Postmortem Intestinal Sampling in Malawi

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Abstract

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Background In LMICs, undernutrition is the leading underlying cause of child mortality, which remains unacceptably high. Environmental enteric dysfunction (EED), a prevalent condition in areas with lack of access to hygiene and safe sanitation could be a significant contributor to undernutrition. To better understand the role of EED in child undernutrition and mortality, accurate cause of death ascertainment is crucial. Minimally invasive tissue sampling (MITS) is a validated technique for assigning cause of death, but the current procedure lacks gastrointestinal interrogation. We aimed to assess the utility of postmortem endoscopically obtained intestinal biopsies, as autolysis may preclude its usefulness, and to adapt an EED histopathologic scoring system to assess upper intestinal disease severity and an inflammatory bowel histopathologic disease scoring system to measure lower intestinal disease severity.

Methods MITS in Malawi (MiM) recruited children who died during inpatient admission at Queen Elizabeth Central Hospital in Blantyre, Malawi. Eligibility criteria included age (1 week-59 months) and admission diagnosis (acute illness or malnutrition). Upon death, the standard MITS procedure was conducted after upper and lower endoscopies to collect three duodenal and one colonic and one rectal sample, respectively. These were formalin fixed, prepared into paraffin blocks, sectioned, and stained with hematoxylin and eosin (H&E). Two independent pathologists assessed degree of autolysis on each H&E slide and a gastrointestinal pathologist applied the 11-parameter upper and 4-parameter lower intestinal histopathologic scoring system to measure intestinal disease severity.

Results Twenty-eight post-mortem endoscopic procedures were completed. We were able to obtain duodenal, colonic, and rectal samples in 25, 6, and 25 cases. Some degree of autolysis was found in nearly all intestinal samples (n=99 (98%)), though complete autolysis was rare (n=11 (11%)). We found increasing tissue autolysis in more distally located intestinal tissues. Tissue autolysis in the rectum was negatively associated with age (PR=0.991; 95%CI: 0.987, 0.995). We found no associations between tissue autolysis and any other variables of interest, including postmortem time interval and body refrigeration time. Chronic inflammation was prevalent in both the upper and lower intestinal tissues (average scores: 1.9 and 2.0,

respectively), although acute inflammation was not present in any of the tissues. We also identified a low density of Paneth and goblet cells in the upper intestine (average scores: 2.5 and 2.2 respectively).
Conclusions Postmortem endoscopic biopsy is feasible, despite high levels of autolysis. Autolysis was not found to be dependent on body refrigeration or postmortem time interval, but did vary based on intestinal site, possibly related to increasing microbial colonization in more distal segments of the intestinal tract. We identified duodenal chronic inflammation and reduced Paneth and goblet cell densities, which are features of EED. Severe rectal chronic inflammation was also seen, including in children as young as 6 weeks old. These data warrant further investigation and confirmation but point to the utility of including intestinal sampling as part of the MITS procedure.

INTRODUCTION

Substantial progress has been made in reducing child mortality over the past two decades, however, child mortality rates remain unacceptably high in most low- and middle-income countries (LMICs), many of which are not on track to reach the Sustainable Development Goal (SDG) child health target of <25 deaths per 1000 live births by 2030. Globally, 5.3 million children under age 5 died in 2018 and the child mortality rate was 38.6 deaths per 1000 live births [1]. In Malawi, 30,226 deaths occurred in the same year and the mortality rate was 49.7 per 1000 live births, 23% above the global average, and based on currently trajectories Malawi is not on target to achieve the SDG child health target [1]. Undernutrition is the leading risk factor for childhood mortality and is a persistent and prevalent problem in LMICs, including Malawi [2, 3]. Undernutrition is an important risk factor for infectious disease incidence and case fatality, particularly due to pneumonia and diarrhea [3, 4]. The causes of undernutrition are multifactorial and include food insecurity and recurrent infections.

Environmental enteric dysfunction (EED), a largely asymptomatic condition, is highly prevalent in settings with poor access to adequate sanitation and hygiene facilities and is thought to be an important cause of child undernutrition. An environmental trigger (e.g, recurrent or persistent enteric infection) leads to an intestinal inflammatory response which then causes characteristic villous blunting and concomitant reduced surface area and malabsorption. A permeability defect is another hallmark and can result in translocation of microbes or microbial products. The intestinal inflammation and translocation can both stimulate a chronic systemic inflammatory state [5]. These pathophysiologic perturbations (malabsorption and systemic inflammation) can lead to poor growth and undernutrition.

In order to prevent child deaths, it's crucial to understand what conditions are causing and contributing to mortality. The most comprehensive and accurate method of assigning cause of death (COD) is full autopsy. However, in many settings, this method is socially unacceptable or too resource-intensive to be performed with any frequency. Verbal autopsy is commonly used instead [6]. Verbal autopsy utilizes clinical history and structured interviews with caregivers to assess the circumstances preceding the child's death [6, 7]. Based on these factors, COD is assigned. This method of assigning COD is often applied in LMICs as it requires fewer resources, has been demonstrated to be widely culturally acceptable, and can be done several days or weeks after death. [8]. Verbal autopsy has been shown to be relatively accurate for some causes such as injury-related deaths. However, verbal autopsy lacks precision for many etiologies which share similar symptoms and situations in which the child was not seen by a clinician. Furthermore, it is often unable to identify underlying factors contributing to death [9]. Comparison studies of verbal autopsy and full autopsy suggest that 10% to 30% of verbal autopsy COD determinations are erroneous, and 40% to 50% are erroneous in cases of HIV positive individuals [10-12].

A validated alternative to full autopsy is minimally invasive tissue sampling (MITS). This method involves sampling blood and cerebrospinal fluid as well as collecting samples from the brain, heart, lungs, liver, and bone marrow. These tissues are collected using transcutaneous needle biopsy and examined for histopathologic and microbiologic findings [9]. MITS has been found to be highly concordant with traditional autopsy in assessment of COD in pediatrics. Specifically, MITS shows high validity in detecting deaths due to disseminated infections and cancers, but lesser validity for more localized causes of death such as CNS infections that localize to very specific regions of the brain, or pulmonary infections that affect only one lobe, and for some congenital malformations. MITS, which leaves no disfigurement of the body, has been demonstrated to be more culturally acceptable than full autopsy [9].

