SM22 a Plasma Biomarker for Human Transmural Intestinal Ischemia

SM22 a Plasma Biomarker for Human Transmural Intestinal Ischemia

Dirk H.S.M. Schellekens, MD,† Kostan W. Reisinger, MD, PhD,*† Kaatje Lenaerts, PhD,*† M'hamed Hadfoune, BSc,*† Steven W. Olde Damink, MD, PhD,*† Wim A. Buurman, PhD,‡ Cornelis H. C. Dejong, MD, PhD,*† and Joep P. M. Derikx, MD, PhD*†§*

Objective: To evaluate the diagnostic potential of smooth muscle protein of 22 kDa (SM22) as plasma biomarker for the detection of transmural intestinal ischemia.

Background: Acute mesenteric ischemia is an abdominal emergency requiring rapid diagnosis and treatment. Especially, detection of transmural damage is imperative because it mandates emergency surgery. Since early clinical and radiological signs are nonspecific, there is an urgent need for accurate biomarkers. SM22 is a potential marker for transmural damage because of its abundant expression in intestinal smooth muscles.

Methods: SM22 concentrations were measured using a newly built enzyme-linked immunosorbent assay. SM22 release was assessed in plasma and intestinal tissue of rats subjected to intestinal ischemia. Blood and tissue were sampled at baseline and followed up to 24 hours of ischemia. Next, organ-specific SM22 arteriovenous concentration differences were studied in both rats and patients. Finally, plasma from patients with intestinal ischemia, other acute abdominal complaints, and healthy volunteers were tested for SM22.

Results: SM22 concentrations were significantly elevated in rats from 4 hours of ischemia onwards. Furthermore, SM22 plasma concentrations closely paralleled the histological increasing degree of intestinal smooth muscle damage. Arteriovenous calculations showed that SM22 was specifically released by the intestines and renally cleared. First data of SM22 release in man demonstrated that patients with transmural intestinal ischemia had significantly higher plasma SM22 levels than patients with only ischemic mucosal injury, other acute abdominal diseases, or healthy controls.

Conclusions: This study shows that SM22 is released into the circulation upon severe ischemia of the intestinal muscle layers. Plasma levels of SM22 are potentially useful for the detection of transmural intestinal damage.

Keywords: biomarker, diagnostic characteristics, human, I-FABP, intestinal ischemia, SM22, transmural intestinal damage

(*Ann Surg* 2018;268:120–126)

Acute intestinal ischemia is a rare but critical abdominal emergency. It is a consequence of acute thromboembolic occlusion of the mesenteric blood supply or nonocclusive mesenteric ischemia secondary to diminished blood flow caused by intestinal strangulation, major surgery, shock, trauma, and sepsis.[1,2] The main risk of intestinal ischemia is severe ischemia of the muscle layers, that is, transmural ischemia (TI), leading to intestinal wall perforation, sepsis, and death.[3,4] TI necessitates prompt surgery to resect nonviable intestine.[5] Early diagnosis and timely intervention are therefore key factors to reduce the high morbidity and mortality rates of 60% to 80% in patients suffering from intestinal ischemia.[6] These rates remain as high as they were 70 years ago, despite the introduction of new imaging techniques.[3,7] Consequently, there is a great need for a diagnostic test that timely detects TI. Currently available biomarkers, including intestinal fatty acid binding protein (I-FABP) and α-glutathione S-transferase (α-GST), released by mature enterocytes upon intestinal ischemia, only diagnose mucosal damage.[8–11] Taking into consideration the relatively large proportion of smooth muscle cells in the outer layer of the gut, a potential diagnostic marker for TI could reflect ischemic damage of the intestinal muscle layers. In this context, smooth muscle protein of 22 kDa (SM22) is of interest. It is a small, water-soluble protein abundantly expressed by intestinal smooth muscle tissue.[12,13] The biological and physiological functions of SM22 remain unclear; it is hypothesized to play a role in the maturation and differentiation of smooth muscle cells.[14,15]

The aims of this study were the following: the development and validation of a new reliable sandwich enzyme-linked immunosorbent assay (ELISA) for quantification of SM22; to investigate the release pattern of SM22 in plasma and the relation of plasma levels with the degree of intestinal damage in a rat model of intestinal ischemia; and to obtain pilot data on the diagnostic potential of SM22 plasma levels to detect severe ischemia of the muscle layers in man.

This study describes the first quantitative ELISA for SM22 and demonstrates SM22 to be a specific marker for severe ischemia of the muscle layers, leading to transmural intestinal ischemia, in both a clinically relevant rat model and in patients with acute intestinal ischemia.

From the *Department of Surgery, Maastricht University Medical Center, Maastricht, the Netherlands; †NUTRIM School for Nutrition, Toxicology and Metabolism, Maastricht University Medical Center, Maastricht, the Netherlands; ‡MHeNs School of Mental Health and Neuroscience, Maastricht University Medical Center, Maastricht, the Netherlands; and §Currently at the Pediatric Surgical Center of Amsterdam, Emma Children's Hospital Academic Medical Center and VU University Medical Center, Amsterdam, the Netherlands.

Author contributions: D.S., K.R., W.B., and J.D. contributed to the research design, conducting experiments, acquiring data, analysis and interpretation of data, writing the manuscript, and gave final approval of the version to be published. M.H. and S.O.D. contributed to acquisition of data, providing reagents, and revised the article critically for important intellectual content, and gave final approval of the version to be published. K.L. and C.D. contributed to conception and design, interpretation of data, and revised the article critically for important intellectual content, and gave final approval of the version to be published. D.S. and J.D. had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis.

Funding: Supported by the Dutch Society for Gastroenterology (Gastrostart 2013-42 to CHC Dejong) and the Dutch Digestive Foundation (MLDS Career development grant CDG13-14 to JPM Derikx).

