Reduced production of sulfated glycosaminoglycans occurs in Zambian children with kwashiorkor but not marasmus1–4


ABSTRACT
Background: Kwashiorkor, a form of severe malnutrition with high mortality, is characterized by edema and systemic abnormalities. Although extremely common, its pathophysiology remains poorly understood, and its characteristic physical signs are unexplained.

Objective: Because kwashiorkor can develop in protein-losing enteropathy, which is caused by a loss of enterocyte heparan sulfate proteoglycan (HSPG), and previous observations suggest abnormal sulfated glycosaminoglycan (GAG) metabolism, we examined whether intestinal GAG and HSPG are abnormal in children with kwashiorkor.

Design: Duodenal biopsy samples collected from Zambian children with marasmus (n = 18), marasmic kwashiorkor (n = 8), and kwashiorkor (n = 15) were examined for expression of HSPG, GAGs, and immunologic markers and compared against reference samples from healthy UK control children. GAG and HSPG expression density and inflammatory cell populations were quantitated by computerized analysis.

Results: The kwashiorkor group was less wasted and had a lower HIV incidence than did the other groups. All duodenal biopsy samples showed inflammation compared with the histologically unflamed control samples. Biopsy samples from marasmic children had greater inflammation and greater CD3+ and HLA-DR (human leukocyte antigen DR)–positive cell densities than did samples from children with kwashiorkor. Expression of both HSPG and GAGs was similar between marasmic and well-nourished UK children but was markedly lower in children with kwashiorkor in both the epithelium and lamina propria. Although underglycosylated and undersulfated, epithelial syndecan-1 protein was normally expressed in kwashiorkor, which confirmed that abnormalities arise after core protein synthesis.

Conclusions: Intestinal HSPG loss occurs in kwashiorkor, which may precipitate protein-losing enteropathy to cause edema. If occurring systemically, impaired HSPG expression could cause several previously unexplained features of kwashiorkor. We speculate that a genetic predisposition to reduced HSPG biosynthesis may offer a contrasting selective advantage, by both diminishing protein catabolism during transient undernutrition and protecting against specific infectious diseases.

INTRODUCTION

Edema that occurs because of malnutrition has been recognized since biblical times (1). The name kwashiorkor is derived from a Ghanaian term for a characteristic disease arising in young children and is clinically distinct from marasmus (1). In addition to edema, features distinguishing kwashiorkor from marasmus include hypoaalbuminemia, skin exfoliation, fatty liver, susceptibility to infection, heart failure, thrombosis, profound apathy, and suppressed appetite (1). In marasmic kwashiorkor, edema coexists with severe wasting. In Zambia, children with marasmic kwashiorkor have mild edema and are clinically closer to marasmus than kwashiorkor (2). Kwashiorkor is both extremely common and the most lethal form of malnutrition—fatality rates for established kwashiorkor remain at ~25% or more in many facilities (3, 4).

Early theories that disease is caused by a low-protein, carbohydrate-based diet (1) have been rendered unlikely by findings of similar protein intakes in children developing kwashiorkor and marasmus (5, 6). Resolution of all signs, including edema, occurs with low-protein treatment regimens, and low-protein diets do not reproduce kwashiorkor in animals (1). Speculation that kwashiorkor might be caused by aflatoxin poisoning (7) has not been supported by analyses of postmortem tissues (1). Although enhanced free radical production occurs, causing lipid peroxidation (1), a large field trial of an antioxidant cocktail did not prevent kwashiorkor (8). Golden et al (1, 9) speculated that sulfated glycosaminoglycans (GAGs) might be disrupted in kwashiorkor on the

1 From the Department of Paediatrics and Child Health, University Teaching Hospital of Lusaka, Lusaka, Zambia (BA and MM); the Centre for Paediatric Gastroenterology, Royal Free & University College School of Medicine, London, United Kingdom (AOF, FT, and CS); the Tropical Gastroenterology and Nutrition Group, University of Zambia School of Medicine, Lusaka, Zambia (PK); the Queen Mary, University of London, London, United Kingdom (PK); the Centre for Gastroenterology and Nutrition, University College London, London, United Kingdom (RD); the University of Aberdeen, Aberdeen, United Kingdom (MHG); the Burnham Institute for Research, La Jolla, CA (EAE and HHF); and the Clinical Sciences Research Institute, Warwick Medical School, University of Warwick, Coventry, United Kingdom (SHM).

