Small intestinal MUC2 synthesis in human preterm infants

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THE MUCUS LAYER FORMS a physical barrier between the underlying epithelium and the lumen of the gastrointestinal tract.(9) It protects the epithelium against noxious agents, viruses, and pathogenic bacteria. The structural component of this mucous layer is secretory mucin 2 (MUC2), which is secreted by goblet cells of the intestine and gives intestinal mucus a high density and viscoelasticity (30). MUC2 is a glycoprotein rich in oligosaccharides, O-linked to threonine and serine residues in the peptide backbone (29). MUC2 contains high amounts of threonine and proline tandem repeat sequences, which together constitute 20–55% of total amino acid composition (33). Threonine is an indispensable amino acid utilized in a large amount by the portal-drained viscera in first pass (28). Recently, we showed that the equivalent of almost 90% of dietary threonine is utilized by the intestine of piglets (25). The high visceral need for threonine presumably reflects the high synthesis rate of secretory mucins, e.g., MUC2. Therefore, threonine might be one of the amino acids essential for maintaining the protective mucus layer and thus intestinal barrier function.

Disturbances in intestinal barrier function, characterized by inappropriate initial bacterial colonization of the intestine and immature epithelial responses to bacteria and their toxins, are thought to be crucial in the development of necrotizing enterocolitis (NEC) in preterm infants (35). NEC is the most devastating intestinal disease in premature infants and a regular indication for neonatal bowel resection (5). Postoperatively, adequate gut adaptation and recovery of the intestinal barrier function are required to avoid malabsorption and pathogenic bacterial insults. Therefore, patients with NEC receive total parenteral nutrition for a variable period of time after surgical intervention. However, lack of enteral nutrition can induce intestinal atrophy and may reduce digestive, absorptive, and protective capacity (3, 12, 23). In these circumstances, the usage of systemically derived substrates such as threonine might be of vital nutritional importance to maintain and restore an intact mucus layer and thus intestinal barrier function.

We recently developed a tracer method to study small intestinal MUC2 synthesis and to determine the fractional synthetic rate (FSR) of MUC2 in human neonates as a potential parameter for intestinal barrier function (24). We found that dietary threonine is incorporated into small intestinal MUC2. However, because the tracer was administered enteraly, we could not verify whether threonine used for MUC2 synthesis was derived directly from the diet and/or after intestinal absorption and thus from the systemic circulation. As already known, the intestine utilizes amino acids in first pass and/or from the systemic site (20, 28, 32, 36). Hence, threonine incorporated into small intestinal MUC2 may be derived from either the lumen or the basolateral site (systemic circulation). Moreover, our study in piglets demonstrated that two-thirds of threonine utilized by the portal-drained viscera was extracted from the systemic circulation during normal protein feeding (25). Thus, in the present study, our main objective was to determine whether a functional small intestine of preterm human infants after bowel resection for NEC was able to incorporate systemically derived threonine into MUC2. Furthermore, the FSR of MUC2 was calculated to determine the capacity of the small intestine to synthesize and secrete MUC2 from systemically derived threonine.

MATERIALS AND METHODS

Patients

The study was conducted at the Department of Pediatrics, Division of Neonatology and the Department of Pediatric Surgery of the Erasmus Medical Center (MC)-Sophia Children’s Hospital (Rotterdam, the Netherlands) after approval from the Erasmus MC Institutional Review Board. Written, informed consent was obtained from the parents.

Eligible for this study were infants who had had a bowel resection for NEC in the neonatal period and received a temporary enterostomy...
during the initial surgery. The diagnosis NEC was made on specific clinical symptoms, namely systemic (e.g., apnea, respiratory failure, lethargy, poor feeding, temperature instability, or hypotension), abdominal signs (e.g., distension, gastric retention, tenderness, vomiting, diarrhea, and hematochezia), and/or characteristic features on an abdominal X-ray according to the Bell staging criteria. NEC was further confirmed at surgery and by histological features characteristic for NEC identified in resected intestinal tissue sent for routine histopathology. We included seven infants in the study. Table 1 lists their main clinical characteristics. All included infants had bowel surgery at the Erasmus MC Sophia Children’s Hospital and were admitted to the Neonatal Intensive Care Unit (NICU) or had bowel surgery at the Erasmus MC Sophia Children’s Hospital. Exclusion criterion for this study and were admitted to the Neonatal Intensive Care Unit (NICU) or had bowel surgery at the Erasmus MC Sophia Children’s Hospital. Exclusion criterion for this study was cystic fibrosis.

