Intestinal capacity to digest and absorb carbohydrates is maintained in a rat model of cholestasis

E. Leonie Los, Henk Wolters, Frans Stellaard, Folkert Kuipers, Henkjan J. Verkade, and Edmond H. H. M. Rings

Pediatric Gastroenterology/Laboratory of Pediatrics, Department of Pediatrics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Submitted 30 April 2007; accepted in final form 9 July 2007

Los EL, Wolters H, Stellaard F, Kuipers F, Verkade HJ, Rings EH. Intestinal capacity to digest and absorb carbohydrates is maintained in a rat model of cholestasis. *Am J Physiol Gastrointest Liver Physiol* 293: G615–G622, 2007. First published July 12, 2007; doi:10.1152/ajpgi.00188.2007.—Cholestasis is associated with systemic accumulation of bile salts and with deficiency of bile in the intestinal lumen. During the past years bile salts have been identified as signaling molecules that regulate lipid, glucose, and energy metabolism. Bile salts have also been shown to activate signaling routes leading to proliferation, apoptosis, or differentiation. It is unclear, however, whether cholestasis affects the constitution and absorptive capacity of the intestinal epithelium in vivo. We studied small intestinal morphology, proliferation, apoptosis, expression of intestinal-specific genes, and carbohydrate absorption in cholestatic (1 wk bile duct ligation), bile-deficient (1 wk bile diversion), and control (sham) rats. Absorptive capacity was assessed by determination of plasma [3H]- and [13C]glucose concentrations after intraduodenal administration of [3H]glucose and naturally enriched [13C]sucrose, respectively. Small intestinal morphology, proliferation, apoptosis, and gene expression of intestinal transcription factors (mRNA levels of Cdx-2, Gata-4, and Hnf-1α, and Cdx-2 protein levels) were similar in cholestatic, bile-deficient, and control rats. The (unlabeled) blood glucose response after intraduodenal administration was delayed in cholestatic animals, but the absorption over 180 min was quantitatively similar between the groups. Plasma concentrations of [3H]glucose and [13C]glucose peaked to similar extents in all groups within 7.5 and 30 min, respectively. Absorption of [3H]glucose and [13C]glucose in plasma was similar in all groups. The present data indicate that neither accumulation of bile salts in the body, nor their intestinal deficiency, two characteristic features of cholestasis, affect rat small intestinal proliferation, differentiation, apoptosis, or its capacity to digest and absorb carbohydrates.

Cholestasis is associated with accumulation of bile salts in the body and by deficiency of bile salts in the intestinal lumen (29). Bile salts facilitate dietary lipid absorption in the intestinal lumen and contribute to cholesterol homeostasis (18). More recently, bile salts have been identified as signaling molecules. Through activation of the farnesoid X receptor (FXR), bile salts regulate various aspects of glucose and lipid metabolism as well as intestinal barrier function (18, 21). Watanabe et al. (42) described a role for bile salts in the regulation of energy metabolism via the G protein-coupled bile acid receptor (GPBAR1). Finally, bile salts can activate MAPK pathways, leading to proliferation or apoptosis (18).

In children, cholestatic liver disease negatively affects nutritional status, growth, and development, which cannot be explained by solely the inability to absorb lipids and lipid-soluble vitamins (4, 5, 12). So far, it has remained unclear to what extent cholestasis, i.e., systemic accumulation and intestinal deficiency of bile salts, affects small intestinal epithelial proliferation, differentiation, or apoptosis and, consequently, absorptive capacity in vivo.

In the small intestinal lumen sucrose is hydrolyzed into glucose and fructose by the brush border membrane enzyme sucrase. Glucose is actively transported across the apical membranes of enterocytes by the sodium-dependent glucose co-transporter SGLT-1. The majority of glucose is passively transported from the enterocyte into the circulation by the facilitated glucose transporter GLUT-2 (38). Intestinal sucrase-isomaltase gene transcription is regulated by the intestine-specific transcription factors Gata binding protein 4 (GATA-4), hepatic nuclear factor 1α (HNF-1α), and caudal type homebox transcription factor 2 (CDX-2) (9).

In the present study, we investigated whether cholestasis affects small intestinal constitution and the absorptive capacity for carbohydrates in rats.