2 BA and AOF are joint first authors.

3 PK was supported by The Wellcome Trust, and HHF and SHM were supported by the Children’s Heart Fund for research into protein-losing enteropathy.

4 Address correspondence and reprint requests to SH Murch, Warwick Medical School, Clinical Sciences Research Institute, Clifford Bridge Road, Coventry CV2 2DX, United Kingdom. E-mail: s.murch@warwick.ac.uk. Received October 13, 2008. Accepted for publication November 22, 2008. First published online December 30, 2008, doi: 10.3945/ajcn.2008.27092.
basis of findings of diffusion of interstitial dye, renal podocyte effacement, resistance of these children to cholera (1), and reports of low urinary excretion of sulfate and sulfated GAGs (10, 11). The generalized nature of this defect in complex carbohydrate metabolism is suggested by findings of striking reductions in brain sulfated GAGs in kwashiorkor, with relative sparing of undersulfated chondroitin sulfate over heparan sulfate proteoglycan (HSPG) (12). These findings agree with magnetic resonance imaging data showing marked brain volume shrinkage that recovers with nutritional rehabilitation (13).

We identified a specific role for HSPG in protein-losing enteropathy (PLE). Infants with congenitally absent enterocyte HSPG suffered fatal PLE (14), whereas mislocalization of enterocyte HSPG in congenital disorder of glycosylation (CDG) caused episodes of life-threatening PLE (15). In vitro studies confirmed a critical role for HSPG in preventing PLE and identified synergy between HSPG loss, cytokines (tumor necrosis factor- \( \gamma \) and interferon- \( \gamma \)), and hydrostatic pressure in promoting intestinal protein leak (16, 17). We confirmed these findings in vivo in transgenic mice, in which cytokines or pressure-induced intestinal albumin leak was markedly increased when gut epithelial expression of proteoglycan syndecan-1 or HSPG is inhibited or HSPG glycan chain synthesis is reduced (18, 19). In view of the similarities between infants with kwashiorkor and infants with abnormal intestinal HSPG expression, we evaluated the hypothesis that there may be abnormal intestinal expression of HSPG in kwashiorkor.

SUBJECTS AND METHODS

All patients were treated, and the investigations were performed on the malnutrition ward of the University Teaching Hospital, Lusaka, Zambia. We examined duodenal biopsy samples that had been taken previously for a study of enteral nutrition therapy in malnourished children, which was performed during 1998–2000 (2, 20). The material came from 3 groups of children: marasmus (\( n = 18 \)), marasmic kwashiorkor (\( n = 8 \)), and classic kwashiorkor (\( n = 15 \)). Their clinical details are shown in Table 1. Follow-up duodenal biopsy samples were studied from 16 of these patients who successfully completed nutritional rehabilitation (5 marasmus, 5 marasmic kwashiorkor, and 6 kwashiorkor).

Children were recruited if they had malnutrition and persistent diarrhea lasting \( \geq 14 \) d (2, 20). Children were excluded if they had features of measles, chicken pox, or neurologic disorder or if they were exclusively breastfed. The study protocol was approved by the Research Ethics Committee of the University of Lusaka, and formal consent obtained from parents or guardians for each procedure. A structured medical history was taken and a complete physical examination was performed, including anthropometric measurements. Malnutrition was classified as underweight, marasmus, kwashiorkor, and marasmic kwashiorkor as previously reported (21), and analyses of infection, malnutrition, mortality, and response to nutrition have been published (2, 20).

Duodenal biopsy samples were collected by BA via endoscopy under sedation with midazolam. No adverse events were identified during or after these procedures, although 2 follow-up endoscopies were cancelled because of paradoxical agitation during sedation.

Biopsy analysis

Avidin-biotin immunohistochemistry (Vectastain Elite; Vector, Peterborough, United Kingdom) was performed after appropriate antigen retrieval and blocking of endogenous peroxidase. Antibodies used included anti-CD3 (1:50), human leukocyte antigen DR-1 (HLA-DR; 1:50), Ki67 (1:50; all Dako, Cambridge, United Kingdom), syndecan-1 (1:50; Serotec, Oxford, United Kingdom), and HSPG (10E4, 1:50; Seigakaku, AMS Biotech, Abingdon, United Kingdom), as previously reported (15, 22). The distribution of sulfated GAGs was studied with a poly-L-lysine probe at pH 1.2, as previously reported (14, 15). Both the HSPG monoclonal and the poly-L-lysine probes have been extensively characterized. Monoclonal 10E4 detects \( N \)-acytelylated and \( N \)-sulfated disaccharides within HSPG glycan chains, and its staining is abrogated by preincubation with heparitinase before application of the monoclonal probe (23). The poly-L-lysine probe is specific for sulfate residues in GAG chains of heparan, deraman, and chondroitin sulfates (14, 15).