The current MITS procedure widely deployed by many research groups does not include gastrointestinal (GI) sampling as trans-abdominal needle sampling is hampered by variability in intestinal anatomy leading to inability to target specific portions of the GI tract. Also, unlike solid organs, the GI tract is not fixed within the abdominal cavity, resulting in slippage of intestinal tissue away from needle tip instead of penetration into the tissue. Though MITS is a validated alternative to full autopsy, lack of GI sampling substantially stymies the understanding of EED and other GI disorders' contribution to childhood deaths worldwide.

One of the primary aims of the MITS in Malawi (MiM) study was to investigate the feasibility of a postmortem endoscopic approach to sample GI tissue. There were a number of potential barriers to postmortem endoscopic sampling such as inability to intubate per os or per anus postmortem (e.g., due to rigor mortis or navigating an unprepared bowel). However, the MiM study has already determined the feasibility of this approach.

Despite the success of the procedure, rapid intestinal tissue degradation after death could render the acquired samples uninformative. Microbes degrade tissues postmortem. This process commences and transpires most rapidly in tissues where blood supply is most reduced in the perimortem process and where microbes are most abundant. It is not surprising that intestinal tissue is the first to degrade postmortem. In order to preserve vital organs (e.g., brain, lungs, heart) blood is shunted away from other organs (e.g., liver, kidney, intestines) in critical medical conditions. Furthermore, the intestinal tract is highly colonized with gut microbes, increasingly so in the distal tract [13].

There are a number of factors that may contribute to the degree of autolysis of intestinal tissue, thus impacting the utility of postmortem intestinal tissue sampling. First, autolysis is an ongoing process so the time interval between death and sample acquisition could be a critical factor and influenced by whether and for how long the body is refrigerated, as refrigeration slows the microbial degradation of organic matter. Specific medical conditions could lead to intestinal mucosal conditions that favor autolysis. For example, children with severe acute malnutrition (SAM) have alterations in the gut microbiome that could impact postmortem tissue degradation [14, 15]. Many infectious conditions, including gastroenteritis and sepsis, similarly disrupt the gut microbiome [16]. Antibiotics that disrupt the gut flora may lead to reduced autolysis [17].

This study is situated within the MiM study and has two aims. First, we aim to determine the degree of autolysis within the intestinal tissues and explore the relationship between clinical, demographic, and postmortem factors with degree of intestinal tissue autolysis to assess the utility of gastrointestinal MITS to improve cause of death determination. Second, we aim to describe the degree of intestinal histopathology among the samples that had not fully autolyzed and explore clinical and demographic factors with the severity of intestinal disease using a histopathologic scoring system adapted for use in this study.

METHODS

Study Design and Setting

MiM is a cross-sectional study utilizing MITS to better understand COD among children aged 1-59 months hospitalized with acute illness and/or malnutrition. Recruitment for the MiM study was conducted at the Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi. QECH serves the whole Southern Region of the country and is a national referral hospital and teaching hospital for the University of Malawi College of Medicine (CoM). This setting was selected for two reasons. First, QECH and CoM were the settings for early pioneering work on minimally invasive brain tissue sampling in the context of cerebral malaria [18]. Second, the CHAIN study (discussed below), the parent study of MiM, enrolled participants at QECH and had already set up infrastructure to collect relevant antemortem clinical data.

Participant Selection

MiM started as a sub-study of CHAIN (see below). CHAIN purposefully over-recruited undernourished children, and because undernutrition is a major risk factor for mortality, it was anticipated that the majority of children in the MiM study will have been undernourished. Recruitment was expanded beyond patients enrolled in CHAIN mid-way through the study due to lower than anticipated enrollment and case fatality among CHAIN patients. Recruitment was expanded to patients enrolled in another study with similarities to CHAIN (see below) and to patients on the QECH pediatric wards who were not enrolled in any other studies. Exclusion criteria for MiM were per CHAIN: traumatic injury, known terminal illness, congenital syndrome, surgery in the past 6 months, age <1 month or >59 months.

Study staff sought to approach the parent or guardian of each eligible child that died on the pediatric ward at QECH. Nurses on the ward were asked to notify MiM staff when an eligible child died. Families were given a respectful amount of time following their child's death before they were provided information about the study and consented. Recruitment for MiM began on August 20, 2018 and was completed three weeks prematurely on April 9, 2020 due to the COVID-19 pandemic, only one case shy of the anticipated sample size of 30. Due to COVID-related factors the endoscopy team was not available for the final MITS procedure, therefore the final number of participants in this analysis was 28:

- The Childhood Acute Illness & Nutrition Network (CHAIN) study was a cohort study assessing risk factors for mortality and other poor outcomes among children

hospitalized with acute illness and/or undernutrition in limited-resource settings. One of these settings was QECH [19]. N= 5

- The CHAIN nutritional sub-study aimed to identify detailed metabolic risk factors in addition to routine CHAIN assessments. N= 3
- The Kusamala study was a cluster-randomized controlled trial investigating the effectiveness of nurse counselling for patients with SAM under 5 years of age. Inclusion criteria were similar to that of CHAIN and participants were recruited from QECH. N= 2
- Pediatric wards at QECH (not enrolled in any study preceding MiM). N= 18

Clinical Data Collection

Identical case report forms were used to capture antemortem data (history, physical examination findings including anthropometry, diagnoses, and treatment/management) for children enrolled in CHAIN, Nutrition substudy, and Kusamala. For children not enrolled in one of the parent studies prior to their death, MiM study staff extracted as much of the data as available from the participant's medical record. Admission diagnoses and treatment interventions were per the ward clinicians (i.e., recorded by but not determined by study staff). Antemortem anthropometrics were measured by study staff for children enrolled in CHAIN, Nutrition substudy, and Kusamala. All participants had postmortem anthropometric measurements performed by MiM staff.

Severe acute malnutrition (SAM), defined as mid-upper arm circumference (MUAC) <11.5 (among those ≥6 months of age), weight-for-length z-score <-3, or nutritional edema, was determined based on postmortem anthropometric measurements as the majority of participants did not have sufficient antemortem measurements. Postmortem assessment of nutritional status has been used in previously published studies [20]. Measurements of MUAC, weight and length were taken by two different MiM study personnel and where discrepancies occurred were remeasured a third time. The average of the two closest measurements were used. WHO Anthro software was used to calculate Z scores.

Children enrolled in CHAIN and the Nutrition substudy had blood samples drawn at admission. Routine HIV testing was performed using both the Determine and Unigold rapid diagnostic tests (RDT). Positive results among children <18 months and inconsistent results between the two tests among children ≥18 months were confirmed by PCR. Malaria testing was performed using the Bioline RDT. For MiM participants not enrolled in these two parent studies, postmortem blood

samples were tested (including malaria thick and thin smears). Antemortem laboratory results ordered for clinical purposes were abstracted from laboratory reports. All MiM participants with successful postmortem blood sampling (n=21 (72%)) had samples sent for culture and sensitivity. Other laboratory investigations on postmortem samples are pending and delayed due to the COVID-19 pandemic (e.g., qPCR analysis on blood, stool, and intestinal biopsies).