**Feeding**

Before onset of NEC, feeding regimens adhered to our feeding protocol for newborns, breast milk and/or formula [Nenatal, Nenatal Breast Milk Fortifier, Nutrilon Premium, or Nutrilon Pepti Junior; all from Nutricia, Zoetermeer, the Netherlands] and parenteral nutrition containing glucose, amino acids (Primene 10%; Clinitec Benelux NV, Brussels, Belgium), and lipids (Intralipid 20%; Fresenius Kabi, Den Bosch, the Netherlands). For patients in whom the diagnosis of NEC was considered, enteral feeding was immediately stopped and switched to full parenteral feeding. Patients recovering from NEC received full parenteral feeding during the first 10 postoperative days.

Next, minimal enteral feeding was added during 4 days, after which enteral feeding was gradually introduced by continuous nasogastric gavage feeding or bottle feeding under simultaneous diminishing of the parenteral nutrition. All patients included received enteral feeding. Six infants had a much higher dietary threonine intake compared with the threonine tracer amount administered intravenously and received no parenteral threonine supplementation at the time of the study. One infant received both enteral and parenteral nutrition at the time of the study (Table 1).

**Study Design**

The study was performed when the infant was clinically stable, i.e., about 4 wk (4 ± 1 wk) following bowel resection. Then, a continuous 12-h infusion (7.43 μmol/kg and 7.43 μmol/kg per h) of [U-13C]threonine (99.47%; Cambridge Isotopes Laboratories, Andover, MA) was administered intravenously. All isotopes were tested and found sterile and pyrogen free. The small intestinal outflow fluid samples were collected in adhesive bags, which were stuck around the enterostomy. At 3-h intervals, adhesive bags including small intestinal outflow fluid were removed by the attending nurse and stored at –80°C for further analysis. Small intestinal outflow fluid samples were collected during 48 h. At time zero (baseline value) and after 9 h and 12 h of tracer administration, blood samples were collected by heelstick. Blood was centrifuged immediately to separate plasma and cells. The plasma was stored at –80°C until further analysis. Figure 1 outlines the tracer-infusion study described.

**Analytical Procedure**

Isolation of MUC2. MUC2 was isolated from the small intestinal outflow fluid using cesium chloride (CsCl) density gradient ultracentrifugation according to the method previously described combined with gravity gel filtration chromatography (24, 30). Briefly, samples were homogenized in homogenization buffer, pH 7.5 [6 mol/l guanidinium HCl (Sigma), 50 mmol/l Tris(hydroxymethyl)aminomethane (Tris, GibCO), 5 mmol/l EDTA (Merck), and 1 mmol/l PMSF (Sigma)]. The disulfide bonds of mucins were chemically reduced by adding 100 mmol/l DTT (Sigma) and stirring overnight at 4°C in the dark. The homogenate was centrifuged to remove the fat layer and to isolate the supernatant at 23,700 g for 10 min at 4°C (Mulfuge 3 S-R, Heraeus, Wehrheim, Germany). Sulfhydryl groups were carboxymethylated through addition of 250 mmol/l iodoacetamide (Sigma) in the supernatant and stirring for another 24 h at 4°C. Mucins were purified by equilibrium ultracentrifugation on a CsCl (Roche, Uppsala, Sweden) density gradient. The gradient had a guanidinium HCl concentration of 4 mol/l with CsCl added to a density of 1.40 g/ml. Isopycnic density gradient centrifugation was performed in a Beckmann ultracentrifuge, 70 Ti rotor at 250,000 g for 72–88 h at 4°C. One-millilitre fractions were collected, and the mucin-containing fractions were analyzed as described below. Mucin-containing fractions were pooled and further purified by gravity gel filtration chromatography using columns (Econo-column; Bio-Rad, Veenendaal, the Netherlands) filled with 20 ml of Sepharose CL-2B resin. The resin was equilibrated with 50 mmol/l Tris, 5 mmol/l EDTA, and 1 mmol/l PMSF, pH 7.5, containing 4 mol/l guanidinium HCl. One milliliter of the mucin-containing fractions of each time point was loaded onto the