Previous studies have established that the probe for sulfated GAGs recognizes predominantly HSPG within the epithelium and a combination of heparan sulfate, deraman sulfate, and chondroitin sulfate within the lamina propria (14). Both methods demonstrate similar epithelial staining in normal small intestine, which is predominantly basolateral, although intracellular staining may be variably seen toward the villus tip (14). Within the lamina propria, the poly-L-lysine probe for sulfated GAGs may demonstrate more extensive interstitial staining in normal patients, which may be variably increased in severe malnutrition (22).


### TABLE 1

Clinical characteristics of the patients at the time of admission

<table>
<thead>
<tr>
<th></th>
<th>Marasmus (( n = 18 ))</th>
<th>Marasmic kwashiorkor (( n = 8 ))</th>
<th>Kwashiorkor (( n = 15 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mo)</td>
<td>16 (12.2, 17.4)(^1)</td>
<td>17 (13.6, 19.1)</td>
<td>19 (16, 19.8)</td>
</tr>
<tr>
<td>Sex [M/F (( n ))]</td>
<td>11/7</td>
<td>4/4</td>
<td>11/4</td>
</tr>
<tr>
<td>Weight-adjusted ( z ) score</td>
<td>–4.8 (–3.2, –5.4)</td>
<td>–4.7 (–4.5, –5.3)</td>
<td>–3.6 (–3.1, –3.9)</td>
</tr>
<tr>
<td>Weight for height ( z ) score</td>
<td>–3.5 (–2.9, –5.2)</td>
<td>–4.5 (–3.4, –5.1)</td>
<td>–2.5 (–2.1, –3.2)</td>
</tr>
<tr>
<td>Height-adjusted ( z ) score</td>
<td>–2.7 (–1.3, –4)</td>
<td>–3.1 (–1.6, –3.8)</td>
<td>–3 (–2, –3.5)</td>
</tr>
<tr>
<td>HIV positive (( n ))</td>
<td>14</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Acid fast bacilli in gastric aspirate (( n ))</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Fever at time of admission (( n ))</td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^1\) Comparisons between groups were made by using an unpaired \( t \) test if probable significant differences were identified by ANOVA and a multiple range test.

\(^2\) Median; 95% CI in parentheses (all such values).

\(^3\) Significantly different from kwashiorkor, \( P < 0.05 \).
than the HSPG monoclonal probe, although many cell types are HSPG immunoreactive, and extracellular HSPG immunoreactivity may also occur because of secretion by fibroblasts or cleavage of cell surface proteoglycans (15, 23).

Digital photomicrographs were prepared from all slides with use of the same microscope (Leica DMLB) and image capture system (Nikon Digital Sight DL-S1). All quantitations were made on unprocessed images with the use of ImageJ software (National Institutes of Health, Bethesda, MD: http://rsb.info.nih.gov/ij/). For quantitation of staining densities, plug-ins for color deconvolution (AC Ruifrok, Netherlands) and for region-of-interest densitometry (J Kuhn, A Padua, USA) were used to separate specific di-aminobenzidine (DAB) or silver staining from haemalum counterstain and to quantify local density of DAB or silver stain. Separate quantitation was done in the lamina propria, excluding crypts, and intraepithelial compartments. For the lamina propria, low-power images were used, and a single field was drawn around the entire compartment excluding crypts. If biopsy morphology prevented measurement of a single compartment, a mean was obtained from separate compartments of roughly equal areas. For the epithelium, high-power images were used, and a mean density was derived for each specimen from quantitation of multiple individual intercellular spaces in well-orientated villi.

Densitometry was performed on an inverse scale (1–250 units), based on light transmission. The staining density index presented is reciprocal [1/transmission intensity $\times 10^4 - 40$ (background subtraction)], but all statistical analyses were performed on unprocessed data. With this derived index, values $\geq 150$ represent intense staining, whereas values $\leq 60$ represent faint staining. Reviewing previously reported specimens (15), an epithelial HSPG expression density of 60 was associated with acute severe PLE, whereas an expression density of 100 was sufficient to prevent intestinal protein leak. Cell densities were quantified by using the Nucleus Counter Plug-in (ImageJ software). Previously stained biopsy samples from UK children, reported in other studies (14, 15, 22), that were found not to have significant gastrointestinal disease were similarly quantified for expression of sulfated GAGs and HSPG.