The authors have no potential conflicts of interest to disclose.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.annalsofsurgery.com).

Reprints: Dirk H.S.M. Schellekens, MD, Department of Surgery, Maastricht University Medical Center, P.O. Box 616, 6200 MD, Maastricht, the Netherlands. E-mail: D.Schellekens@maastrichtuniversity.nl.

ISSN: 0003-4932/17/26801-0120

DOI: 10.1097/SLA.0000000000002278

METHODS

Study Approval

Animal experiments were approved by the Animal Ethics Committee at the Maastricht University. Clinical studies were approved by the Medical Ethics Committee of Maastricht University Medical Centre. Written informed consent was obtained from all participants, parents, or caretakers. The study was carried out according to the revised declaration of Helsinki (October 2008, Seoul).

Animals

Male Sprague–Dawley rats were purchased from Charles River Laboratories and housed under controlled conditions with unrestricted access to chow and drinking water. Rats were randomly divided into 8 groups of 6 animals that either underwent jejunal ischemia or sham operation. Animals were anesthetized with isoflurane (induction 4%, maintenance 1.5%) and bupivacaine was used for analgesia.

Experimental Rat Model for Occlusive Intestinal Ischemia

Animals were divided into groups based on the duration of intestinal ischemic injury that was used. After midline laparotomy, baseline blood samples were obtained from the inferior caval vein. Only the mesenteric blood supply (arteries and veins) to a 16-cm midjejunal segment was ligated (tied and cut) for 2, 4, 6, 8, 12, or 24 hours. During surgery, body temperature was maintained at 37°C using heat pads and rectal temperature control. To counteract fluid loss due to evaporation and lack of physical activity, rats were resuscitated with 3 mL normal saline injected subcutaneously. After ligation, the abdomen was closed and animals were allowed to recover from anesthesia. Twelve animals were sham-operated. In these groups, samples were obtained at 6 hours (n = 6) or 24 hours (n = 6) after laparotomy without intestinal ischemia.

At the end of each ischemic period, rats were again anesthetized according to the previously described protocol and the incision was re-opened. The renal vein, portal vein, hepatic vein, and inferior abdominal aorta (just above the bifurcation) were catheterized to study organ-specific SM22 release and clearance. A 25-gauge needle (BD Microlance, Becton Dickinson Medical, Breda, the Netherlands) fixed in a silicone tube (Silclear Medical Grade tubing, 0.020'' × 0.037'', MEDNET, Münster, Germany) was used for catheterization, and fixed with cyano-acrylate as previously described.[16] All catheters were filled with heparinized saline solution (20 U/mL) until blood sampling. Next, blood was collected from all catheters simultaneously and directly transferred to prechilled EDTA vacuum tubes (BD Vacutainer, Becton Dickinson Diagnostics, Breda, the Netherlands) and kept on ice. Samples were centrifuged at 3500 rpm at 4°C for 15 minutes to obtain plasma. Plasma was immediately stored in aliquots at −80 °C until analysis. At sacrifice, a segment of ischemic jejunum was resected, together with a proximal part of jejunum, which remained untreated during surgery, serving as internal control tissue. Tissue samples were immediately snap-frozen or formalin-fixed for future analysis (Supplementary Fig. 1, http://links.lww.com/SLA/B220).

Study Participants

Twenty-two patients who were known to have intestinal ischemia using standard diagnostic procedures and therefore underwent emergency abdominal surgery were consecutively enrolled in this study. During routine laboratory tests, an extra 2 mL of plasma was stored for further analysis. Forty patients presenting at the Emergency Department with other acute abdominal complaints were included to study, whether plasma SM22 allows differentiation between intestinal ischemia and other acute abdominal pathology. To study the renal clearance in man, arterial blood from the radial artery was collected simultaneously with blood drawn from the right renal vein by direct puncture from 10 patients undergoing major upper abdominal surgery. Fifty healthy volunteers were included in this study to obtain reference values for plasma concentrations of SM22 and I-FABP.

After inclusion, plasma was collected, transferred into multiple cryopreservation tubes, and frozen at −80°C until analysis. All necessary clinical information was retrospectively collected from the electronic health record system of the participating patients, including radiological data, intraoperative findings, and histopathological examinations. To compare the studied markers with conventional markers to diagnose intestinal ischemia, plasma lactate level, white blood cell (WBC) count, and arterial pH were also retrospectively collected in the group of patients with histopathological proven intestinal ischemia.

Development of SM22 ELISA

Plasma concentrations of SM22 were measured using a newly developed in-house Sandwich ELISA. Two mouse monoclonal antibodies (mAbs), kindly provided by Dr. A. Chiavegato,[13] were used to select the best combination of mAbs against SM22 for an ELISA. These antibodies were raised in mice using purified native SM22 from porcine stomach as antigen.[17] A suitable antibody combination was selected by checking the immunoreactivity signal against recombinant SM22 protein (ATGen co., Ltd, Gentaur, Eersel, the Netherlands) and the assay procedure was optimized. The developed ELISA was then characterized by determination of the lower limit of detection, intra-assay and interassay coefficient of variation, dilutional linearity, and recovery (see Supplementary material, http://links.lww.com/SLA/B220). The ELISA was performed in 96-well microtiter plates (Greiner Bio-one, Alphen a/d Rijn, the Netherlands). The immunoreactive SM22 concentrations in samples were calculated by reference to the calibration curve (see the supplemental data for details regarding these studies).