**MATERIALS AND METHODS**

*Rats and housing.* Male Wistar rats, weighing 270–300 g at the beginning of the study, were obtained from Harlan (Horst, The Netherlands). They were individually housed in Plexiglas cages (25 × 25 × 30 cm) on a layer of wooden shavings under controlled temperature and humidity and on a 12:12-h light-dark cycle. Water and chow diet (Hope Farms, Woerden, The Netherlands) were available ad libitum. All experiments were approved by the Animal Experiments Ethical Committee of the University of Groningen.

**Materials.** 6,6-[2H]glucose, 98% 2H was obtained from Isotec (Miamisburg, OH). Isotopic purity was confirmed by GC-MS. Cane sugar (Caribbean Gold, Amstelveen, The Netherlands) was used as naturally enriched [13C]sucrose.

**Surgery.** All rats were equipped with permanent catheters in the jugular vein and duodenum, as described by Kuipers et al. (25). Bile duct-ligated (cholestatic; n = 5) and bile-diverted (bile-deficient; n = 7) rats were compared with sham-operated rats (control; n = 6). The experimental model allows for physiological studies in unanesthetized rats with bile duct ligation and diversion without the interference of stress or restraint. These models have been proven useful to analyze intestinal absorption capacity (22, 26, 27). After surgery, the rats were allowed to recover for 1 wk.

**Experimental procedures.** Feces were collected for 24 h, between days 5 and 6 after surgery. Cholestatic, bile-deficient, and control rats

[Address for reprint requests and other correspondence: E. H. H. M. Rings, Pediatric Gastroenterology/Research Laboratory of Pediatrics, Univ. Medical Center Groningen, Univ. of Groningen, P.O. Box 30.001, 9700 RB Groningen, The Netherlands (e-mail: e.h.h.m.rings@bkk.umcg.nl).]

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
were subjected to a combined [3H]glucose-[13C]sucrose absorption test at 1 wk after surgery. On the day of the experiment, the rats received an intraduodenal bolus of 1 mg [3H]glucose and 0.25 g [13C]sucrose in 1 ml water, after an overnight fast. At \( t = 0, 7.5, 15, 30, 45, 60, 90, 120, \) and 180 min, blood samples were taken for determination of blood glucose concentrations and plasma [3H]- and [13C]glucose enrichments. At the end of the experiment, the rats were killed and the small intestine was collected for analysis. The small intestine was flushed with ice-cold PBS and was divided into the duodenum, the jejunum, and the ileum. Material was harvested for histology and gene expression. Small intestinal mucosa was scraped for the determination of enzyme activity.

**Analytical methods.** Plasma bile salts were determined as described previously (19). Fat ingestion, fecal fat excretion, and net fat absorption were measured and calculated as described previously (22).

**Stable isotope test.** Blood glucose concentrations were measured with a Lifescan EuroFlash glucose meter (Lifescan Benelux, Beerse, Belgium). The sample preparation procedure of plasma [3H]glucose enrichment and plasma [13C]glucose enrichment was as described by Vonk et al. (40). The 2H enrichment was measured by GC-MS (Trace MS, Interscience, Breda, The Netherlands) (41). The 13C-t0-12C isotope ratio measurement of the glucose penta-acetate derivative was determined by gas chromatography-combustion-isotope ratio mass spectrometry by using a Delta Plus instrument (Thermo Finnigan, Bremen, Germany) (40). Concentrations were calculated as described by Vonk et al. (41).

**Disaccharidase activity.** Enzyme activity levels of sucrase were measured in freshly scraped intestinal mucosa as described by Dahlqvist (11). Activity levels were normalized to protein levels, measured by the BCA method as described by the manufacturer (Pierce, Rockford, IL).

**RNA isolation and measurement of mRNA levels by real-time PCR (Taqman).** mRNA expression levels in duodenum, jejunum, and ileum were measured by real-time PCR, as described previously (15). PCR results were normalized to \( \beta \)-actin mRNA levels. The sequences of the primers and probes are listed in Table 1.