Serum analysis

HIV serology was performed in all patients as previously reported (2). Isoelectric focusing for analysis of transferrin glycosylation was performed in 9 HIV-negative patients (15).

**Statistical analysis**

Data were assessed for normal distribution by analysis of standardized skewness and standardized kurtosis by using Stata graphical 5 Plus software (Manugistics Inc, Rockville, MD). Data were compared initially by analysis of variance (ANOVA) or Kruskal-Wallis ANOVA and multiple range testing. When potentially significant differences were identified, comparison was made between individual groups by using an unpaired $t$ test (clinical groups) or a Mann-Whitney $U$ test (all histochemical and immunohistochemical measurements), with appropriate adjustment for multiple comparisons. $P$ values $\leq 0.05$ were considered statistically significant.

**RESULTS**

There were some differences between the groups at presentation (Table 1). Other than a lower incidence of tuberculosis (acid fast bacilli detected in gastric aspirate) in marasmus and of HIV in kwashiorkor, infectious complications were similar between groups. This lower prevalence of HIV in kwashiorkor was representative of the overall study population of 200 children previously reported (2).

No significant differences in biopsy samples by conventional histology were observed between the groups (Table 2). However, mucosal architecture was markedly abnormal compared with that in healthy UK children, who had a median villus:crypt ratio of $\approx 2:1$ (22). Villous height was lower and crypt depth increased to a similar extent as in UK specimens in all malnutrition groups, with minimal change after nutritional rehabilitation (Table 2).

**Mucosal inflammation in marasmus and kwashiorkor**

Mucosal inflammatory responses were different between the clinical groups (Figures 1, Table 3), although no significant difference in overall lamina propria cell density was observed between the groups (Table 2). All groups had a significantly higher CD3$^+$ intraepithelial lymphocyte (IEL) density than did UK control children, as previously noted in Gambian children (22). This was significantly higher in the children with marasmic kwashiorkor than in all other groups, with IEL density reaching levels seen in celiac disease (Table 3, Figure 1). Within the lamina propria, all groups had a 4–5-fold higher total CD3$^+$ T cell density (Table 3) than did UK children, which had an overall cell density similar to that in malnourished Gambian children (22). The children with kwashiorkor had significantly lower lamina propria T cell counts than did those with marasmus (Table 3).

**TABLE 2**

<p>| Mucosal characteristics by hematoxylin and eosin staining of biopsy samples, by group |
|-----------------------------------------------|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Marasmus ($n = 18$)</th>
<th>Marasmic kwashiorkor ($n = 8$)</th>
<th>Kwashiorkor ($n = 15$)</th>
<th>Nutritional rehabilitation ($n = 15$)</th>
<th>UK controls ($n = 19$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villous height (µm)</td>
<td>241.9 ± 10.8$^2$</td>
<td>243.2 ± 25.8</td>
<td>282.4 ± 27.5</td>
<td>250.8 ± 12.9</td>
</tr>
<tr>
<td>Crypt depth (µm)</td>
<td>263.4 ± 25.4</td>
<td>263.0 ± 23.4</td>
<td>274.4 ± 18.2</td>
<td>245.8 ± 15.9</td>
</tr>
<tr>
<td>Villus:crypt ratio</td>
<td>1.12 ± 0.15</td>
<td>1.14 ± 0.17</td>
<td>1.00 ± 0.16</td>
<td>1.01 ± 0.11</td>
</tr>
<tr>
<td>Lamina propria cell density</td>
<td>7290 (5780, 8801)$^3$</td>
<td>7584 (6696, 8472)</td>
<td>6842 (5175, 8509)</td>
<td>8202 (6930, 9474)</td>
</tr>
</tbody>
</table>

$^1$ No significant differences were found between the study groups in villous height, crypt depth, villus:crypt ratio, or lamina propria cell density by ANOVA and multiple range testing; thus, further Mann-Whitney testing was not performed. Normal data for comparison of villus and crypt architecture with UK control children were obtained from previous studies (22). The nutritional rehabilitation group includes some patients from each group in the first 3 columns, for whom biopsy had been repeated after a period of nutritional therapy.

$^2$ Mean ± SE (all such values).