Plasma Measurements

The SM22 concentrations were measured using a newly built ELISA. Human I-FABP was measured using in-house available assay.[18] The ELISA was developed to measure I-FABP in plasma samples with rabbit polyclonal antibodies, using recombinant human I-FABP as standard. This assay is based on the sandwich principle with a working time of 3.5 hours. The detection range for human I-FABP is 12.5 to 800 pg/mL. Samples were assayed by trained laboratory technicians at the specialized laboratory facilities of the Department of General Surgery. The laboratory personnel were unaware of the final diagnosis concerning the patient samples or to which group the animals were allocated. Samples were run in duplicate, and a variability of 5% between sample duplicates was accepted.

Plasma lactate level, WBC count, and arterial pH were determined as routine patient care by the clinical chemistry laboratory and were taken at the same time-point as the samples for SM22 and I-FABP measurement.

Calculations

Arteriovenous concentration differences were calculated to study the contribution of the intestines (portal vein minus artery) and the liver [hepatic vein minus (30% × artery + 70% × portal vein)] to systemic SM22 release. Renal clearance was calculated by dividing the arteriovenous concentration gradient (renal vein minus artery) by

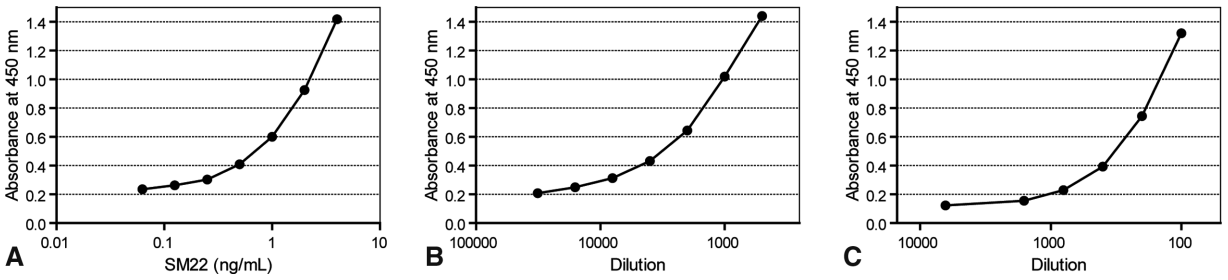

FIGURE 1. Representative curves from different species of the SM22 sandwich ELISA. (**A**) Recombinant SM22 with concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 ng/mL. (**B**) Native human SM22-containing solution diluted 1/500 to 1/3200. (**C**) Native rat SM22-containing solution diluted 1/100 to 1/6400. Each point represents the mean of duplicate measurements. ELISA, enzyme-linked immunosorbent assay; SM22, smooth muscle protein of 22 kDa.

the arterial concentration (uptake/influx).[19] This quotient was multiplied by the percentage of blood flowing through the kidney to calculate fractional plasma clearance. In rat, the renal blood flow per minute is approximately 25% of the total blood volume. Renal blood flow per minute in humans is to be estimated at 22%.[20]

Intestinal Tissue Extracts

Snap-frozen tissue samples were homogenized in lysis buffer. Samples were centrifuged at 27,000*g* for 15 minutes at 4°C. The resulting supernatants were centrifuged for a second time at 50,000*g* for 15 minutes. Protein concentration of the supernatants was determined using a BCA protein assay kit (Pierce). The SM22 concentration was measured using the newly developed ELISA. SM22 concentrations were expressed as nanograms SM22 per milligram total protein.

Immunohistochemistry for SM22

The sources of antibodies are listed in supplemental methods. Tissue samples were formaldehyde-fixed and cut at 4 μm before blocking. Mouse anti-SM22 antibody was incubated with the sample overnight at 4°C. A biotine conjugated rabbit anti-mouse antibody was used as the secondary.

Statistics

Analysis was performed using Graphpad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Normality was tested by Kolmogorov–Smirnov test. Results are presented as mean ± standard error of mean (SEM). Comparisons of 2 groups were performed by Mann–Whitney *U* test. For more than 2 groups, Kruskal–Wallis test with a Dunn post hoc test was used. Arteriovenous concentration gradients were tested versus a theoretical mean of zero using a Wilcoxon signed-rank test. $P \leq 0.05$ was considered statistically significant (for more detailed information please see the online-only Data Supplement, http://links.lww.com/SLA/B220).

RESULTS

ELISA

The selected combination of mAbs was suitable for development of a sandwich ELISA to quantifying both human and rat SM22 concentrations. Figure 1 shows typical examples of standard curves of SM22 for this sandwich ELISA, which were prepared by plotting the absorbance of each standard solution (mean of 2 measurements) against standard of recombinant human SM22 ranging from 0 to 4 ng/mL (Fig. 1A), a native human SM22-containing solution (diluted 1/500–1/3200; Fig. 1B), or a native rat SM22-containing solution (diluted 1/100–1/6400; Fig. 1C). These data show the usefulness of the ELISA for both human and rat SM22 measurements.

The basic validation features including a lower detection limit, reproducibility, dilution linearity, and recovery of this sandwich ELISA were assessed using different human plasma samples. The lower detection limit for SM22 in the ELISA was determined to be 62.5 pg/mL. The intra-assay and interassay coefficient of variation (CV%) ranged from 6.2% to 14.8% and from 4.9% to 16.3%, respectively (Supplementary Table 1, http://links.lww.com/SLA/B220). The assay showed dilutional linearity (Supplementary Fig. 2, http://links.lww.com/SLA/B220), and the average recovery of the samples ranged from 93.2% to 108.6% (Supplementary Table 2, http://links.lww.com/SLA/B220). Similarly, the usefulness of the ELISA was investigated for rat plasma samples (data not shown).

The analytical performances of the SM22 ELISA indicate that the assay can be used for measurement of SM22 levels in both rat and human plasma samples.