Acidosis was defined as antemortem blood pH below 7.35 and metabolic acidosis based on a blood bicarbonate level less than 21 mEg/L [21]. Anemia was defined using age-based hemoglobin standards (e.g., <110g/L for children $\geq$6 months of age) based on antemortem hemoglobin results [22, 23]. Nearly all (n=26 (93%)) children were on antibiotics during admission, and all were given broad spectrum antibiotics. Based on advisement from an infectious disease physician on the study team, ceftriaxone and ciprofloxacin were considered as the most gut microbiome disruptive antibiotics amongst those with which the children were treated.

The time interval from death to refrigeration to endoscopy were recorded by study staff following MITS procedure. Pathologists and additional study staff reported postmortem findings. Stomach contents were recorded upon initiation of the MITS procedure. Stomach pH was measured during the MITS procedure. Tissue autolysis was recorded for all tissues in the core MITS procedure as well as gastrointestinal MITS. Liver steatosis was reported by pathologists in their assessment of MITS samples.

Body Handling and Sampling Procedures

The study team strived to conduct the MITS procedure as soon as practically possible after consent was obtained. Efforts were made to place the body in the morgue refrigerator in the immediate postmortem period. At the beginning of the study, this was not routine practice at QECH for pediatric deaths; however, it became part of standardized clinical workflow thereby facilitating study procedures.

The MITS procedure commenced with the endoscopy which was performed by one of two study members (a gastroenterologist and an endoscopist). Gastric and duodenal fluid were aspirated when possible as ambient samples and when not possible after flushing with buffered saline. Mucosal biopsies were obtained using pinch forceps from the stomach, upper intestine (including the 1st part of the duodenum (D1), 2nd part of the duodenum (D2), and most distal accessible portion of the duodenum and jejunum (which are difficult to anatomically distinguish via

endoscopy and henceforth referred to as D3/D4). Lower endoscopy was also performed to obtain rectal samples and attempt colonic sampling as proximally as possible. Rectal brushes were used to obtain stool samples. In addition, one of two CoM pathologists trained in the MITS procedure performed the "core" procedure to obtain blood, cerebrospinal fluid, and urine as well as brain, heart, lung, liver, and bone marrow biopsies. Procedures for core sampling for MITS have been described elsewhere [24].

All tissues, including from the GI tract, are paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E). H&E slides were scanned and uploaded to a telepathology platform.

Assessing Tissue Autolysis

We analyzed the degree of autolysis as assessed in five anatomical locations for all successfully obtained biopsies: D1, D2, D3/D4, colon, and rectum. Two pathologists (a general pathologist in Malawi, pathologist A, and a gastrointestinal pathologist in the U.S., pathologist B) independently rated each GI slide for the degree of autolysis on a scale of 1-4 corresponding to <50% autolysis, 50-75%, >75% but some intact tissue, and complete autolysis, respectively. At the time of this interim analysis only pathologist A's ratings were complete as shipment of slides for scanning and access by pathologist B has been hindered by the COVID-19 pandemic. Interrater reliability comparing the two pathologists' autolysis rating was performed using Cohen's Kappa statistic and Gwet's AC. Gwet's AC was chosen in addition to Kappa as it has been shown to be more reliable, particularly when there is an uneven prevalence within scoring categories [25].

To explore how autolysis varied by intestinal site, we utilized graphical visualizations and confirmed any relationships using paired t-tests. We a priori hypothesized that certain variables could be associated with tissue autolysis. Most variables of interest were coded as binary yes/no variables for analysis. These include diagnoses of sepsis, gastroenteritis, and acute respiratory infection [1], nutritional status, blood culture results, HIV and malaria test results, antibiotic use, and postmortem stomach contents. A few variables were analyzed continuously: age, ambient stomach fluid pH [2], time from death to initiation of endoscopy, and time the body was refrigerated. Associations between binary variables and autolysis rating were assessed using Fisher's exact

[1] Based on admission diagnoses for this interim analysis. Future planned analyses will use cause of death diagnoses which are not yet available at this time.

[2] Only examined in association with postmortem tissue autolysis in D1.

test. Association between continuous variables and autolysis rating were determined using univariate linear regression. Assuming a 25% prevalence of refrigeration time greater than 6 hours, this study was initially powered to assess a 1.5-fold increase in tissue autolysis with postmortem refrigeration time and time interval from death to initiation of endoscopy. Because these variables were of particular interest, they were assessed together using bivariate logistic regression.

Assessing Histopathologic Disease Severity

For samples without complete autolysis, the GI pathologist determined severity of upper intestinal histopathology using a scoring system developed and deployed by the Environmental Enteric Dysfunction Biopsy Initiative (EEDBI) [26]. Lower intestinal disease severity was adapted from a histological index used for ulcerative colitis [27]. The EEDBI scoring system utilizes 11 upper intestinal histologic criteria and the ulcerative colitis scoring system assesses 4 lower intestinal criteria [26, 27]. Each criterion has a maximum score of either 3 or 4, and higher scores indicate more severe pathology. Because of autolysis, the number of tissues available for histopathological scoring varies by participant. Furthermore, autolysis may render certain histologic criteria not scorable. Therefore, intestinal disease for each participant is determined as a percentage of total score for each tissue based on the criteria that were scorable; this is referred to as “total score percent”.

The degree of intestinal disease severity was first compared across intestinal tissue types to determine whether disease was more common in any part of the intestine. Disease severity scores were also compared across criteria to explore whether severity was being driven by any criteria in particular.

Most of the variables hypothesized to be associated with intestinal histopathologic disease severity were coded as binary yes/no variables with the exception of age and ambient stomach fluid pH. The association between all variables and disease severity was assessed using univariate linear regression.

In all instances, significance was determined using a two-tailed alpha level of 0.05, but visual trends were also used to draw conclusions due to the small sample size of the study. The pathologists directly entered their data into Redcap database report forms. All other data were recorded on paper report form and then double-entered and checked for accuracy. Statistical

analyses were conducted using Stata/SE 16.1, and graphics were designed using RStudio version 1.2.5019.