$^3$ Mean; 95% CI in parentheses (all such values).
There was a strongly up-regulated expression of the interferon-γ-induced class II major histocompatibility complex molecule HLA-DR on surface epithelium in all Zambian cases. This indicated a Th1-dominated mucosal response in these children initially enrolled into the nutritional intervention study (2, 20), which has also been observed in both malnourished and well-nourished Gambian children (13), although in UK children such a finding is considered abnormal. On formal quantitation of staining intensity, no overall difference in HLA-DR expression in the epithelium was detected between clinical groups (Table 3). In contrast, there was a significantly lower density of HLA-DR+ cells in the lamina propria in the children with kwashiorkor than in the other groups (Table 3).

We found a strikingly higher expression of the crypt epithelial nuclear proliferation marker Ki67 in all groups compared with the UK control children (21) (Table 3, Figure 1). Although there were no differences identified between the malnourished groups, there was a reduction in crypt epithelial Ki67+ cell density in

**FIGURE 1.** Mucosal inflammatory responses. Distribution of CD3+ T cells, cells expressing human leukocyte antigen DR (HLA-DR), and the proliferative antigen Ki67 in Zambian children. Immunoreactive cells are stained brown. The top row shows slides from children with marasmus (Maras), the second from children with marasmic kwashiorkor (M-K), the third from children with kwashiorkor (Kwas), and the bottom from children participating in a nutritional rehabilitation program (Nutr rehab). All were taken from specimens near to overall mean values (data shown in Table 2).

**TABLE 3**

Results of immunohistochemical analysis of mucosal biopsy samples, by group

<table>
<thead>
<tr>
<th></th>
<th>Marasmus</th>
<th>Marasmic kwashiorkor</th>
<th>Kwashiorkor</th>
<th>Nutritional rehabilitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ IEL (per 100 enterocytes)</td>
<td>30 (26.4, 37.7)</td>
<td>45.5 (37.4, 58.3)</td>
<td>31 (26, 37.6)</td>
<td>30 (22.5, 36.9)</td>
</tr>
<tr>
<td>Epithelial HLA-DR staining intensity</td>
<td>118.7 (90.7, 202.6)</td>
<td>118.7 (80.5, 190.9)</td>
<td>120 (70.4, 194.2)</td>
<td>150.5 (113.2, 190.9)</td>
</tr>
<tr>
<td>Ki 67+ cells within crypts (%)</td>
<td>76.4 (69.5, 89.5)</td>
<td>76 (68.2, 84.4)</td>
<td>75 (57.6, 85.8)</td>
<td>68.3 (66.6, 71.4)</td>
</tr>
<tr>
<td>Lamina propria CD3+ cells (per mm²)</td>
<td>1953 (1708, 2054)</td>
<td>1842 (1358, 2457)</td>
<td>1318 (1187, 1862)</td>
<td>1893 (1527, 2060)</td>
</tr>
<tr>
<td>Lamina propria HLA-DR+ cells (per mm²)</td>
<td>2522 (2005, 2862)</td>
<td>2347 (1938, 3181)</td>
<td>1907 (1462, 2208)</td>
<td>2527 (1972, 2819)</td>
</tr>
<tr>
<td>Lamina propria area HLA-DR+ (%)</td>
<td>0.7 (0.41, 0.85)</td>
<td>0.61 (0.36, 0.95)</td>
<td>0.35 (0.18, 0.57)</td>
<td>0.52 (0.28, 0.89)</td>
</tr>
<tr>
<td>Lamina propria Ki67+ cell density (per mm²)</td>
<td>716 (311, 991)</td>
<td>845 (386, 1253)</td>
<td>488 (394, 632)</td>
<td>810 (504, 1119)</td>
</tr>
</tbody>
</table>

1 All values are medians (95% CIs). Reference data for healthy UK control children were obtained from previous studies (22): median <20 for CD3+ intraepithelial lymphocytes (IEL; per 100 enterocytes), median <30 for Ki 67+ cells within crypts (%), and 290–390 for lamina propria CD3+ cells (per mm²). HLA-DR, human leukocyte antigen DR. Differences that were potentially significant by ANOVA or Kruskal-Wallis test were examined further by a Mann-Whitney U test.

2,3 Significantly different from marasmic kwashiorkor: 2P < 0.05, 3P < 0.01.