Transmural Ischemic Damage in Rats

First, the release pattern of SM22 was investigated throughout a 24-hour period in a rat model for transmural small intestinal ischemia by ligation of the mesenteric blood supply to a 16-cm jejunal segment. After 4 hours of ischemia, SM22 levels were significantly increased in the systemic circulation (from 0.15 ± 0.03 ng/mL to 8.46 ± 1.76 ng/mL; $P < 0.001$; Fig. 2A). The peak concentration was reached at 6 hours (11.20 ± 3.82 ng/mL; $P < 0.001$). Thereafter, the values returned toward baseline. They were no longer significantly elevated after 12 and 24 hours of ischemia (Fig. 2A). As expected, plasma SM22 levels remain low in the sham-operated animals throughout the entire experiment (Fig. 2A). Next, we studied the relation between SM22 plasma release and the extent of smooth muscle damage using immunohistochemistry for SM22 in the smooth muscle layers. Damage of the longitudinal muscular layer of the intestinal wall became apparent in jejunum exposed to 6 hours ischemia compared with either control or sham (Fig. 2D). This was marked by loss of nuclei and atrophy of the longitudinal muscle layer with pale SM22 staining, indicating that a major part of the SM22 protein had leaked out of the smooth muscle cells. As ischemia duration further increased, reduced intensity of staining revealed a further decrease in concentration or even total absence of SM22 protein in the muscular layers, whereas the mucosa had disappeared completely (Fig. 2D). Furthermore, in the late stages, lysis, separation, and thinning of the circular and longitudinal layers of the muscularis externa were observed. There was no remarkable histological change throughout the experiment in sham-operated animals (Fig. 2D). In line, the SM22 content measured by ELISA of extracts of jejunal tissue obtained after onset of ischemia was significantly diminished in jejunal tissue of rats subjected to ischemia, when

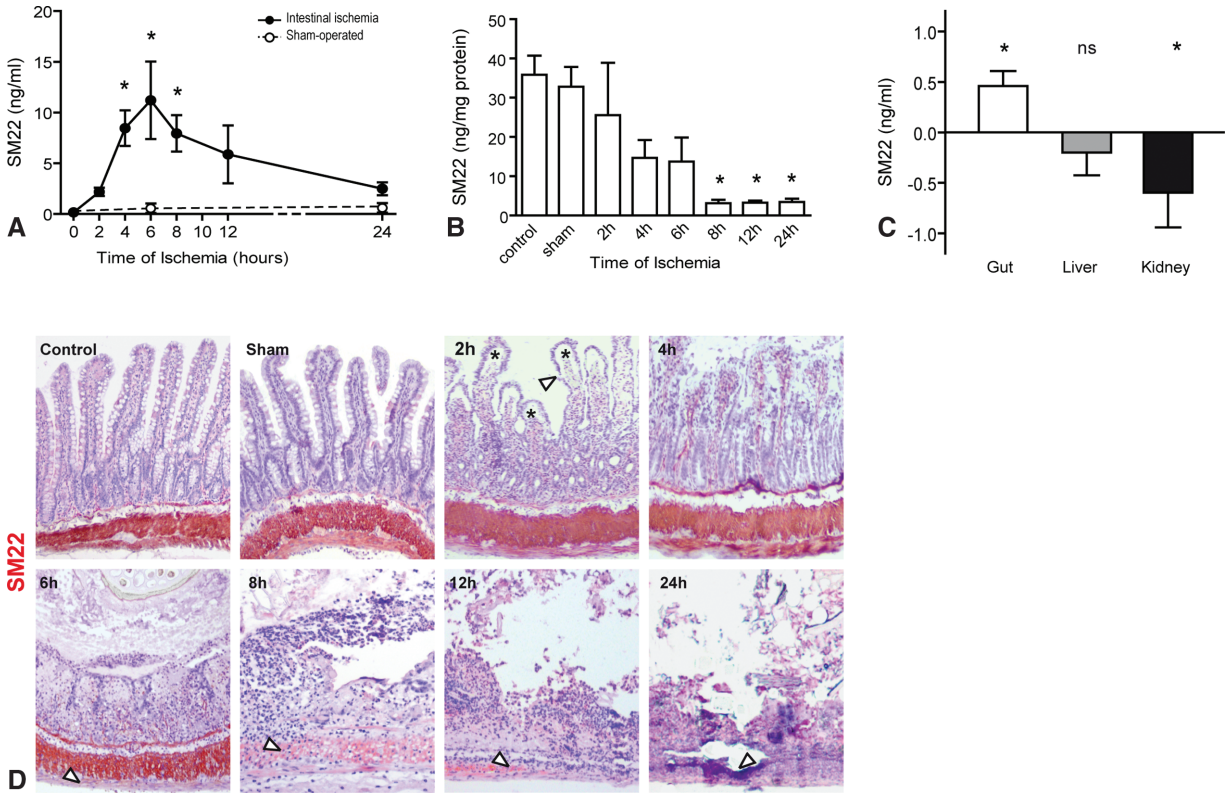

FIGURE 2. SM22 concentrations of plasma samples and jejunal extracts in rats subjected to intestinal ischemia compared with their histological perturbations. (**A**) Plasma SM22 levels from rats undergoing intestinal ischemia or sham-operation. SM22 plasma levels becomes elevated after 4 to 8 hours of ischemia, and is significantly higher compared with $t = 0$ or sham ($^*P < 0.001$). (**B**) The SM22 content of jejunal extracts was diminished in rats subjected to ischemia compared to control or sham operated animals from 8 hours onwards ($^*P < 0.05$). (**C**) Arteriovenous concentration gradients of SM22 across the intestines (portal vein minus artery), the liver (hepatic vein minus portal vein), and the kidney (renal vein minus artery) show that SM22 is released from the intestine and excreted renally ($^*P < 0.05$ compared with 0). (**D**) Histology revealed large subepithelial spaces at the villus tips (asterisk) with disruption of the epithelial lining (arrowhead) after 2 hours of ischemia. Complete disintegration of the villi was seen following 4 hours of ischemia. SM22 immunohistochemistry demonstrated necrosis of the longitudinal muscular layer of the intestinal wall in jejunum exposed to 6 hours ischemia (arrowhead). Next, staining showed a further decrease in intensity after 8 hours ischemia (arrowhead) or even total absence of SM22 protein in all muscular layers after 12 and 24 hours ischemia. In addition, in the late stages of lysis, separation and thinning of the circular and longitudinal layers of the muscularis externa were observed (arrowheads). There was no remarkable histological change throughout the experiment in sham-operated animals. SM22, smooth muscle protein of 22 kDa.