Ethical Approval

This study has received ethics approval from the Malawi National Health Sciences Research Committee (NHSRC 1913), Oxford University Ethics Committee (OxTREC 34-16), and an exemption from the University of Washington IRB (STUDY00003689). Written informed consent was obtained from parents/guardians of the deceased children enrolled in MiM. Assistance with coffin purchase, transportation, and grief support was offered to all parents/guardians approached for participation in the study regardless of whether they agreed to participate.

RESULTS

During the study period, there were 76 eligible deaths at QECH, see Figure 1. Inability to reach a key study team member and not being alerted by the ward nurses were the primary reasons that a family was not approached for participation. Fifty-eight were approached and 29 consented to participation. The primary reasons for denied consent was lack of perceived benefit to participation and preference to take the child's body home. Due to COVID-related circumstances, intestinal procedures could not be undertaken for the last case. All remaining 28 study participants had at least one intestinal sample successfully biopsied. Of 28 participants, 25 had D1 sampled, 21 had D2, 24 had D3/D4, 6 had colon, and 25 had successful rectum sampling. The paucity in successful colon samples is due to stomach contents (e.g., milk) and stool in unprepared lower bowel interfering with equipment blockage reducing capacity to sufficiently insufflate.

Tissue Autolysis

Pathologist A assessed autolysis of all samples while Pathologist B has only had access to 19 participants' slide images and has therefore assessed autolysis in 19 D1, 16 D2, 17 D3/D4, 6 colon, and 19 rectum samples. Pathologist agreement on tissue autolysis was generally low, but pathologist A systematically rated autolysis one point higher than pathologist B on average (mean rating by pathologist A = 2.77, mean rating by pathologist B = 1.77, $p<0.005$). To statistically assess agreement, two measures were used. The kappa statistic was calculated as 0.01 and Gwet's AC is 0.34. Figure 2 displays a comparison of the two pathologists' autolysis ratings.

Autolysis was common among tissues sampled for this study, as some degree of autolysis was found in 99 tissue samples (98%). However, only 11 tissue samples (11%) were completely autolyzed. The degree of autolysis varied by intestinal site, as displayed in Figure 3. Due to having so few colon samples, these tissues were eliminated from further analyses. D1 was the least autolyzed (mean autolysis score = 2.3) followed by D2 (mean autolysis score = 2.6). Mean autolysis was similar between D3/D4 and the rectum which were the most autolyzed (mean ratings of 2.9 and 2.8, respectively). There was pattern of worse autolysis in more distal intestinal sites. A paired t-test comparing the upper intestine (D1, D2, D3/D4) to the rectum results in a p-value of 0.09. Similarly, comparing each upper intestinal site to each other continues the trend described above (D1 vs. D2 p=0.45, D2 vs. D3/D4 = p=0.29, D1 vs. D3/D4 p=0.06). D1 tissue was significantly less autolyzed than rectal tissue (p=0.03).

Only one statistically significant association was found between tissue autolysis and the independent variables of interest. These results are displayed in Table 1. Age was significantly associated with rectal tissue autolysis. For each week age increased, the degree of rectal autolysis was 0.9% lowered (PR=0.991; 95%CI: 0.987, 0.995). The relationship between autolysis and the time interval from death was of primary interest to this study, and duration of body refrigeration was also of interest. However, neither variable was significantly associated with autolysis. Six hours is an oft-cited postmortem interval after which sampling of intestinal tissue is thought to be without merit due to complete or near complete degradation [28-30]. We did not find a statistically significant association between autolysis of tissue obtained prior to or after 6 hours postmortem. Because postmortem interval and duration of body refrigeration were of particular interest, they were included together in multivariate logistic regression to assess whether there was any relationship between intestinal tissue autolysis and the two variables together. No statistically significant relationships were found. Small sample size precluded an assessment of any interaction between postmortem time interval and refrigeration time. Figure 4 graphically displays the relationship between these two variables and tissue autolysis.

In addition to the variables presented in Table 1, the relationship between intestinal autolysis and malaria test results, HIV exposure (by history), acidosis (by antemortem blood gas), and postmortem ambient stomach fluid pH, and non-intestinal tissue autolysis were also of interest. However, the small sample size did not allow for an analysis of these variables. Of those participants with intact tissue samples available for analysis (n=28), only one had blood gases assessed, only one (of 24) had a positive malaria test, and HIV exposure data was only available

for 6 (4 of whom were exposed). Ambient stomach fluid was aspirated from 10 participants, all of whom had pH measured. Only one participant had autolysis in non-intestinal tissue: the central nervous system.

Intestinal Histopathologic Disease Severity

Pathologist B is scoring intestinal disease severity on H&E slide images that are not completely autolyzed. At the time of this writing, the pathologist has scored samples from 19 participants including: 19 D1, 16 D2, 17 D3/D4, 5 colon, and 18 rectal samples.

Disease severity did not differ substantially by intestinal site. D2 and D3/D4 samples had the highest disease severity scores (mean total percent score = 27% and 26%, respectively). Though there were very few samples, the colon tended to score better than other tissues (mean = 15.6%). Figure 5 shows the average total percent disease severity score for each intestinal site. Of the upper intestinal scoring parameters, Paneth cell depletion was the most severely abnormal (mean score = 2.5 (out of 3)), followed closely by goblet cell depletion (mean score = 2.2 (out of 4)), and increased chronic inflammation (mean score = 1.9 (out of 3)). Intraepithelial lymphocytes, shortened villus architecture, Brunner gland density, enterocyte injury, and epithelial detachment were scorable in very few tissues and had lower mean scores (0.55, 1.75, 1.2, 1, and 1.7, respectively). Acute inflammation, eosinophilic infiltration, and foveolar metaplasia were not seen in any upper intestinal tissue examined in this study. In the lower intestine, there was no evidence of neutrophils in the lamina propria, neutrophils in the epithelium, or ulceration. Notably, chronic inflammation was the only abnormality seen among the four lower intestinal criteria (mean score = 2.0 (out of 3)). Table 2 summarizes the average severity of each criterion for both the upper and lower intestine. A visual representation of disease severity among different intestinal tissue types within each child shows some variation, see Figure 6. No participants have perfect consistency across tissue types, but many participants' tissue scores are either consistently high or consistently low. When the remaining tissue slides are scored, this question will be explored further and accompanied by statistical analysis.

Exploring the relationships between the variables of interest and intestinal disease severity did not reveal any significant associations. These results are summarized in Table 3. No statistically significant relationship was detected between age and disease severity, though a pattern arises visually as shown in Figure 7. In the upper intestine, disease severity is worse when age is higher. Conversely, disease severity is lower when age is higher in the lower intestine. No relationship

was found between disease severity and sex, nutritional status or liver steatosis (a sign of severe malnutrition), admission diagnoses of sepsis or gastroenteritis, anemia (which can be a consequence of malabsorption), or HIV-infection (as determined by RDTs).