4 Significantly different from nutritional rehabilitation, P < 0.01.

5,6 Significantly different from kwashiorkor: 5P < 0.05, 6P < 0.01.
Reduced mucosal HSPG expression in classic kwashiorkor

Mucosal findings in children with marasmus (Figures 2 and 3), using monoclonal 10E4 for HSPG glycan chains and poly-L-lysine probe for sulfate residues in GAGs, were as previously reported in intestinal specimens for both stains (14, 15, 24, 25). Epithelial staining was generally maximal on the basolateral surface, although more diffuse intracellular staining was seen in some epithelial cells, maximal toward the villous tip. The significance of this finding is unclear. Lamina propria staining was more extensive. The strongest staining occurred in basement membranes and vascular endothelium. Many cell types, including plasma cells and lymphocytes, showed strong pericellular staining, whereas macrophages and fibroblasts additionally showed diffuse intracellular staining. Some additional stromal staining from shed HSPG ectodomains were identified. Staining was slightly stronger when the cationic probe for sulfated GAGs was used, which also detects chondroitin and dermatan sulfates. Overall, the staining patterns appeared similar to those seen in well-nourished UK control children, although some cases showed reduced staining intensity. No significant group differences were identified in mucosal GAG or HSPG expression density between well-nourished UK control children and Zambian children with marasmus or marasmic kwashiorkor (Figure 2). In contrast, there was a significant reduction in GAGs within both the epithelium and lamina propria in the kwashiorkor group (Figure 2A, Figure 3). Both cationic probe histochemistry for sulfate residues and immunohistochemistry for HSPG glycan chains showed markedly reduced epithelial staining intensity in kwashiorkor specifically, although with some overlap in individual cases. However, staining with a monoclonal antibody recognizing the core protein of the proteoglycan syndecan-1 showed that this was normally localized and preserved in kwashiorkor (Figure 2A, Figure 3). Thus, the protein itself appeared normally expressed but had underglycosylated and undersulfated GAG chains attached, indicating a possible posttranslational defect.

Within the lamina propria, we also found a reduced expression of both sulfated GAGs and HSPG in the epithelium of the kwashiorkor group (Figure 2B, Figure 3), although again with some overlap. Therefore, an impaired expression of both sulfated GAGs and HSPG occurred overall in the group with kwashiorkor throughout the small intestinal mucosa, despite less mucosal inflammation. Our evidence confirming a central role of intestinal HSPG in preventing protein leakage (16–18) predicts that PLE is a feature of kwashiorkor. Several patients with kwashiorkor had a loss of epithelial HSPG and GAGs similar to that identified previously to be associated with acute PLE (14, 15). Because lost HSPG synergizes strongly with proinflammatory cytokines and hydrostatic pressure (16–19), it will be exacerbated if infection or kwashiorkor-associated heart failure develops.

Analysis of the relatively few biopsy samples taken after nutritional rehabilitation showed more normal appearances in those who had survived kwashiorkor, although epithelial HSPG and GAGs still showed reduced staining density compared with samples from survivors of the other malnutrition disorders. However, available numbers were small, and the results showed wide scatter, although not statistically significant. These data are thus too preliminary to determine whether there may be constitutive epithelial HSPG underglycosylation in children who develop kwashiorkor, independent of nutritional status.
DISCUSSION

Our findings support data from other organs, which indicates a generalized loss of sulfated GAGs in kwashiorkor (1, 9–12). We acknowledge limitations imposed by studies of biopsy samples, too small for in-depth glycobiological analysis with current technology. Analysis of staining intensity, even with computerized densitometry, is a relatively crude approach to a complex biosynthetic process. Potential sources of artifact may arise during tissue handling or staining. However, using 2 well-characterized stains specific for glycan carbohydrates (15) and sulfates (14, 15), we identified a similar pattern of reduced sulfated GAG expression throughout the small intestinal mucosa. Children with marasmus and persistent diarrhea generally showed HSPG expression similar to that of normal European children. Thus, it is unlikely that nutritional deficiencies causing wasting and stunting per se induce this loss. Because sulfated GAGs are now identified as critical in many aspects of physiology (24), their disruption may play a role in the signs and pathophysiology of kwashiorkor.

HSPG loss promotes albumin leakage and reduced tissue turgor (9, 14–18, 24, 25). Although urinary protein loss is rare in kwashiorkor (9), PLE has been reported and may precipitate disease (26, 27). Although we found normal HSPG in marasmic kwashiorkor, IEL density was high, which indicated a likely recent infection. We previously showed that reglycosylation capacity limits PLE after HSPG loss (16–18) and suggests that these previously marasmic children may have suffered an acute episode of PLE on infection but were then able to reglycosylate to restore the epithelial barrier.