**Statistical analysis.** Values represent means ± SE for the indicated number of rats per group. Using SPSS version 12.0.2 statistical software (Chicago, IL), we calculated significance of differences. Significance of differences was calculated with regard to the treatments as well as the different intestinal segments. Body weight and food intake were normally distributed and tested with the one-way ANOVA. When the one-way ANOVA resulted in a significant difference among the groups \( (P < 0.05) \), the two-tailed \( t \)-test was used to calculate differences among the treatments or intestinal segments separately. All other parameters were not normally distributed and therefore were tested with Kruskal-Wallis \( H \) test and subsequently with the Mann-Whitney \( U \)-test for differences among the treatment groups or intestinal segments when a significant difference \( (P < 0.05) \) was observed.

### RESULTS

**Characterization of the model.** Plasma bile salt concentration was significantly higher in cholestatic rats compared with control and bile-deficient rats (187 ± 20 vs. 10 ± 4 and 1.0 ± 0.5 \( \mu \)mol/l, respectively) and significantly lower in bile-deficient rats compared with control rats \( (P < 0.01) \).
In accordance with the localization of intestinal bile salt reabsorption, expression of the apical sodium-dependent bile acid transporter (Asbt) and the ileal bile acid binding protein (Ibabp) was restricted to the ileum (3, 10). Asbt expression was not quantitatively changed in cholestatic or bile-deficient rats, whereas Ibabp expression was reduced in cholestatic rats (by ~35%) and significantly reduced in bile-deficient rats compared with control rats (by ~60%; P < 0.05, Fig. 1). Expression of the short heterodimer partner (Shp) was similar in all three segments and significantly reduced in cholestatic and bile-deficient rats in the duodenum by ~60 and ~45%, respectively (both P < 0.05), in the jejunum by ~75 and ~70%, respectively (both P < 0.01), and in the ileum both by ~95% (both P < 0.01, Fig. 1). Expression of Gpbar1 was similar in duodenum, jejunum, and ileum and was not affected by cholestasis or bile diversion (Fig. 1).

After surgery the body weight decreased in all groups, but to a greater extent in cholestatic and bile-deficient rats compared with the sham-operated controls (93.7 ± 0.6 and 92.7 ± 0.7 vs. 97.5 ± 0.6% of initial weight at day 2 after surgery; both P < 0.01). Body weight of control rats remained stable over the experimental period, whereas body weight of bile-deficient rats increased over time to the level of control rats at day 7. Body weight of the cholestatic rats remained stable over the experimental period and was significantly lower than body weight of control rats over the entire experimental period (approximately −4%, P < 0.05). Average food intake during the experimental period was similar in control and cholestatic rats (4.5 ± 0.2 and 4.1 ± 0.1% body wt). Average food intake of bile-deficient rats was significantly higher than that of cholestatic rats (4.9 ± 0.1 vs. 4.1 ± 0.1% body wt, P < 0.01) (25).

Fat balance was measured from day 5 to 6 after surgery (Fig. 1). Fat ingestion was slightly but not significantly higher in bile-deficient rats compared with control rats, as previously found (22). Fecal fat excretion was significantly higher in cholestatic and bile-deficient rats compared with control rats (1.2 ± 0.1 and 1.2 ± 0.1 vs. 0.2 ± 0.0 mmol fatty acids/day, respectively, each P < 0.01). The resulting net fat absorption was significantly lower in cholestatic rats compared with control rats (2.9 ± 0.1 vs. 3.7 ± 0.2 mmol/day, respectively, P < 0.05), whereas net fat absorption in bile-deficient rats was not significantly different from the control rats. Cholestatic and bile-deficient rats had significantly lower coefficients of fat absorption than control rats (71.8 ± 0.5 and 73.1 ± 0.9 vs. 93.9 ± 0.2% of ingested amount, respectively, each P < 0.01).