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex</th>
<th>GA, wk</th>
<th>BW, g</th>
<th>AS, days</th>
<th>Cause</th>
<th>Enterostomy</th>
<th>Residual Small Intestine</th>
<th>Enteral Threonine Intake, μmol/kg per h</th>
<th>Parenteral Threonine Intake, μmol/kg per h</th>
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<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>28</td>
<td>695</td>
<td>11</td>
<td>NEC (small bowel)</td>
<td>Ileostomy</td>
<td>Small bowel excluding 7 cm ileum</td>
<td>55</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>30</td>
<td>930</td>
<td>33</td>
<td>NEC (small bowel)</td>
<td>Jejunostomy</td>
<td>Small bowel excluding 5 cm jejunum and 15 cm ileum</td>
<td>90</td>
<td>—</td>
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<tr>
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<td>M</td>
<td>25</td>
<td>940</td>
<td>15</td>
<td>NEC (small bowel)</td>
<td>Ileostomy</td>
<td>Small bowel excluding 24 cm ileum and the ileocaecal valve</td>
<td>69</td>
<td>—</td>
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<tr>
<td>4</td>
<td>M</td>
<td>30</td>
<td>885</td>
<td>30</td>
<td>NEC (small bowel)</td>
<td>Ileostomy</td>
<td>Small bowel excluding 10 cm ileum and the ileocaecal valve</td>
<td>55</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>26</td>
<td>950</td>
<td>44</td>
<td>Intestinal obstruction after conservative treatment for NEC</td>
<td>Ileostomy</td>
<td>Total small bowel</td>
<td>10</td>
<td>31</td>
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<tr>
<td>6</td>
<td>M</td>
<td>32</td>
<td>995</td>
<td>11</td>
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<td>Ileostomy</td>
<td>Small bowel excluding 10 cm ileum</td>
<td>45</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>33</td>
<td>1445</td>
<td>5</td>
<td>NEC (colon)</td>
<td>End-Ileostomy</td>
<td>Total small bowel</td>
<td>55</td>
<td>—</td>
</tr>
</tbody>
</table>

| Median   | 30   | 940   | 15    | 5        | NEC (colon) | End-Ileostomy | Total small bowel | 55                               | —                               |
| Min-Max  | 25–33 | 695–1445 | 5–44 | —        | NEC (small bowel) | Ileostomy | Small bowel excluding 10 cm ileum | 10–90                             | —                               |

M, Male; GA: gestational age; BW: birth weight; AS: age at surgery; NEC: necrotizing enterocolitis.
columns. Mucins were eluted in the void volume of the column with 50 mmol/l Tris, 5 mmol/l EDTA, and 1 mmol/l PMSF, pH 7.5, containing 4 M guanidinium HCl. The mucin-containing fractions were extensively dialyzed against distilled water at 4°C and stored at −20°C until further analysis.

**Analysis of MUC2.** Samples of dialyzed fractions were analyzed on reducing polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecylsulfate (SDS-PAGE) (14). Gels were composed of 3% stacking gel and 4% running gel using a stock solution containing 30% acrylamide and 2% bis-acrylamide. Prestained precision plus protein standards were purchased from Bio-Rad (ranging from 10 kDa to 250 kDa). Before SDS-PAGE analysis, samples were boiled for 4 min in buffer containing 1% (vol/vol) 2-mercaptoethanol (Bio-Rad) and 2% (wt/vol) SDS. Gels were stained with periodic acid-Schiff reagent (PAS, Sigma) according to Konat et al. (13), with Phast Gel Blue R (Coomassie, Amersham, Roosendaal, the Netherlands), and with Silver Staining according to the manufacturer’s protocol. Because mucins have a characteristic buoyant density of 1.45 g/ml, the density of CsCl gradient fractions was measured by weighing 1 ml of each fraction using a calibrated pipette. Hexose assay was performed by measuring the optical density at 540 nm. Mucin-containing fractions, i.e., fractions that contained a high molecular weight PAS-positive band at 150 kDa after SDS-PAGE, had a buoyant density of 1.45 g/ml, contained relatively high hexose levels, were pooled, and stored at −20°C for further analysis.