In addition to the variables presented in Table 3, HIV exposure, stomach pH, acidosis, and were of interest to examine as potentially related to disease severity, but we were unable to assess due to small sample sizes.

DISCUSSION

Inter-pathologist Agreement on Autolysis Ratings

The difference noted between the two pathologists' autolysis ratings was not unexpected. Pathologist B is a gastrointestinal pathologist and pathologist A is a general pathologist, so some disagreement was anticipated. The kappa coefficient indicates slight agreement between the two pathologists' ratings and Gwet's AC indicates fair agreement. Another consideration is the pattern of pathologist A rating autolysis approximately one score more severe than pathologist B on a fairly consistent basis. The systematic nature of the differences between these ratings is not well captured by Kappa or Gwet's AC. This question will be explored further once COVID-19 restrictions lift and pathologist B is able to rate the remaining samples.

Intestinal Tissue Autolysis

A comparison of the mean autolysis rating by intestinal site shows a pattern of increasing autolysis in more distally located tissues, see Figure 3. The degree of autolysis was statistically significantly different in D1 compared to the rectum, though no other pairwise comparisons were found to be significant. As breakdown of tissue, including intestinal tissue, is caused by degradation by bacteria, it is not unexpected that autolysis was worse in more distal segments of the intestine since colonization with microbes also increases the more caudal the intestinal tract. Confirmation of the association of autolysis with distal location in future studies would be helpful. While the rectum may be the most accessible segment, especially for scale-up of postmortem sampling, if this segment is too degraded relative to the upper intestine, the utility of rectal samples may be minimal.

We assessed the relationship between various demographic, clinical, and postmortem variables with tissue autolysis. Only the relationship with age was statistically significant, in an inverse

direction. The magnitude of this effect is small, a 0.9% reduction in rectal autolysis with each increasing week of age, thus it is likely not clinically significant. Furthermore, the distribution of age in this study was highly right skewed with few participants older than 18 months. These, along with lack of strong biological plausibility, suggest that this result may be a product of chance rather than a true, meaningful relationship.

Of particular interest to this study was the potential relationship between tissue autolysis with postmortem time interval and refrigeration time. At least some degree of autolysis was seen in all intestinal slides, even in a case where sampling had occurred within a few hours postmortem. Autolysis occurred to a variable degree that we could not associate with postmortem time or body refrigeration time intervals. Though previous studies suggest that decreased postmortem time interval and increased refrigeration time are important for minimizing tissue degradation, we could not identify a relationship either visually or statistically [30-33]. Heimesaat et al. demonstrate that there are substantial changes to the intestinal microbiome between 12 - 24 hours postmortem in that the bacterial composition changes and translocation occurs [31]. Additional studies suggest that degradation renders tissue histopathologically uninformative beyond 24 hours after death [33]. The MiM study team strived to conduct the procedure as rapidly as possible; we did not have any cases where sampling was conducted more than 20 hours post-mortem, and we had few cases of completely autolyzed tissue. It is possible that sample size precludes us from identifying significant relationships, though our study was initially powered to detect a 50% change in tissue autolysis with each additional hour postmortem. Our results may suggest that post-mortem and refrigeration time intervals may not be as important to tissue degradation if sampling is done prior to 24 hours with refrigeration. It is also possible that the autolytic process begins in the perimortem period in severe illness where perfusion is shunted to vital organs and away from the intestines, liver, and kidney. While we did not identify hepatic or renal autolysis, intestinal tissue degradation during agonal events would not be unexpected given the combination of hypoperfusion or hypoxia in an organ naturally exposed to a microbial milieu. Comparison to postmortem tissue from persons who died suddenly (e.g., from overwhelming injury) would help clarify this question.

It is possible that nutritional status could play an important role in tissue autolysis, as malnutrition can have a substantial effect on the gut microbiome, including compositions that differ between children with different forms of malnutrition (e.g., edematous SAM (or kwashiorkor), non-edematous SAM) [15]. We did not, however, identify a relationship between autolysis and nutritional status although we were not powered to detect such associations.

Our study also looked at the relationship between tissue autolysis and sepsis and gastroenteritis diagnoses as they could disrupt the microbiome and are conditions where circulatory shunting from “non-vital” organs due to shock (e.g., septic or hypovolemic) would be particularly common. As this is an interim analysis, definitive cause-of-death results are not yet available so most diagnoses are based on clinician notes at the time of admission to hospital. We did not identify a relationship, nor with HIV-infection which may affect autolysis by contributing to intestinal dysbiosis [34]. Larger studies will be needed to fully explore these factors.

Intestinal Histopathologic Disease Severity

Despite autolysis prevalence, many intestinal disease features were discernible. Not surprisingly, the epithelial surface is the first to degrade, hence parameters related to the epithelium (e.g. villus architecture) were generally not assessable. However, inflammatory response regardless of white cell type, was scored in 81% and 72% of upper and lower intestinal samples, respectively. All tissues were devoid of acute inflammation. This was consistent with findings from biopsies of children with EED in Zambia, Pakistan, and Bangladesh, as was our finding of chronic inflammation in duodenal lamina propria and reduced Paneth cell and goblet cell density [26, 35]. This suggests that chronic inflammation and low density of Paneth and goblet cells may be indicative of intestinal disease, and perhaps EED. Paneth and goblet cells play important roles in antimicrobial activity and mucin production, respectively. Depletion of these cells may be due to higher rates of cell turnover or possibly an abnormality in stem cell differentiation [36]. Further studies are needed to conclusively interpret this finding.

Evaluation of rectal tissue was a novel feature of our study – as in the upper intestine, chronic inflammation was prevalent while acute inflammation was not seen. While further research is needed to corroborate these findings, they do suggest that rectal tissue, which is more accessible than upper intestinal tissue, may offer opportunities to assess for EED more widely in living patients. Furthermore, rectal biopsy offers opportunities for easier scale-up of intestinal assessment within MITS procedures. However, increased prevalence of autolysis in the rectum compared to the upper intestine may limit its utility in postmortem histologic assessments, although microbiologic and other interrogations should not be as autolysis-dependent.

