activity corresponded with lower mRNA levels of lactase, as shown by quantitative PCR (fig 4B). EFA deficiency was not associated with decreased activity (3.6±1.6 vs. 2.8±0.7 nmol/μg protein in controls, NS) or mRNA expression (0.9±0.1 vs. 0.7±0.1 in controls, NS) of another disaccharidase in the mid small intestine, sucrase, indicating a distinct specificity of EFA deficiency on lactase. We determined if reduced lactase mRNA expression levels were regulated by the transcription factors at the transcriptional level. The mRNA expression of transcription factors involved in regulation of the lactase mRNA expression, namely Cdx-2, Gata-4 and Hnf-1α (fig 4C), was not different between EFA-deficient and control animals. This indicates that the regulation of lactase is at least not regulated at the transcriptional level of the transcription factors Cdx2, GATA-4 and HNF-1α.

**Decreased lactase activity and mRNA expression are associated with low LA concentrations in the mid small intestine**

EFAs are involved in regulation of membrane fluidity and alterations in membrane lipid matrix. Therefore, it has been proposed that EFAs indirectly influence normal conformation and functioning of the proteins embedded in the membrane.(41) For this reason we tested if lactase activity in the mid segment of the small intestine correlated with LA levels. LA concentrations
were highest in the mid part of the small intestine in control mice. Interestingly, LA concentration was significantly lower in the mid part of the small intestinal mucosa of EFA-deficient compared to control mice (26 mol% vs. 16 mol%, respectively, p<0.01) (fig 5A). LA concentrations in proximal and distal part were similar in both groups. In the mid small intestine LA concentrations positively correlated with lactase activity (r=0.77, p<0.01) and with mRNA expression of lactase (r=0.79, p<0.01) (fig 5B and 5C, respectively). However, decreased mRNA levels of lactase clearly indicate that the intestinal impairment can not exclusively be the result of alterations in membrane composition and fluidity. There was no relationship, however, between LA concentrations in the mid small intestine on the one hand and activity (r=-0.08, NS) or mRNA expression (r=-0.03, NS) of sucrase on the other hand (fig 5D and 5E, respectively).
Discussion

Our previous studies suggested that EFA deficiency in mice affects fat absorption at the level of the small intestinal mucosa.\(^{45}\) We now explored the effects of EFA deficiency in mice on mucosal histology and physiological function of the small intestine, carbohydrate digestion and absorption. Our data demonstrate that EFA deficiency is not only associated with fat malabsorption, but also with impaired lactose digestion in the murine model of EFA deficiency. The effect of EFA deficiency is mainly on lactase mRNA transcription or stability, since the impaired lactose digestion coincided with an ~50% reduced lactase activity and mRNA expression in mid small intestine of EFA-deficient mice. Interestingly, the reduction in enzyme activity during EFA deficiency was specific for lactase. In addition, intestinal lactase activity and mRNA expression strongly correlated with mucosal linoleic acid concentrations, which were depressed in EFA deficiency, particularly in mid-intestine.

Our findings seem to be in concordance with a study of Clark et al. which showed the most marked delay in fat transport during EFA deficiency in the mid portion of the small intestine.\(^{8}\) We analyzed whether regional differences in the severity of EFA deficiency could contribute to the observations. However, the severity of EFA deficiency was similar in the different parts of the small intestine of EFA-deficient mice (data not shown). Based on pathophysiology we suggest 2 possibilities for this phenomenon: EFA deficiency in the mid intestine is associated with either increased (local) oxidation of LA or with increased turnover/export of LA. However, further studies need to be performed in order to investigate the specific effect of EFA deficiency on fatty acid composition and lactase activity in the mid small intestine.

As expected from previous studies, our murine model of EFA deficiency was clearly deficient, as indicated by elevated triene/tetraene ratios in erythrocytes and fat malabsorption. In addition, we measured the bile production by the collection of bile via gallbladder cannulation as described previously.\(^{19}\) Conform previous observations, the mice fed the EFA-deficient diet have
increased bile flow, biliary bile salt and phospholipid secretion rates (data not shown), as well as higher plasma levels of triene/tetraene ratio (0.55±0.20 vs. 0.01±0.00, p<0.01), compared with control mice. Although one would expect that EFA deficiency associated fat malabsorption would lead to a lower body weight, we did not observe this in our present or previous studies. Therefore, we assume that prolongation of the EFA-deficient state would indeed be expected to result in lower body weight.