compared with control at 8, 12, and 24 hours of ischemia (36.05 ± 2.56 ng/mg protein vs 4.64 ± 1.67 ng/mg protein, 3.48 ± 0.38 ng/mg protein or 3.28 ± 0.58 ng/mg protein, respectively; $P < 0.05$) (Fig. 2B). Importantly, SM22 plasma concentrations closely paralleled the increasing degree of intestinal transmural damage upon progression of the duration of ischemia. These data indicate SM22 to be a potential plasma marker for detection of severe ischemic intestinal damage of the muscle layers.

Source and Fate of SM22 in Rat and Man

To investigate whether SM22 was specifically released from the intestines, arteriovenous concentration gradients of SM22 across the intestines were assessed in rat. Subtracting the arterial plasma concentration of SM22 from the concentration measured in the portal vein revealed that SM22 was specifically released from the intestine ($P < 0.05$ vs 0; Fig. 2C). To clarify its clearance from the circulation and potential hepatic metabolism, arteriovenous concentration gradients of SM22 across the liver [hepatic vein minus (30% × artery + 70% × portal vein)] and the kidney (renal vein minus artery) were determined. We showed that SM22 was removed by the kidneys from circulation ($P < 0.05$ vs 0) without being primarily metabolized by the liver (Fig. 2C). The renal fractional extraction rate (arteriovenous gradient/arterial concentration × 100%) was approximately 20%. Renal plasma SM22 clearance was calculated to be 5.0%/min (20% × 25%/min), resulting in a plasma half-life of approximately 14 minutes.

To obtain data on the clearance of SM22 in humans, we included 10 patients undergoing major upper abdominal surgery from whom radial arterial blood was sampled simultaneously with blood drawn from the right renal vein. The fractional extraction rate of SM22 in humans was calculated to be approximately 11%. Plasma SM22 clearance was calculated to equal 2.4%/min (11% × 22%/min), leading to a plasma half-life of about 23 minutes. This short circulating half-life of SM22 allows detection of intestinal transmural damage to the intestinal muscular layers in patients without renal failure.

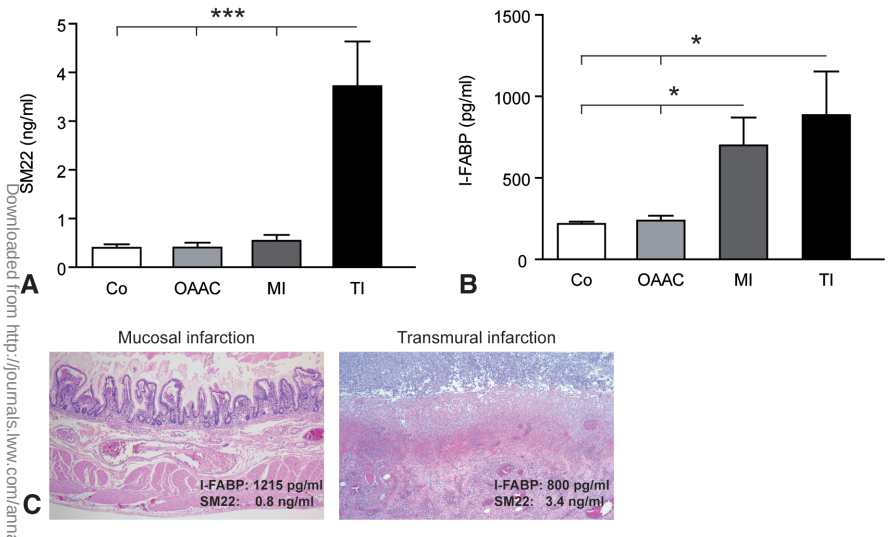

FIGURE 3. SM22 and intestinal fatty acid-binding protein (I-FABP) levels in plasma samples obtained from patients suffering from intestinal ischemia, other acute abdominal complains, or healthy controls. (**A**) Plasma SM22 levels are only significantly elevated in patients suffering from transmural ischemic (TI) damage compared with patients with mucosal ischemic (MI) damage and healthy controls (Co) or patients with other acute abdominal complains (OAAC). (**B**) Plasma I-FABP levels were significantly increased in both patients with MI and TI damage compared with Co and OAAC. No difference in I-FAPB levels was apparent between MI and TI. (**C**) The typical histopathology of patients with either MI or TI is shown. The former is of a patient with only MI showing enhanced plasma I-FABP levels of 1200 pg/mL with normal plasma SM22 value, whereas the TI patient (right panel) shows both elevated I-FABP and SM22 plasma levels ($^{*}P < 0.05$, $^{***}P < 0.01$).

Translation to the Clinical Situation

Plasma SM22 levels were investigated in 22 patients diagnosed with intestinal ischemia undergoing surgery and compared with 50 healthy controls. The patients did not have renal failure. Eleven patients suffered from acute intestinal ischemia due to mesenteric occlusion or intestinal strangulation, 9 patients as a complication of aortic surgery, and 2 as a result of sepsis accompanied by hypoperfusion. Nine of these patients were diagnosed by the pathologist with only mucosal infarction (MI), whereas 13 patients were diagnosed with TI. In patients with proven transmural ischemia, SM22 plasma levels were significantly higher (3.72 ± 0.91 ng/mL) compared with patients with only mucosal ischemic injury (0.45 ± 0.08 ng/mL; $P < 0.001$) or healthy controls (0.40 ± 0.07 ng/mL; $P < 0.001$) (Fig. 3A).