Comparing disease severity between tissues resulted in no significant differences, excluding the colon. Disease severity in the colon was less severe than in other intestinal tissues; however, only

five colon samples were scored which limits our ability to draw definitive conclusions. EED is thought to be a patchy disease of the upper intestine, thus we expected to see variation in histopathologic severity of intestinal samples within the same child. While some samples were too autolyzed to assess histologic characteristics, an average of 2.9 intestinal biopsies was available per child. Our preliminary assessment suggests that histopathologic severity varied some, but not widely, within each child. This question will be reexamined upon lifting of COVID-19 restrictions when additional intestinal slides can be scored.

EED is thought to be an acquired disease, as Chacko et al (1969) demonstrated that fetuses and neonates have few or no changes in villus architecture but villus changes became more severe as age increased [29]. Our results disagree in that we see no significant relationship between age and intestinal disease, but we see markers of severe intestinal disease in infants as young as 6 weeks old. We are unable to conclude why we see severe intestinal inflammation in young infants, though these children may have been given breastmilk substitutes or contaminated fluids or foods which could put them at higher risk for EED. We did not have sufficient data to answer this question.

In addition to age, no other relationships were found between clinical or demographic factors and disease severity. SAM has been associated with an enteropathy, especially among children with kwashiorkor, which may be unique from EED [37, 38]. Furthermore, EED can cause malabsorption and a chronic systemic inflammatory state, leading to malnutrition. However, no relationship was found in this study between disease severity and anthropometric status. A comparison of disease severity in children with kwashiorkor to those with non-edematous SAM and to those with healthy anthropometrics is important to further understand whether a relationship exists between nutritional status and intestinal disease severity.

Diagnoses were not associated with intestinal disease severity or with degree of autolysis, but admission diagnoses per the admitting clinician were utilized in this interim analysis. A formal cause of death analysis will be conducted once COVID-19 restrictions allow for completion of assessments of MITS samples which will offer a more accurate and complete inventory of diagnoses.

Limitations

This study has a number of limitations. Study size is a substantial limitation in this analysis. The MiM study was primarily designed to assess the feasibility of postmortem endoscopy which had never, as far as we are aware, been previously performed. The MITS procedure is invasive and highly socially sensitive. For all of these reasons, a larger sample size was not required nor practical for the primary aim of the study. The endoscopic approach has been demonstrated to be feasible, and it was an important next step to assess the prevalence and degree of autolysis and disease severity, and factors related to them even though the study was not powered with these outcomes in mind. While we were powered to detect a 1.5-fold increase in effect size of postmortem tissue autolysis and time interval postmortem, most other variables of interest in this study only had power to detect relationships with effect sizes ranging from 2 to 4-fold at minimum. However, it was crucial to leverage the data generated to explore whether postmortem gastrointestinal sampling can provide useful data to further the understanding of the role of GI pathophysiology in child deaths as a means to develop interventions to reduce mortality. Another limitation is that the results of this study may not be generalizable to all children in LMICs or even sub-Saharan Africa. The parent study from which many MiM participants were recruited purposefully oversampled undernourished children in order to better understand risk factors for mortality related to nutritional status. Furthermore, the participants in this study died while admitted to QECH in Blantyre. Although QECH does serve as a national referral hospital, patients are more likely to be from lower socioeconomic backgrounds and more likely to have resided in or near Blantyre. However, community-based deaths were not assessed in our study. A limitation specific to this interim analysis is that, although all specimens have been obtained, specimen processing is still ongoing. Laboratory analyses, transfer of slides, and slide scoring have all been substantially slowed by the COVID-19 pandemic and therefore could not be included in this thesis. However, once these data are available, they will be used in updated analyses.

Conclusions

Postmortem intestinal tissue autolysis is common and does not appear to be impacted by time since death nor time that the body was refrigerated. Tissue autolysis did vary by intestinal site, with more autolysis in the more distal intestinal regions, supporting the hypothesis that autolysis increases with increasing bacterial concentrations. Despite this, we were able to apply a novel intestinal histopathologic disease severity scoring system which identified features of EED in the duodenum – chronic inflammation without acute inflammation and reduced Paneth and goblet cell densities. Chronic inflammation was also identified in the rectum, and unexpectantly even among

children as young as 6 weeks of age. Future studies with larger sample size are necessary to further confirm the trends seen in this study and determine factors associated with tissue autolysis and disease severity.

TABLES AND FIGURES

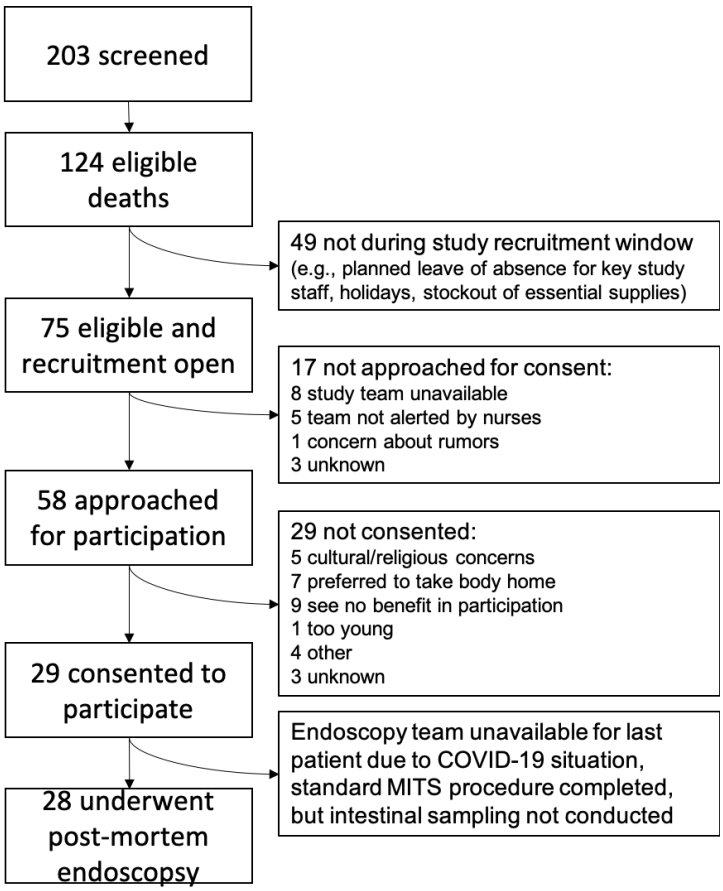

Figure 1: participant recruitment scheme including reasons eligible cases were not enrolled in the MiM study.