**Western blot analysis.** The mucin-containing pooled fractions (16 μl) were loaded and run on a SDS-PAGE gel. The separated proteins were transferred to nitrocellulose membranes (Protran BA 83, 0.2 μm) and blocked for 1 h at room temperature in blocking buffer containing 50 mmol/l Tris, pH 7.8, 5% (wt/vol) nonfat dry milk powder (Campina Melkunie, Eindhoven, the Netherlands) 2 mmol/l CaCl2, 0.05% (vol/vol) Triton X-100, and 0.01% Antifoam B (Sigma-Aldrich). Blots were incubated overnight at 4°C with rabbit polyclonal antibody human colonic mucin (HCM)-1 (1:2,500 in blocking buffer) (30). After being washed with PBS containing 0.2% Tween-20, blot-bound antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (HCM-1, 1:1,000 in blocking buffer) and SuperSignal West Femto Luminol Enhancer kit (Pierce, Eiten-Leur, the Netherlands).

**Mass Spectrometry**

**Analysis of [U-13C]threonine.** After gravity gel filtration chromatography, aliquots of 3–4 ml of the pooled MUC2 fractions were dialyzed, freeze dried, and prepared for mass spectrometric analysis. The dried samples were hydrolyzed by adding 1 ml of 6 mol/l HCl and incubating at 110°C in sealed tubes for 24 h. The protein hydrolysates (1,000 μl) were dried under a nitrogen stream at 55°C, and the residue was dissolved in 0.5 ml water. The amino acids in the residue were bound to a 1 ml AG50 W-X8, H+ cation exchange column (Bio-Rad, Richmond, VA). Amino acids were eluted with 3 ml 6 mol/l NH4OH and dried under nitrogen at 30°C. Threonine was converted to its N-ethoxycarbonylthyletherester derivative according to the method used in our previous work (25). The [13C/12C] ratio of threonine in isolated MUC2 was measured by a Thermo Electron Delta-XP isotope ratio GC linked online with a trace gas chromatography (GC) (Thermo Electron, Bremen, Germany) and a combustion interface type 3 (Thermo Electron). Aliquots of 1 μl of the ethyl acetate suspension containing the amino acid derivatives were introduced into the GC system by a CTC PAL autosampler (CTC Switzerland). Chromatographic conditions were like those described previously. After separation using capillary GC, amino acids were combusted online at 940°C and introduced as CO2 into the isotope ratio mass spectrometer (MS), where the [13C/12C] ratio was measured. Enrichment was expressed in molar percent excess (MPE).

**Analysis of [U-13C]threonine in blood samples.** Small aliquots (50 μl) were prepared to determine threonine enrichment and concentration by GC-MS. Although aminoacyl-tRNA-bound threonine derived from intestinal biopsies is the best representative to determine the true threonine precursor pool, plasma threonine enrichment is used as a substitute for the true intracellular threonine enrichment of goblet cells. For obvious ethical reasons and the vulnerability of the intestine after surgical intervention, we were not able to sample additional intestinal tissue to determine the true threonine precursor pool.