Jejunal morphology, proliferation, and apoptosis are not affected in cholestatic rats. Jejunal sections of control, cholestatic, and bile-deficient rat intestines were stained with hematoxylin and eosin, Ki-67, and TUNEL to assess morphology, proliferation, and apoptosis, respectively (Fig. 2). Villus and crypt morphology, proliferation, and apoptosis did not differ between the groups. Villus length was similar in control, cholestatic, and bile-deficient rats (394 ± 36, 445 ± 26, and 510 ± 27 μm, respectively, Table 2), however, villus length in bile-deficient rats showed a trend toward being higher than villus length in control rats (Kruskal-Wallis H: P = 0.064; Mann-Whitney U of bile-deficient rats vs. control rats: P = 0.032) (27). Crypt-to-villus ratios were similar in control, cholestatic, and bile-deficient rats (0.29 ± 0.02, 0.30 ± 0.02, and 0.28 ± 0.00, respectively, Table 2).
Expression of intestinal transcription factors is maintained in cholestatic rats. The expression of intestine-specific transcription factors was determined to assess effects of the experimental manipulations on enterocyte differentiation. CDX-2, HNF-1α, and GATA-4 are known to cooperatively regulate sucrase-isomaltase gene transcription (9). Hnf-1α expression was similar in duodenum, jejunum, and ileum, whereas Gata-4 expression was significantly lower in the ileum compared with the duodenum and jejunum (both P < 0.01; Fig. 3) (8). No significant differences in Hnf-1α or Gata-4 expression were found among the groups. Cdx-2 expression increased slightly from the proximal to the distal part of the intestine of control rats [not significant (NS); Fig. 3]. No significant differences were found in duodenum, jejunum, and ileum among all groups. Cdx-2 protein expression was similar in all groups (Fig. 3).

Blood glucose response is delayed in cholestatic rats. The blood glucose concentration in response to the intraduodenal bolus of labeled glucose and sucrose was delayed in cholestatic rats compared with bile-deficient and control rats and peaked at

![Fig. 2. Jejunal morphology, proliferation, and apoptosis in control, cholestatic, and bile-deficient rats. Intestinal morphology was assessed by hematoxylin and eosin (HE) staining, proliferation by Ki-67 staining, and apoptosis by TUNEL staining. Crypts and villi are indicated in the pictures. Cells positively stained with Ki-67 are indicated by the arrow.](image)

**Table 2. Villus length and crypt-to-villus length ratio**

<table>
<thead>
<tr>
<th>Group</th>
<th>Villus Length, μm</th>
<th>Crypt-to-Villus Length Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>394 ± 36</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>Cholestatic</td>
<td>445 ± 26</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>Bile-deficient</td>
<td>510 ± 27</td>
<td>0.28 ± 0.00</td>
</tr>
</tbody>
</table>

![Fig. 3. Expression of intestine-specific transcription factors in control (open bars), cholestatic (solid bars), and bile-deficient (shaded bars) rats. Duodenal, jejunal, and ileal expression of Hnf-1α (A), Gata-4 (B), and Cdx-2 (C), all normalized to β-actin levels. D. jejunal Cdx-2 protein expression. Cells positively stained with α-Cdx-2 are indicated by the arrow. Data represent means ± SE of 5–7 rats per group. Gata-4 expression was higher in the duodenum and jejunum compared with the ileum (P < 0.01).](image)
15 min rather than at 7.5 min, respectively. Figure 4 shows significantly higher glucose concentrations in cholestatic rats compared with control and bile-deficient rats at 30 and 45 min after bolus administration. The area under the curve was not significantly changed in cholestatic rats compared with control and bile-deficient rats (1.108 ± 51 vs. 1.050 ± 40 and 1.024 ± 28 mmol·l⁻¹·min⁻¹, respectively, NS).

**Monomeric glucose absorption is maintained in cholestatic rats.** Monomeric glucose absorption was assessed by determination of plasma appearance of [²H]glucose after its intraduodenal administration. Plasma [²H]glucose concentrations peaked at 7.5 min in control, cholestatic, and bile-deficient rats (38 ± 4, 39 ± 6, and 41 ± 3 μmol/l, respectively; Fig. 5). Areas under the curve were similar in cholestatic, control, and bile-deficient rats (2.220 ± 363, 1.956 ± 315, and 1.763 ± 222 μmol·l⁻¹·min⁻¹, respectively, NS).

In all groups, expression of the apical glucose transporter Sglt-1 and that of the basolateral glucose transporter Glut-2 were slightly lower in the ileum compared with the duodenum and jejunum (all P < 0.05; Fig. 5). Jejunal Sglt-1 expression was significantly increased in bile-deficient rats compared with control rats (P < 0.01). Glut-2 expression was not significantly different between the three groups.