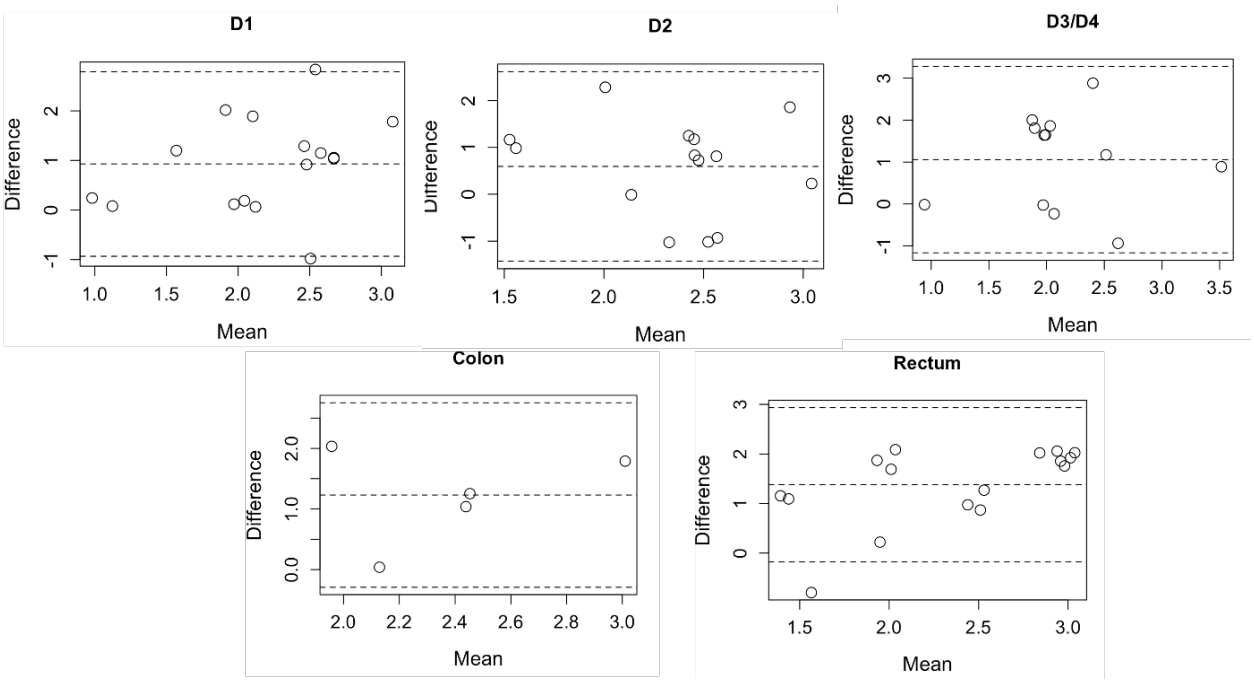

Figure 2: The difference in autolysis rating by intestinal site, as calculated by pathologist B's rating subtracted from pathologist A's rating.

Abbreviations: D1: first portion of the duodenum, D2: second portion of duodenum, D3: third portion of duodenum. D4: 4th portion of the duodenum.

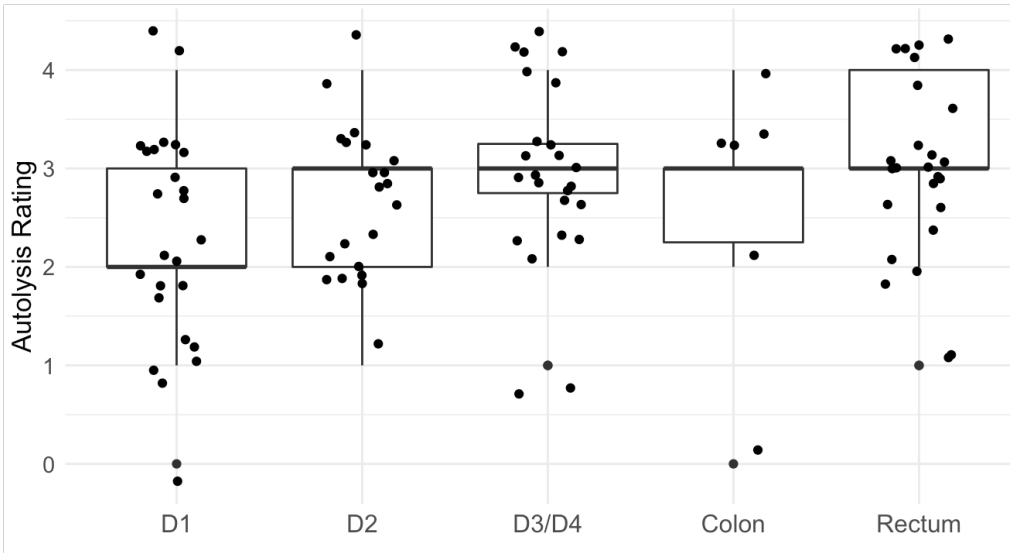

Figure 3: Tissue autolysis in each intestinal site, as boxplots. Each dot represents one tissue sample.

Abbreviations: D1: first portion of the duodenum, D2: second portion of duodenum, D3: third portion of duodenum. D4: 4th portion of the duodenum.