EFA deficiency in mice did not affect morphology or proliferative capacity of the small intestine. As far as we know, our study is the first to describe the effects of EFA deficiency on the intestinal morphology in mice. Christon et al. have shown that low dietary linoleic acid levels were associated with alterations in villi and crypt sizes in rats. We did not observe differences in villus length between EFA-deficient and control mice in the proximal-to-distal axis of the small intestine. These results indicate that EFA deficiency associated malabsorption of fats and disaccharides is not associated with morphological alterations in small intestine of mice.

To assess small intestinal function in EFA-deficient mice, we studied carbohydrate absorption, using stable isotope methodology. The advantage of stable isotope methodology is that it can easily be extrapolated to patient studies. EFA-deficient mice had higher total blood glucose levels from 60 min. after the administration of the glucose/lactose bolus. High total blood glucose levels could theoretically be explained by lower blood glucose clearance (slower postprandial uptake of glucose by the peripheral tissues), rather than by disturbed intestinal absorption. This finding is associated with higher insulin concentrations at the end of the experiment in EFA-deficient mice, which is not the result of an impaired glucose disposal, as this appears to be normal. This observation is in accordance with previous studies suggesting a relationship between EFA deficiency and insulin resistance. However, it is not clear what the exact reason is for higher insulin levels in EFA-deficient mice. We cannot exclude that the increased content of saturated fats in the EFA-deficient diet contributes to this phenomenon, independently from EFA deficiency.
Measurement of the absorption of $^{13}$C$_6$-glucose, originating from the administered U-$^{13}$C-glucose, revealed similar appearance and disappearance of the labeled glucose in both groups. This observation indicates that EFA deficiency does not affect the monosaccharide glucose absorption in mice. The plasma appearance of $^{13}$C-glucose originating from lactose, however, was significantly delayed in EFA-deficient mice. The discrepancy in the effect of EFA deficiency on glucose and lactose absorption could be explained by the diverse intestinal fates of these carbohydrates. Unlike glucose, which is directly transported by the glucose transporters across the brush border membrane of the enterocyte, lactose first needs to be hydrolyzed by the enzyme lactase.$^{(18; 36)}$ In order to investigate whether our functional results corresponded with altered lactase activity or expression, we measured these parameters in EFA-deficient and control mice. Lactase is a critical disaccharidase during early postnatal life and a sensitive intestinal marker for functional changes occurring in the small intestine of the adult animal. Its activity relatively decreases during weaning to low adult levels, thus the total lactase activity remains the same during the adulthood.$^{(31; 32)}$ The delayed lactose digestion corresponded with an approximate 50% reduction in both lactase activity and mRNA expression in EFA-deficient mice. The mRNA levels of relevant transcription factors for lactase mRNA expression were unaffected in EFA-deficient mice. Due to the unaltered mRNA levels of the transcription factors, we conclude that the regulation of lactase is at least not regulated at the transcriptional level of the transcription factors Cdx2, GATA-4 and HNF-1α.

In order to assess the specificity of the reduced enzyme activity associated with EFA deficiency for lactase, we measured the enzyme activity of another disaccharidase, sucrase. Enzyme activity of sucrase was not decreased in the three parts of the small intestine of EFA-deficient mice. These data clearly demonstrated that EFA deficiency does not generally affect disaccharidase function and activity, but rather specifically affects the mRNA expression and activity of lactase.
Under physiological conditions phospholipids of the small intestinal mucosa contain considerable amounts of LA (C18:2n-6) and its long-chain polyunsaturated fatty acid metabolite arachidonic acid (AA, C20:4n-6).(16) During EFA deficiency LA levels are decreased in intestinal mucosa.(13; 22) We observed LA deficiency in mucosal phospholipids, particularly in the mid part of the small intestine, which strongly correlated with reduced lactase activity and mRNA expression. It is tempting to speculate that low LA levels in phospholipids of cellular membranes lead to structural and physiological changes in the lipid membrane. A study in pigs (6) suggested that EFA-deficiency reduces membrane fluidity and affects membrane protein behavior in the enterocyte membranes. Theoretically, these structural changes in the cellular membrane of the enterocytes during EFA deficiency could also be the cause of functional alterations in the membrane anchoring of lactase. However, since not only lactase activity but also its mRNA expression was decreased in EFA deficiency and since the lactase hydrolytic portion of the enzyme is at a considerable distance from the membrane, (40) it is likely that altered membrane fluidity is not the (single) factor involved.