HSPG deficiency is consistent with known associations of kwashiorkor. Its frequent onset after displacement from the breast may relate to both infections and substrate deficiency, because breast milk is rich in N-acetyl glucosamine (GlcNAc) and sulfur-containing amino acids (28). It is intriguing that consumption of insects, high in chitin GlcNAc, appears to protect infants against kwashiorkor, which suggests the ironic possibility that Western missionaries may have increased the incidence of kwashiorkor in the early 20th century because of their campaign against entomophagy (29). Aflatoxin liver damage is associated with endothelial HSPG loss, and heparin is strongly protective (30). The known state of oxidative stress (1) may also relate to HSPG deficiency, because the constitutive antioxidant function of superoxide dismutase is dependent on HSPG binding (31) and complex carbohydrates are a target for oxidative damage.

Even under conditions of extreme privation, it is unusual for more than ~10% of children to develop kwashiorkor. Why then do 90% of children living in the same household and eating the same diet fail to develop kwashiorkor? A potential genetic predisposition was postulated by Gopalan (3). We note in this context that mice with congenitally reduced HSPG biosynthesis appear normal but show exaggerated enteric protein leakage on cytokine challenge (11, 32) or under conditions of undernutrition (L. Bode, H. Freeze, unpublished observations, 2008).

Undernutrition induces competition between metabolic pathways for limited nutritional substrates. Low glycosylators may then better maintain body mass, because reduced consumption of substrates in glycosylation reduces the need for protein catabolism. There is indeed evidence that protein breakdown is reduced (33) and body mass is preserved (1, 2) in children with kwashiorkor. However, an infectious enteric challenge that causes inflammatory HSPG breakdown (25), mandating rapid epithelial reglycosylation for repair (15–18), may cause HSPG deficiency sufficient to induce PLE. Chronic enteric protein losses would then exacerbate nutritional inadequacy, causing a vicious circle.

Because of the multiple roles of HSPG (24), deficient production during starvation may explain several features of kwashiorkor in addition to oxidative stress (31). Fatty liver, caused by low hepatic lipoprotein lipase expression and high serum free fatty acids (34), is explicable on the basis that hepatic lipoprotein lipase localization and fatty acid uptake are HSPG dependent (35, 36). Hepatic knockdown of GlcNAc N-deacytelyase/ N-sulfotransferase 1 (Ndst1) in mice markedly inhibits lipoprotein clearance (36). Exfoliative dermatitis may develop because HSPG glycosylation of CD44 is important for keratinocyte adhesion (37). Vascular thrombosis is a known consequence of lost HSPG-dependent antithrombotic mechanisms (24, 27), whereas myocardial contractility is decreased by protamine, which blocks GAGs (38) and is increased by heparin (39). Reduced glycosylation of syndecans (as for syndecan-1) may induce hypophagia, because hypothalamic syndecan-3 GAG chains mediate the normal hunger response (40) and may contribute to increased infectious mortality, as observed in lipopolysaccharide–challenged, syndecan-4–deficient mice (41). We thus suggest that inadequate HSPG synthesis under malnourished conditions may underpin several major features of kwashiorkor. Such loss of GAGs may also have therapeutic implications, particularly because blood–brain barrier integrity is HSPG dependent (24). In view of the major loss of brain HSPG in kwashiorkor (12), drugs that are highly toxic if they cross the blood–brain barrier, notably ivermectin (used widely in Africa to treat onchocerciasis), may provide particular risks for those with nutritional edema.

Polymorphisms reducing HSPG glycosylation or sulfation may have benefits other than limiting protein catabolism. A further predisposition to evolutionary advantage would be protection against infective diseases, because many pathogens bind specifically to domains within sulfated GAGs. Plasmodium falciparum erythrocyte membrane protein-1 induces intravascular sequestration in infected erythrocytes by binding to endothelial HSPG (42). HSPG binding avidity determines pathogenicity (43), and exogenous GAGs reverse microvascular complications (42). It is thus notable that kwashiorkor protects against severe P. falciparum malaria and that cerebral malaria is almost never seen in this disorder. Vibrio cholerae survives in water by specifically binding to GlcNAc residues in crustacean chitins and then infects host cells by binding to glycosylax GlcNAc (44). Patients with kwashiorkor showed a remarkable 25-fold reduction in cholera infection compared with those with marasmus during an outbreak in a therapeutic feeding center (1).
HIV entry across endothelial barriers is mediated by interaction between gp120 and HSPG (45), and transplacental infection is thus likely to be HSPG dependent. The finding of 50% less HIV seropositivity in kwashiorkor in the overall cohort of 200 children from this unit (2) is in line with previous findings of 3-fold lower HIV seropositivity in Malawian children (46), 4-fold lower HIV seropositivity in Indian children with kwashiorkor (47), and 2–4-fold lower HIV seropositivity in numerous other African countries. Thus, vertical transmission of HIV appears to be reduced in infants who later develop kwashiorkor. We speculate that genetic polymorphism or polymorphisms in GAG expression by the mother or infant may protect the infant from both intrauterine and breast-milk transmission of the virus.