**Sucrose digestion is maintained in cholestatic rats.** Sucrose digestion was assessed by appearance of plasma [¹³C]glucose derived from [¹³C]sucrose. Plasma [¹³C]glucose concentrations peaked at 30 min in control, cholestatic, and bile-deficient rats (3.4 ± 0.2, 4.1 ± 0.5, and 3.2 ± 0.3 mmol/l, respectively; Fig. 6). Area under the curve was not significantly changed in cholestatic rats, compared with control and bile-deficient rats (346 ± 42 vs. 312 ± 17 and 266 ± 21 mmol·l⁻¹·min⁻¹, respectively, NS). Sucrase enzyme activity was highest in the jejunum and lowest in the ileum in control rats (all P < 0.01; Fig. 6). Duodenal sucrase enzyme activity was significantly lower in bile-deficient rats compared with control rats (6.9 ± 1.0 vs. 8.3 ± 0.8 μmol·mg protein⁻¹·h⁻¹, respectively, P < 0.05). The expression pattern of *sucrase-isomaltase* was less pronounced than that of sucrase enzyme activity, but jejunal expression was also significantly higher than ileal expression (P < 0.05). No differences were found among the groups.

**DISCUSSION**

In this study, we investigated whether cholestasis, i.e., the combination of systemic accumulation of bile salts and the deficiency of bile salts in the intestinal lumen, affects the constitution and absorptive capacity of the rat small intestinal epithelium. We compared cholestatic rats with bile-deficient rats, without systemic accumulation, and with control rats. We found that short-term extrahepatic cholestasis in rats does not affect intestinal morphology, proliferation, or apoptosis, nor the functional capacity of the intestine to digest sucrose and to absorb glucose.

Several studies describe the effect of the absence of bile components in the intestinal lumen on intestinal bile salt transporter expression in rats. They report that rat Asbt expression is not regulated by bile salts, whereas rat Ibabp expression is positively regulated by bile salts via Fxr activation (3, 13, 17, 20, 23, 33, 36). In accordance with the literature, our data indicate that Asbt expression is not affected in cholestatic and bile-deficient rats. *Ibabp* expression was decreased in cholestatic rats and significantly decreased in bile-deficient rats. The increased expression of *Ibabp* in cholestatic rats compared with

![Fig. 4. Blood glucose concentration in response to a 1-ml intraduodenal bolus containing [¹³C]sucrose (0.25 g) and [²H]glucose (1 mg) in control (open circles), cholestatic (solid circles), and bile-deficient (shaded circles) rats. Data represent means ± SE of 5–7 rats per group. *P < 0.05 vs. control group. **P < 0.01 cholestatic vs. bile-deficient group.](http://ajpgi.physiology.org/)

![Fig. 5. Glucose absorption in response to a 1-ml intraduodenal bolus containing [¹³C]sucrose (0.25 g) and [²H]glucose (1 mg) in control (open circles/bars), cholestatic (solid circles/bars) and bile-deficient (shaded circles/bars) rats. Data represent means ± SE of 5–7 rats per group. **P < 0.01 cholestatic vs. bile-deficient group.](http://ajpgi.physiology.org/)
bile-deficient rats may be explained by the occurrence of retrograde transport of bile salts from the blood compartment into the epithelial layer of the small intestinal lumen. However, expression of bile salt-sensitive Shp was markedly decreased in both cholestatic and bile-deficient rats. The strongest reduction was observed in the ileum, coinciding with the highest Fxr expression (16). The bile duct-ligated rats had strongly elevated plasma bile salt levels and significantly reduced net fat absorption, in accordance with cholestasis. As previously described (25), cholestatic rats lost slightly more weight than bile-deficient and control rats, despite similar food intakes. The weight loss may be related to the decreased net fat absorption in cholestatic rats (26), compared with unchanged net fat absorption in bile-deficient rats fed a chow diet (22). It is well known that the composition of chow is variable between batches. Therefore we used only one batch to feed all rats before and during our experiments. Usage of a purified diet, such as AIN-93M, is not possible in this experimental setting, because the cornstarch in the diet is naturally enriched in $^{13}$C, as is the sucrose (cane sugar) we used to assess sucrose digestion. Pilot experiments showed that the enrichment of $[^13]$C-glucose in the plasma is immeasurable, because of the high baseline enrichment in rats fed a diet containing cornstarch (unpublished observations).