Next, we measured the plasma concentrations of I-FABP, a small cytosolic protein exclusively present in mature enterocytes and rapidly released into the circulation upon damage. It is a highly sensitive marker for intestinal ischemia with levels already significantly elevated in the systemic circulation within 60 minutes of ischemia of only 1% to 2% of small intestine.[18] Previous research revealed that I-FABP has a short half-life of about 11 minutes.[21]

Mean plasma I-FABP levels were significantly increased in both patients with only MI and patients with TI when compared with healthy controls (699 ± 171.4 pg/mL or 952.0 ± 259.2 vs 217.8 ± 13.6 pg/mL; $P < 0.05$, for both; Fig. 3B). Interestingly, we observed no differences in plasma I-FABP concentrations between the MI patients and TI patients ($P = 0.77$; Fig. 3B).

In addition, none of the classically used laboratory markers for intestinal ischemia (plasma lactate levels, WBC count, and arterial pH) could discriminate patients with transmural ischemia from patients with mucosal ischemic damage (lactate: 3.56 ± 0.92 vs 4.77 ± 0.87 mmol/L; $P = 0.36$; WBC: $15.2 \pm 2.8 \times 10^9$/L vs $20.3 \pm 4.0 \times 10^9$/L; $P = 0.21$; pH: 7.36 ± 0.03 vs 7.28 ± 0.04; $P = 0.16$). To further analyze the clinical potential diagnostic value of SM22, we investigated the SM22 plasma levels of 40 patients with other acute abdominal complaints (OAAC), divided into 5 diagnostic categories: acute appendicitis, diverticulitis, gynecological/urological pathology, gastroenteritis, and nonspecific abdominal pain (all $n = 8$). SM22 concentrations of all categories of OAAC patients were not significantly different from healthy controls or patients with MI (data not shown). Plasma SM22 concentrations were 0.33 ± 0.07 ng/mL in all patients with OAAC combined. In line, SM22 plasma levels of above mentioned patients suffering from TI were significantly higher when compared with patients with OAAC ($P < 0.001$; Fig. 3A). Mean I-FABP plasma levels were 228.4 ± 28.4 pg/mL in patients with OAAC, which was significantly lower when compared with patients with intestinal ischemia ($P < 0.05$; Fig. 3B). These data are consistent with observations from previous studies.[8,22]

DISCUSSION

This study shows that SM22 is a potential plasma marker for the detection of severe ischemia of the intestinal muscle layers. There is currently no plasma marker of intestinal ischemia that might indicate transmural ischemia. Recently reported potential new biomarkers for intestinal ischemia, including I-FABP, only detect mucosal intestinal damage. Severe ischemia of the muscle layers, however, leads to intestinal perforation, sepsis, and death. It is imperative to diagnose patients suffering from this condition early, since it warrants prompt emergency surgery.

First, we set out to develop and validate a new sandwich ELISA to quantify SM22. Combinations of different mouse monoclonal antibodies were used to select the best pair of mAbs against SM22. These antibodies were raised in mice using purified native SM22 from porcine stomach as antigen. As both gene and protein sequences of SM22 are highly conserved across species,[23] the antibodies are known to specifically react with SM22 in smooth muscle tissue of different mammalian and avian origin, including rat, rabbit, guinea pig, chicken, and human.[17] The quantitation range of our SM22 ELISA covers a SM22 value in the expected interval for most patients plasma. As reported in supplementary Table 1 (http://links.lww.com/SLA/B220), the values for interassay and intra-assay precision and accuracy are within current guidance limits of 20% for method acceptance.[24] Based on the analytical performance of the SM22 ELISA, we show that this assay can be used for measuring concentration levels of SM22 in both rat and human plasma.

Next, we studied the release pattern of SM22 from the intestine in plasma and the relation of plasma SM22 levels with the degree of intestinal damage in an experimental animal model of intestinal ischemia. This model of intestinal ischemia is well-characterized and results in histological damage to the intestine,

including transmural damage.[25] We found that SM22 is rapidly released in plasma upon ischemic damage with peak concentrations at 6 hours. Thereafter, its plasma levels returned towards baseline. The latter was considered a consequence of the reduced leakage of smooth muscle cytosolic mass caused by the ischemic degradation of the intestinal muscle combined with the renal clearance of SM22.[25] Therefore, we assessed the damage of intestine in the same model histologically by SM22 immunohistochemistry, and SM22 plasma clearance was studied by calculating AV-concentration gradients across the intestines, liver, and kidney. Plasma SM22 marker levels agreed well with the histological findings. SM22 plasma concentrations are significantly elevated at 4 hours of intestinal ischemia in the rat compared with baseline or sham animals as is shown in Fig. 2. Immunohistochemistry demonstrated no degradation of the muscle layers at this time point, but there is a trend to a significant decline in the concentration SM22 per mg protein of the tissue extracts of the same time point (Fig. 2B). This shows that the transition of mucosal ischemic damage into transmural damage is around 4 hours of ischemia, which is supported by others.[25] The decrease in plasma SM22 at 8, 12, and 24 hours after ischemia could well be explained by the muscle degeneration observed microscopically in corresponding specimens. We found that the content of SM22 per milligram of protein in the intestinal tissue was diminished after ligation of its blood supply, which indicated that the presence of SM22 in the circulation in this model resulted from leakage of the protein. However, active synthesis of SM22 by stressed cells, or a combination of synthesis and leakage, cannot be ruled out by these data.