Briefly, 20 μl of internal standard (1 μmol/ml [13N]threonine) was added to 50 μl plasma and deproteinized with sulfosalicylic acid 6% (wt/vol). After centrifugation for 10 min at 4°C and 20,000 g, the amino acids in the supernatant were isolated by cation exchange separation as described above. The eluate was dried overnight at 30°C under a stream of nitrogen. Finally, t-butyldimethylsilyl derivates were formed by adding 25 μl of dimethylformamide and 25 μl of N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) to the dried residue and heated at 60°C for 1 h. Standard curves were prepared by mixing aqueous solutions of natural and labeled threonine to determine the true threonine enrichment and concentration in plasma. GC-MS analyses were performed in selective ion-monitoring mode on a Carlo Erba GC8000 gas chromatograph connected to a Fisons MD-800 mass spectrometer (Interscience BV, Breda, the Netherlands). One microliter of the derivative was injected in split mode (1:20) on a DB-17, 30 m × 0.25 mm capillary column (Agilent). Selective ion monitoring was carried out at m/z 403.4, 405.3, and 408.3. Enrichment was expressed in MPE.
MUC2 secretion time. MUC2 secretion time was defined as the
time interval between start of intravenous \([U-^{13}C]\)threonine administration
and the appearance of enriched MUC2 in small intestinal
outflow fluid.

FSR. The FSR of MUC2 is expressed as percentage of the total
MUC2 pool synthesized per day. Plasma threonine enrichment is used
as precursor.

FSR was calculated by the equation \([\text{slope of the linear hourly increase of } [U-^{13}C]\text{threonine enrichment of MUC2}/[U-^{13}C]\text{threonine enrichment in plasma}]) \times 24 \text{ h}.

Statistics

Data are presented as the means ± SD or as medians (minimum-
maximum).

RESULTS

Patients

The seven infants studied had median gestational age of 30
(25–33) wk and median birth weight of 940 (695–1445) g
(Table 1). Surgery was performed at median 15 (5–44) days
after birth, with creation of a jejunostomy \((n = 1)\) or ileostomy
\((n = 6)\). At the time of the study \((mean \pm 1 \text{ wk})\) following
bowel resection \(\) patients had mean enteral protein intake of
\(2.6 \pm 1.2 \text{ g/kg per day}\.\) Enteral nutrition consisted of a
combination of breast milk and/or formula feeding \((\text{Nenatal or}
\text{Nutrilon Pepti Junior})\.\) Median dietary threonine intake was 55
\((10–90) \text{ µmol/kg per hour}\.\). Moreover, two infants had a
grade infusion, and one infant received both enteral and
parenteral nutrition during the study (Table 1).

Isolation and Characterization of Mucins

MUC2 was isolated from the intestinal outflow fluid using
CsCl density gradient ultracentrifugation and was character-
ized by PAS staining, hexose assay, buoyant density, and
Western blot analysis as described previously \((24, 30)\.\). The
mucin-containing fractions were pooled and further purified by
gravity gel filtration chromatography. The fractions 5–8 con-
tained the purified mucin MUC2. These fractions contained a
high molecular mass band of \(\pm 550 \text{ kDa}\.\), which was PAS
positive on SDS-PAGE (Fig. 2A). Moreover, this molecular
mass band corresponded with a peak in hexose assay
(Fig. 2C). Each mucin fraction was free of contaminating
proteins detected by Coomassie Blue staining as well as silver
staining (data not shown). Furthermore, Western blot analysis
using anti-HCM-1, an antibody specific for MUC2, revealed
that the pooled fractions 5–8 after Sepharose CL-2B gravity
gel filtration contained MUC2 (Fig. 2B).

Threonine Enrichment

The \([U-^{13}C]\)enrichment of plasma threonine reached a
steady state of 3.63 ± 0.56 MPE in all patients between \(t = 9\)
and \(t = 12 \text{ h}\), seeing that slopes of enrichment between these
time points did not significantly differ from zero. Figure 3
shows the values of MUC2-bound threonine enrichment and
the time curve of systemic tracer incorporation into MUC2 for
two representative patients. Threonine enrichment rose almost
linearly in isolated MUC2 \([\text{maximal threonine enrichment}
(1.06 \pm 0.50 \text{ MPE})]\) and gradually decreased after mean 21 ±
2 h. Time needed to absorb systemic threonine and to synthe-
size and secrete MUC2 into the small intestinal lumen was
within 3 h in four patients, between 3–6 h and between 9–12 h
in the other two patients (Table 2). Median FSR of small
intestinal MUC2 was 67.2 (44.3 - 103.9)% per day (Table 2).