It remains unclear how EFA deficiency specifically affects lactase; several factors could be involved. Theoretically, EFA deficiency could inhibit the differentiation of the enterocytes, accompanied by reduced expression of lactase (32) Lactase and sucrase genes have different mechanisms of transcriptional regulation, what could lead to differential transcription during EFA deficiency. (37; 38) Alternatively, under certain conditions, for example during malnutrition, (30) stability of lactase mRNA is more profoundly decreased than that of sucrase. Our present results do not allow to discriminate between these theoretical options.

Our present results indicate that EFA deficiency has functional consequences for small intestinal function in mice, and it provides indirect support for the hypothesis that reduced mucosal function is involved in fat malabsorption in EFA deficiency. EFA deficiency in (pediatric) cholestatic patients seems primarily caused by fat malabsorption due to bile deficiency. Recently, we reported that cholestasis per se does not affect carbohydrate digestion or
absorption in a rat model of short-term cholestasis.(26) Our present study indicates, however, that EFA deficiency aggravates the malabsorption of fat, and decreases the small intestinal capacity to digest lactose. Decreased levels of LA in the mid part of the small intestine seem to, at least partially, play a pathophysiological role in the diminished mucosal function in EFA deficiency. Our findings imply that dietary interventions for patients encountering EFA deficiency should accommodate the decreased capacity to absorb fat and the reduced capacity to digest lactose.
Acknowledgements

The authors would like to thank Rick Havinga, Ingrid Martini, Juul Baller, Theo Boer, Henk Wolters and Renze Boverhof for excellent technical assistance and helpful suggestions.

Grants

This study was supported by the Dutch Digestive Foundation (MLDS). E.H.H.M. Rings is supported by a fellowship of the Royal Netherlands Academy of Arts and Sciences (KNAW).
Reference List


34. Socha P, Koletzko B, Światkowska E, Pawlovska J, Stolarczyk A and Socha J.


Higher expression of jejunal LPH gene in rats fed the high-carbohydrate/low-fat diet compared with those fed the low-carbohydrate/high-fat diet is associated with in vitro binding of Cdx-2 in nuclear proteins to its promoter regions. *Life Sciences* In Press, Corrected Proof.


39. van Dijk T, Boer T, Havinga R, Stellaard F, Kuipers F and Reijngoud D.

40. Wacker H, Keller P, Falchetto R, Legler G and Semenza G. Location of the two catalytic sites in intestinal lactase-phlorizin hydrolase. Comparison with sucrase-


Figure legends

**Figure 1**

A Fat absorption of total dietary fat, and of major dietary fatty acids (16:0, 18:0, 18:1n-9 and 18:2n-6) in EFA-deficient and control mice. Feces were collected after a 72h period in which the food intake was monitored by weighing food containers. Absorption was calculated by subtracting fecal excretion of these fatty acids after 72h from their dietary intake in 72h and then multiplying the result by 100. B Fatty acid composition of erythrocyte lipids of EFA-deficient and control mice. Fatty acid concentrations are in mol% of total fatty acids. Data represent means ± SD of 7 mice per group. *p< 0.05 and for EFA-deficient vs. control mice.

**Figure 2**

A Ki67 staining of sections of the three parts of the small intestine (proximal, mid and distal) from EFA-deficient and control mice. B Morphometry of the villus length in the three segments of the small intestine (proximal, mid and distal) of EFA-deficient and control mice. Data represent means ± SD of 4-6 mice per group. No significant differences were found between the two groups.