Immediate SM22 release in the circulation after small intestinal ischemia shows that reperfusion is not necessary for SM22 to be released into the circulation. The results from our transorgan AV-concentration gradients show that that SM22 was specifically released from the intestine in our rat model of intestinal ischemia. Furthermore, assessment of SM22 clearance in rats showed that kidneys are fully responsible for its clearance without involvement of the liver. Moreover, we reported a short half-life time of 14 minutes in rats and 23 minutes in humans, which is equivalent with its molecular weight of 22 kDa.[26] This short circulating half-life of SM22 is favorable for usage in daily clinical practice, since plasma measurements of SM22 reflect actual damage of the intestinal muscular layers without a cumulative effect. The results demonstrate the potential utility of SM22 as plasma marker of severe ischemia of the intestinal muscle layers.

Next, we translated the results to the human situation. Of the 22 patients with histologically proven intestinal ischemia, 13 had transmural ischemia and 9 suffered from mucosal damage. Increased SM22 plasma levels were specific for the patients with transmural intestinal ischemia in contrary to the intestinal mucosal damage marker I-FABP and the classical markers lactate, WBC count, and arterial pH, data which were supported by others.[1,10,22] The small intestinal mucosa, especially the mature enterocytes at the tips of the villi, is most vulnerable to ischemia. Transmural ischemia is therefore always associated with mucosal ischemia. As I-FABP is solely expressed by mature enterocytes, it is not surprising that there is no difference in I-FABP concentrations for mucosal and transmural ischemia. Furthermore, plasma levels of SM22 could also differentiate between patients with transmural intestinal ischemia and other causes of acute abdominal complaints, including acute appendicitis, diverticulitis, gynecological/urological pathology, gastroenteritis, and nonspecific abdominal pain.

Although SM22 is never been used as a biomarker, its family protein calponin was investigated as a potential diagnostic marker for aortic dissection.[27] In addition, the detection of smooth muscle proteins using the BB isoform of creatine kinase (CK-BB) and smooth muscle actin (SMA) during acute intestinal ischemic diseases was shown to be useful in both experimental animal studies and in humans.[28–31] Major concern of the studies using CK-BB is that CK-BB is located in all layers of the intestine since creatinin kinase isoenzymes are also present in the mucosa.[28] Furthermore, SMA was measured using only a semiquantitative manner, namely by Western blotting.

A potential drawback of this study is the fact that SM22 is not specific for intestinal tissue, since it is also expressed by other visceral smooth muscle tissue.[13] There are, however, rarely diseases in which acute degradation of smooth muscle cells is part of the pathophysiology other than in the intestinal tract. Furthermore, the addition of plasma I-FABP measurement, which is solely expressed by enterocytes, ensures the detection of intestinal damage. This is in agreement with the findings of others.[10,22]

It is also of clinical importance to determine whether patients suffer from focal intestinal ischemia or whether the ischemic damage is affecting the whole of the superior mesenteric artery territory. This would require another approach and determines whether the ischemia is survivable with resection. Unfortunately, to our knowledge, there is not such a marker. However, we were able to find SM22 as a marker to timely detect severe ischemic damage to the intestinal muscle layers, selecting which of the patients with mesenteric ischemia should undergo emergency surgery.

Future research needs to be conducted before SM22 can be applied in the clinic. First, to validate SM22, large prospective phase 3 to 4 clinical studies are required using patients suspected for intestinal ischemia. Phase 3 to 4 diagnostic studies answer the question whether, in patients in whom it is clinically sensible to suspect intestinal ischemia, the level of SM22 distinguishes those with and without the target disorder and whether patients who undergo this diagnostic test fare better, in their ultimate health outcomes, than similar patients who do not. As it is impossible to perform a bowel resection on all patients suspected for intestinal ischemia, the endpoint for these clinical studies will be a biphasic multidetector computed tomography (MDCT) with intravenous contrast as the current gold standard for the detection of intestinal ischemia.[5,32] Next, on the research agenda would be the creation of a rapid test for SM22, since the current ELISA takes 3.5 hours to conduct, limiting the use of SM22 in daily practice.

In conclusion, we show that assessment of plasma SM22 levels allows detection of severe ischemia of the muscle layers in patients. The combination of a marker for intestinal mucosal damage (I-FABP) and severe damage to the muscle layers (SM22) may help us in daily clinical practice to gain a more complete picture of the potential level of intestinal injury and to identify patients with severe ischemia of the intestinal muscle layers, in need of emergency surgery and patients suffering from mucosal ischemia solely, which is often reversible and in need of a different therapeutic strategy.

ACKNOWLEDGMENTS

We thank Dr A. Chiavegato for providing the mouse anti-SM22 hybridoma cell lines for this study to Professor W.A. Buurman, PhD. We also like to thank B. Boonen and J. Robijns for their advice and technical assistance regarding the development of the SM22 ELISA. Further, we thank B. de Vries, MD, PhD, of the Department of Pathology, for his help regarding the histopathological analysis of the patients suffering from intestinal ischemia.

REFERENCES

1. Oldenburg WA, Lau LL, Rodenberg TJ, et al. Acute mesenteric ischemia: a clinical review. *Arch Intern Med.* 2004;164:1054–1062.
2. Fukatsu K, Sakamoto S, Hara E, et al. Gut ischemia-reperfusion affects gut mucosal immunity: a possible mechanism for infectious complications after severe surgical insults. *Crit Care Med.* 2006;34:182–187.