In light of numerous in vitro data in the literature, we anticipated cholestasis to induce either proliferation or apoptosis in the small intestinal epithelium. Conjugated bile salts in concentrations found during cholestasis induce proliferation in the rat small intestinal cell line IEC-6, and in the human colon carcinoma cell line Caco-2, which gains small intestinal epithelial features upon differentiation (1, 39, 43). In contrast to enterocytes, relatively low concentrations (50 $\mu$M) of conjugated bile salts induce apoptosis in human hepatoma cell lines and primary rat hepatocytes (14, 32, 34). Bile salts in cholestatic concentrations can also be indirectly implicated in inhibition of differentiation and function of small intestinal epithelial cells. Suh and Traber (37) demonstrated that the intestine-specific transcription factor Cdx-2 is an important regulator of differentiation in the small intestine. Differentiation is regulated by phosphorylation and subsequent activation of Cdx-2 via the MAPK/ERK route. Phosphorylated Cdx2 inhibits transcription of the sucrase-isomaltase gene (30). MAPK/ERK, in turn, appears to be activated by various bile salts (2). However, our present results indicate that cholestatic concentrations of bile salts do not affect proliferation, apoptosis, or differentiation in rat small intestinal epithelium in vivo. The discrepancy between the in vitro and in vivo data can possibly be explained by the fact that enterocytes might be more resistant in an in vivo setting, related to protective environmental factors. Besides, the absorption of nutrients and of bile salts takes place in different intestinal segments. In cholestatic rat models, enterocytes are exposed to bile salt concentrations only at their basolateral membrane, whereas intestinal cell lines are exposed to both sides or are not (completely) polarized. Bile salts do not need to enter the cells to activate cellular signaling routes. Kawamata et al. (24) reported that membrane-type G protein-coupled receptor Gpbar1 is expressed in the intestine. In our rats, Gpbar1 was expressed in similar amounts in duodenum, jejunum, and ileum. It is unknown whether Gpbar1 is expressed at apical or basolateral membrane domains. Finally, several in vitro studies have addressed intestinal cell exposure to high concentrations of unconjugated bile salts in the context of colon cancer. Small intestinal cells in vivo are, however, normally not exposed to free bile salts in those concentrations and compositions.

Data addressing to what extent cholestasis or intestinal bile deficiency affect small intestinal function in vivo is scarce. In accordance with our current results, several rat models of cholestasis or intestinal bile deficiency have been reported to have unchanged small intestinal function. Borges et al. (7) reported that obstructive jaundice did not affect jejunal absorption of glucose in rats. Sucrase enzyme activity was shown to be unaffected in cholestyramine-fed and bile-diverted rats (35). Corresponding with unaffected or minimally affected epithelial integrity, we have previously shown that fat absorption in 1 wk bile-diverted rats could be quantitatively reconstituted within hours by using the infusion of model bile (28).
Malnutrition in children with cholestasis has a profound effect on mortality rate of children with end-stage cholestatic liver disease (4, 6, 12). The association between cholestatic liver disease and fat malabsorption is well known. Previous studies in our group have demonstrated that cholestatic rats and bile-deficient rats have severe fat malabsorption (22, 26, 27), as confirmed in the present study. Rings et al. (31) showed that the absorption of free fatty acids but not fat digestion was rate limiting for overall fat absorption in neonates that are known to have a mild “physiological” cholestasis during the first months of life. Our data indicate that intestinal deficiency of bile salts, with or without systemic accumulation of bile salts, does not impair intestinal carbohydrate digestion or absorption in rats. These observations suggest that increasing the dietary carbohydrate intake above conventional levels could be used in preventive or therapeutic fashion for a poor nutritional status induced by fat malabsorption in cholestatic children.

ACKNOWLEDGMENTS

The authors thank Rick Havinga, Theo Boer, Klaas Bijsterveld, Juul Bailer, Marion Priebe, Els Jonkers, Fjodor van der Slijus, and Renze Boeverhof for excellent technical assistance and helpful suggestions.

GRANTS

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

REFERENCES