Delayed clinical progression of HIV also occurs in children who develop kwashiorkor (48).

We speculate that HSPG loss in kwashiorkor may represent a genetically determined reduction in biosynthesis, promoting disease if dietary substrates are inadequate and GI infections necessitate reglycosylation, but limiting body protein catabolism in undernutrition and protecting against some infectious diseases. Protection against HIV clearly predates the development of kwashiorkor. If HSPG biosynthesis is indeed genetically altered in children at risk of kwashiorkor, those who survive it should show a subclinical reduction in HSPG glycan chain length or sulfation at sites of rapid cellular turnover. This is testable, and, if confirmed, would suggest the need for studies of functional

**FIGURE 3.** Distribution of sulfated glycosaminoglycans (GAGs) and heparan sulfate proteoglycan (HSPG) within the mucosa. A: High-power (original magnification ×40) images of histochemical staining for sulfated GAGs (left column) and immunohistochemical staining for HSPG and the proteoglycan syndecan-1 in the villous of children with marasmus (Maras) or kwashiorkor (Kwash). The arrows indicate basolateral epithelial localization of sulfated GAGs and HSPG in marasmus. Similar basolateral localization occurs in well-nourished UK children, as reported previously (14, 15) and is functionally important in preventing intestinal protein leakage (16–18). Basolateral epithelial localization of sulfated GAGs and HSPG was reduced in kwashiorkor, and the subepithelial basement membrane was less well defined. In contrast, the core protein of epithelial syndecan-1 was normally expressed on the basolateral epithelial border in kwashiorkor as in marasmus. The findings differ from those observed in congenital disorders of N-glycosylation, where the core protein is mislocalized intracellularly (15). Syndecan-1 is also expressed in plasma cells within the lamina propria. B: Low-power (original magnification ×20) images showing overall mucosal expression of sulfated GAGs and HSPG in serial sections from children with marasmus, marasmic kwashiorkor (M-K), or kwashiorkor. Expression of GAGs and HSPG was also reduced within the lamina propria in kwashiorkor. Similar staining patterns can be seen in the serial sections stained for GAGs and HSPG (lower row). Staining density index data for the images shown in A and B were similar to the mean values for the overall groups (data shown in Figure 2).
polymorphisms in genes involved in GAG glycan chain synthesis and sulfation (49). Our findings also have potential therapeutic implications. It was shown that mucosal GAG synthesis increased in inflammatory bowel disease after enteral administration of GlcNAc (50) and PLE was inhibited in animals by a heparin analog (18, 19).

We thank all the scientists and clinicians who contributed to the initial part of the nutritional intervention study, particularly John Walker-Smith and Mike Thomson for their contributions to the study design and to SHS International Ltd for a grant that facilitated the study. We particularly wish to express gratitude to members of the team of nurses who worked so hard to achieve a high standard of clinical care and record keeping, including Elwyn Chomba, Chifumbe Chintu, Stella Sinyangwe, Justina Hachungula, Bernice Simpito, Evelyn Mukuka, Angela Watuka, Nyatio Sitwala, Harriet Ngulube, Sandra Chipopo, Asled Banda, Euphrosinia Bwalya, Nalishebo Muyunda, Joyce Nganga, and Eugenia Kachenjela. We are grateful to Saqib Ansari and Paul Ashwood for help with the initial biopsy assessment and to Anatoly Shinyogel for generous guidance with the ImageJ analysis.

The authors’ responsibilities were as follows—BA, MM, and PK—performed the clinical studies, including collection of the tissue samples and the clinical data; AOF, FT, CS, and RD—performed the laboratory analysis of tissue specimens and contributed to the quantitation; MHG—designed and led the laboratory analysis of tissue specimens, performed quantitative and data analysis, and drafted the paper; and HHF and SHM: developed the program of protein-losing enteropathy analysis. None of the authors are aware of any potential conflicts of interest.

REFERENCES