**Figure 3**

A Total blood glucose response in EFA-deficient and control mice measured at different time points (7.5, 15, 30, 45, 60, 90, 120 and 180 min.) after the intragastric glucose/lactose bolus. B Plasma appearance of $^{13}$C$_6$-glucose originating from the administered U-$^{13}$C-glucose in EFA-deficient and control mice after an intragastric administration of glucose/lactose bolus. C Plasma appearance of $^{13}$C-glucose originating from the administered 1-$^{13}$C-lactose in EFA-deficient and control mice after an intragastric administration of glucose/lactose bolus. Data represent means ± SD of 7 mice per group. *p< 0.05 for EFA-deficient vs. control mice.
**Figure 4**

A Enzyme activity of lactase in the three segments of the small intestine of EFA-deficient and control mice. Enzyme activity is expressed as glucose production after 1 hour of incubation of intestinal mucosa with the substrate of lactase. Data represent means ± SD of 6 mice per group. **p< 0.01 for EFA-deficient vs. control mice.**

B mRNA expression levels of lactase gene, normalized to β-actin, in the three segments of the small intestine of EFA-deficient and control mice. Data represent means ± SD of 6 mice per group. **p< 0.01 for EFA-deficient vs. control mice.**

C Relative mRNA expression of the transcription factors Cdx-2, Gata-4 and Hnf-1α (involved in regulation lactase expression) in the mid part of the small intestine. Data represent means ± SD of 5-6 mice per group. No significant differences were found between EFA-deficient and control mice.

**Figure 5**

A LA (C18:2n-6) concentration in small intestinal mucosa along the proximal-to-distal axis of EFA-deficient and control mice. Concentrations are indicated in mol% of total fatty acids. Data represent means ± SD of 6 mice per group. **p< 0.01 for EFA-deficient vs. control mice.**

B Relationship between the mucosal LA concentration (mol%) and enzyme activity of lactase (nmol/µg protein/h) in the mid part of the small intestine. There is a positive correlation (r=0.77, p<0.01) between the LA concentration and lactase activity in mucosa.

C Relationship between the mucosal LA concentration (mol%) and relative mRNA expression of lactase in the mid part of the small intestine. There is a positive correlation (r=0.79, p<0.01) between the LA concentration and lactase mRNA expression in mucosa.

D Relationship between the mucosal LA concentration (mol%) and enzyme activity of sucrase (nmol/µg protein/h) in the mid part of the small intestine. There was no significant correlation (r=-0.08, NS) between the LA concentration and sucrase activity in mucosa.

E Relationship between the mucosal LA concentration (mol%) and relative mRNA expression of sucrase isomaltase gene in the mid part of the small intestine.
of the small intestine. There was no significant correlation ($r=-0.03$, NS) between the LA concentration and mRNA expression of sucrase isomaltase.
Figure 1A
Figure 1B
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EFA-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal</td>
<td><img src="image" alt="a" /></td>
<td><img src="image" alt="b" /></td>
</tr>
<tr>
<td>Mid</td>
<td><img src="image" alt="c" /></td>
<td><img src="image" alt="d" /></td>
</tr>
<tr>
<td>Distal</td>
<td><img src="image" alt="e" /></td>
<td><img src="image" alt="f" /></td>
</tr>
</tbody>
</table>

Figure 2A
Figure 2B
Figure 3A
Figure 3C
Figure 4A
Figure 4C
Figure 5A
$R = 0.77$
Figure 5C
Sucrase activity (nmol/μg protein) vs. LA (mol%). The data points indicate a negative correlation (R=-0.08) with control and EFA-deficient groups distinguished by filled and open squares, respectively.
Figure 5E

- **Figure Description:**
  - The figure displays a scatter plot comparing Relative sucrase mRNA expression against LA (mol%) for Control and EFA-deficient groups.
  - The plot includes data points for both groups, indicated by black squares for Control and white squares for EFA-deficient.
  - A correlation coefficient, $R=-0.03$, suggests a weak negative correlation between Relative sucrase mRNA expression and LA (mol%).

- **Axes:**
  - **Y-axis:** Relative sucrase mRNA expression
  - **X-axis:** LA (mol%)