3. American Gastroenterological Association Medical Position Statement: guidelines on intestinal ischemia. Gastroenterology 2000; 118:951–953.
4. Kozuch PL, Brandt LJ. Review article: diagnosis and management of mesenteric ischaemia with an emphasis on pharmacotherapy. *Aliment Pharmacol Ther.* 2005;21:201–215.
5. Acosta S, Bjorck M. Modern treatment of acute mesenteric ischaemia. *Br J Surg*. 2014;101:e100–e108.
6. Kougias P, Lau D, El Sayed HF, et al. Determinants of mortality and treatment outcome following surgical interventions for acute mesenteric ischemia. *J Vasc Surg*. 2007;46:467–474.
7. Schoots IG, Koffeman GI, Legemate DA, et al. Systematic review of survival after acute mesenteric ischaemia according to disease aetiology. *Br J Surg*. 2004;91:17–27.
8. Kanda T, Fujii H, Tani T, et al. Intestinal fatty acid-binding protein is a useful diagnostic marker for mesenteric infarction in humans. *Gastroenterology.* 1996;110:339–343.
9. Delaney CP, O'Neill S, Manning F, et al. Plasma concentrations of glutathione S-transferase isoenzyme are raised in patients with intestinal ischaemia. *Br J Surg*. 1999;86:1349–1353.
10. Matsumoto S, Sekine K, Funaoka H, et al. Diagnostic performance of plasma biomarkers in patients with acute intestinal ischaemia. *Br J Surg*. 2014;101: 232–238.
11. Evennett NJ, Petrov MS, Mittal A, et al. Systematic review and pooled estimates for the diagnostic accuracy of serological markers for intestinal ischemia. *World J Surg*. 2009;33:1374–1383.
12. Lees-Miller JP, Heeley DH, Smillie LB, et al. Isolation and characterization of an abundant and novel 22-kDa protein (SM22) from chicken gizzard smooth muscle. *J Biol Chem*. 1987;262:2988–2993.
13. Chiavegato A, Roelofs M, Franch R, et al. Differential expression of SM22 isoforms in myofibroblasts and smooth muscle cells from rabbit bladder. *J Muscle Res Cell Motil*. 1999;20:133–146.
14. Thompson O, Moghraby JS, Ayscough KR, et al. Depletion of the actin bundling protein SM22/transgelin increases actin dynamics and enhances the tumourigenic phenotypes of cells. *BMC Cell Biol*. 2012;13:1.
15. Solway J, Seltzer J, Samaha FF, et al. Structure and expression of a smooth muscle cell-specific gene, SM22 alpha. *J Biol Chem*. 1995;270: 13460–13469.
16. Hallemeesch MM, Ten Have GA, Deutz NE. Metabolic flux measurements across portal drained viscera, liver, kidney and hindquarter in mice. *Lab Anim*. 2001;35:101–110.
17. Duband JL, Gimona M, Scatena M, et al. Calponin and SM22 as differentiation markers of smooth muscle: spatiotemporal distribution during avian embryonic development. *Differentiation*. 1993;55:1–11.
18. Schellekens DH, Grootjans J, Dello SA, et al. Plasma intestinal fatty acid-binding protein levels correlate with morphologic epithelial intestinal damage in a human translational ischemia-reperfusion model. *J Clin Gastroenterol*. 2014;48:253–260.
19. van de Poll MC, Siroen MP, van Leeuwen PA, et al. Interorgan amino acid exchange in humans: consequences for arginine and citrulline metabolism. *Am J Clin Nutr.* 2007;85:167–172.
20. Riddez L, Hahn RG, Brismar B, et al. Central and regional hemodynamics during acute hypovolemia and volume substitution in volunteers. *Crit Care Med*. 1997;25:635–640.
21. van de Poll MC, Derikx JP, Buurman WA, et al. Liver manipulation causes hepatocyte injury and precedes systemic inflammation in patients undergoing liver resection. *World J Surg*. 2007;31:2033–2038.
22. Thuijls G, van Wijck K, Grootjans J, et al. Early diagnosis of intestinal ischemia using urinary and plasma fatty acid binding proteins. *Ann Surg*. 2011;253:303–308.
23. Assinder SJ, Stanton JA, Prasad PD. Transgelin: an actin-binding protein and tumour suppressor. *Int J Biochem Cell Biol*. 2009;41:482–486.
24. Andreasson U, Perret-Liaudet A, van Waalwijk van Doorn LJ, et al. A Practical Guide to Immunoassay Method Validation. *Frontiers in neurology*. 2015;6:179.
25. Kanda T, Nakatomi Y, Ishikawa H, et al. Intestinal fatty acid-binding protein as a sensitive marker of intestinal ischemia. *Dig Dis Sci*. 1992;37:1362–1367.
26. Yamaoka T, Tabata Y, Ikada Y. Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice. *J Pharmaceut Sci*. 1994;83:601–606.
27. Suzuki T, Distante A, Zizza A, et al. Preliminary experience with the smooth muscle troponin-like protein, calponin, as a novel biomarker for diagnosing acute aortic dissection. *Eur Heart J*. 2008;29:1439–1445.
28. Graeber GM, Wolf RE, Harmon JW. Serum creatine kinase and alkaline phosphatase in experimental small bowel infarction. *J Surg Res*. 1984;37: 25–32.
29. Evennett N, Cerigioni E, Hall NJ, et al. Smooth muscle actin as a novel serologic marker of severe intestinal damage in rat intestinal ischemia-reperfusion and human necrotising enterocolitis. *J Surg Res*. 2014;191: 323–330.
30. Samaniego Munoz M, Sanchez Navarro MR. [Serum CK-BB. A biochemical marker in neonatal necrotizing enterocolitis]. *An Esp Pediatr.* 1996;44: 577–580.
31. Fried MW, Murthy UK, Hassig SR, et al. Creatine kinase isoenzymes in the diagnosis of intestinal infarction. *Dig Dis Sci*. 1991;36:1589–1593.
32. Tilsed JV, Casamassima A, Kurihara H, et al. ESTES guidelines: acute mesenteric ischaemia. *Eur J Trauma Emerg Surg*. 2016;42:253–270.