DISCUSSION

Tracer studies in mammals showed that amino acids in the
intestine derived from both dietary and arterial sources and are
used for mucosal protein synthesis and energy generation \((20,
26–28, 31, 32, 36)\.\). Because MUC2 is the predominant secre-
tory mucin in the intestine and its peptide backbone is rich in
threonine, we speculated that absorbed threonine is mainly
used for MUC2 synthesis to maintain the protective mucus
layer and thus gut barrier function. Because threonine cannot
be synthesized de novo, the epithelial cells are offered high
quantities of threonine from both the intestinal lumen \([i.e., the
diet or proteolysis (recycling of threonine)]\) and the systemic
circulation during enteral nutrition. The question arises whether
threonine is utilized differently when presented to epithelial cells
via the lumen or via the circulation. In piglets, we previously
showed that two-thirds of threonine utilized by the intestine
derived from the systemic circulation during normal protein
feeding \((25)\.\). However, dietary, rather than systemic threonine
was preferentially utilized for constitutive protein synthesis in
the small intestinal mucosa of these piglets. The present study
demonstrated that even during enteral feeding the intestine
utilizes systemically derived threonine for MUC2 synthesis.
We therefore suggest that dietary threonine is preferentially
taken up by enterocytes and is used for constitutive protein
synthesis, whereas goblet cells might extract threonine from

Fig. 2. Isolation and characterization of human mucin 2 (MUC2) in the
intestinal outflow fluid. A: reducing 4% stacking and 3% running SDS-PAGE
gel containing dialyzed mucin fractions purified by gradient density ultracen-
trifugation followed by Sepharose CL-2B gravity gel filtration. B: periodic
acid-Schiff-positive fractions 5–8 after the gravity gel filtration chromatogra-
phy were pooled and subjected to Western blot analysis (3 time points of
different representative patients). C: hexose content (■) of the same samples as
represented in the gel.
The importance of enteral intake of nutrients in maintaining gut morphology and integrity has been reported by several authors (4, 12, 23). However, after bowel resection for NEC, most infants are fed parenteral nutrition solely for a certain period of time. Although parenteral nutrition is known to induce gut atrophy and thereby loss of absorptive capacity, knowledge on its effect on mucin synthesis in humans is lacking. Thus far, only studies in rats showed that parenteral nutrition is associated with compromised intestinal barrier function because of diminished mucin production (11). Furthermore, the intestinal threonine requirement of parenterally fed piglets is significantly lower than that of piglets fed enterally, which might suggest diminished MUC2 synthesis during parenteral feeding (2). Our present study showed that systemic threonine is utilized for intestinal MUC2 synthesis in preterm infants who were predominantly fed enteral nutrition. However, it is yet unknown whether systemic threonine is utilized similarly in infants fed full parenteral nutrition since other factors such as bowel movements, enteric nerve stimulation, or hormone production related to the presence of nutrients within the gastrointestinal tract might be important.

In conclusion, systemically derived threonine is rapidly incorporated into small intestinal MUC2, and, thereby MUC2 synthesis rate of the small intestine is high.

ACKNOWLEDGMENTS

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GRANTS

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![Fig. 3. Threonine enrichment in MUC2 sampled from small intestinal outflow fluid of 2 representative patients. Threonine enrichment in purified small intestinal MUC2 in 1 representative patient with an ileostomy (A) and the single patient with a jejunostomy (B). These data allow us to determine the secretion time and to calculate the fractional synthetic rate of MUC2. Dashes represent infusion time; MPE, mole percent excess.](http://ajpgi.physiology.org/)

Table 2. Kinetic parameters of small intestinal MUC2

<table>
<thead>
<tr>
<th>Patients</th>
<th>MUC2 Secretion Time, h</th>
<th>FSR, %/day</th>
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<tbody>
<tr>
<td>1</td>
<td>3-6</td>
<td>103.9</td>
</tr>
<tr>
<td>2</td>
<td>&lt;3</td>
<td>85.7</td>
</tr>
<tr>
<td>3</td>
<td>9-12</td>
<td>44.3</td>
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</tr>
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MUC2, mucin 2; FSR: fractional synthetic rate; *not determined.