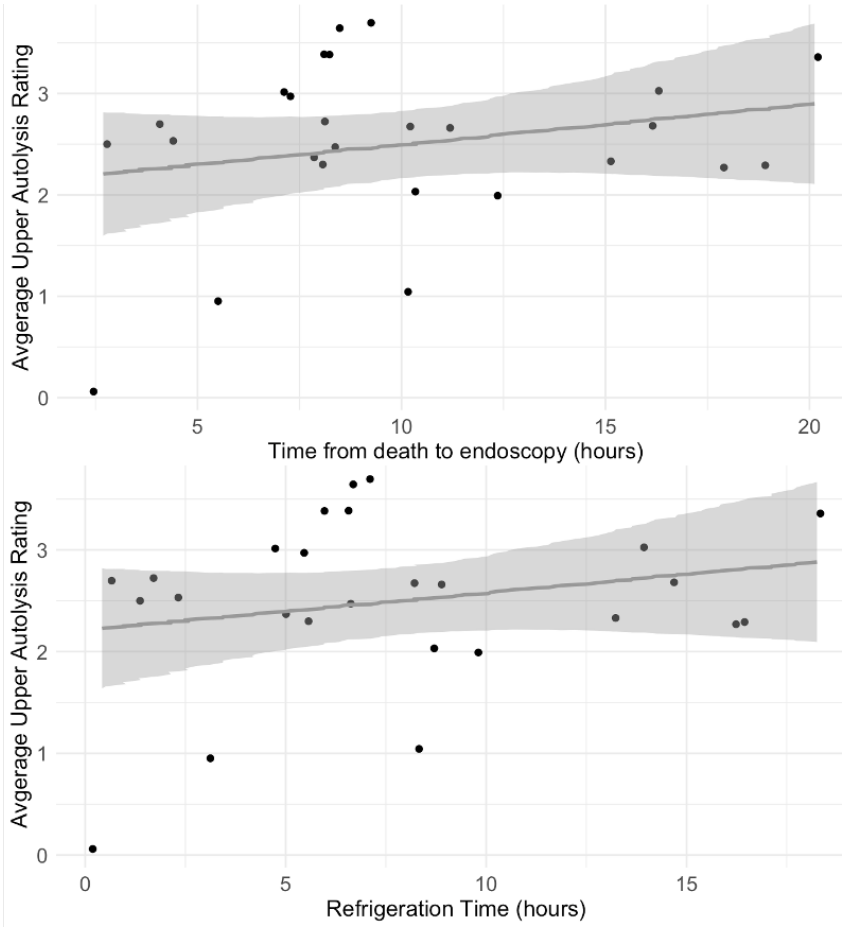

Figure 4: The relationship between tissue autolysis in the upper intestine with time from death to endoscopy and refrigeration time, both in hours, as scatterplots. The regression line is surrounded by 95% confidence intervals in gray.

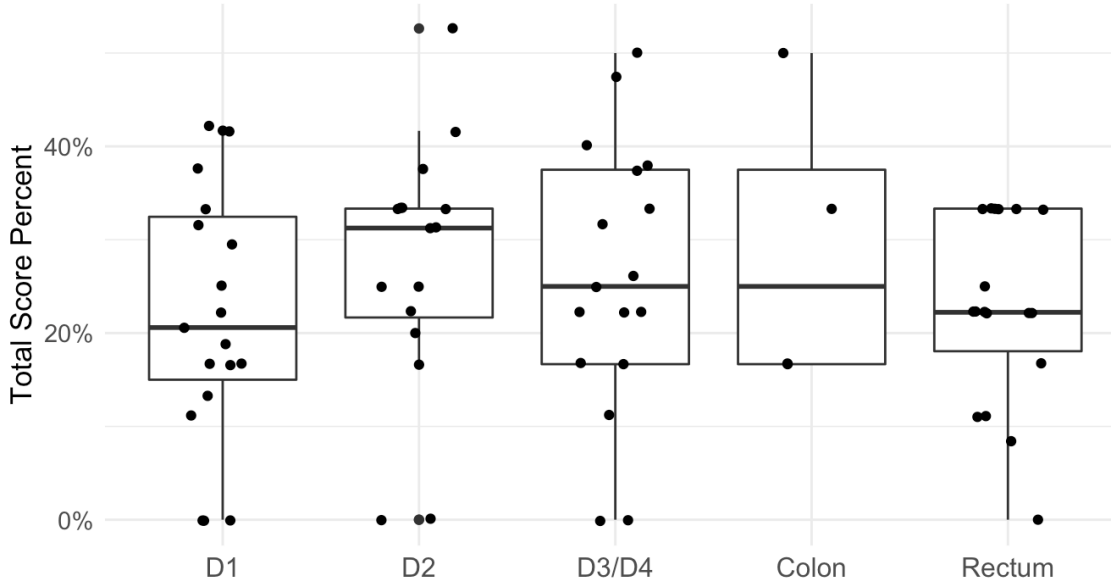

Figure 5: boxplot comparison of intestinal disease severity by tissue site. Each point represents a tissue sample from one patient.

Abbreviations: D1: first portion of the duodenum, D2: second portion of duodenum, D3: third portion of duodenum, D4: 4th portion of the duodenum

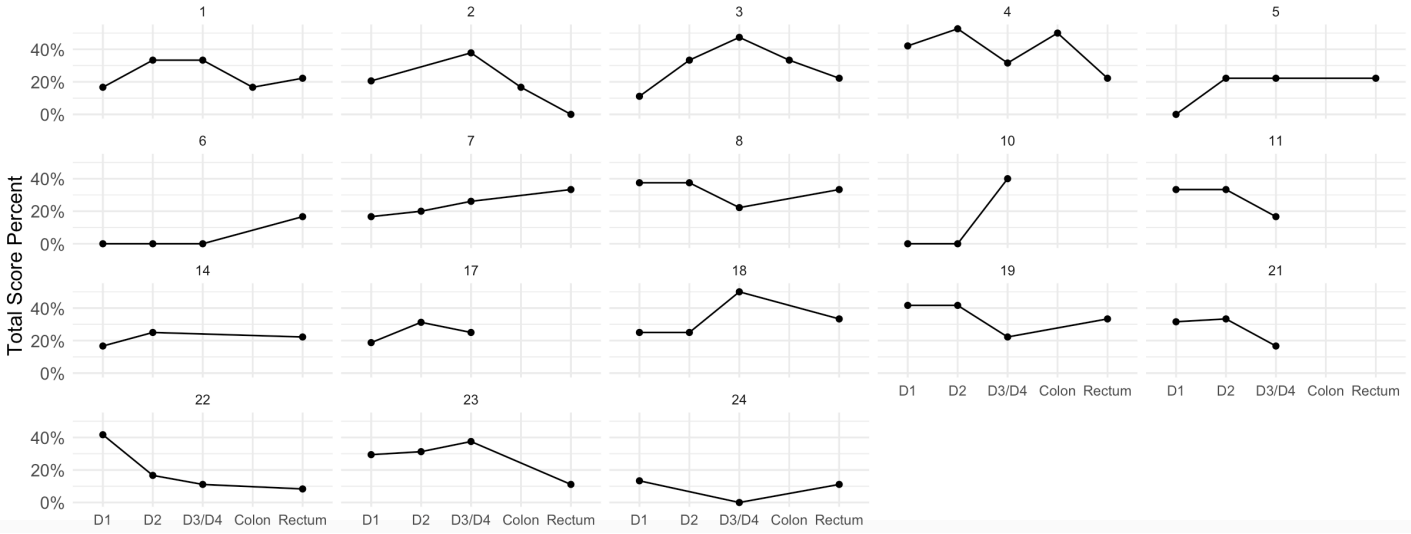

Figure 6: Intraparticipant variation of intestinal disease severity total score percent. Each facet represents one participant, labelled by case number. This plot only shows participants with more than one scored tissue type. For each participant, available tissue scores are shown as points.

Abbreviations: D1: first portion of the duodenum, D2: second portion of duodenum, D3: third portion of duodenum, D4: 4th portion of the duodenum

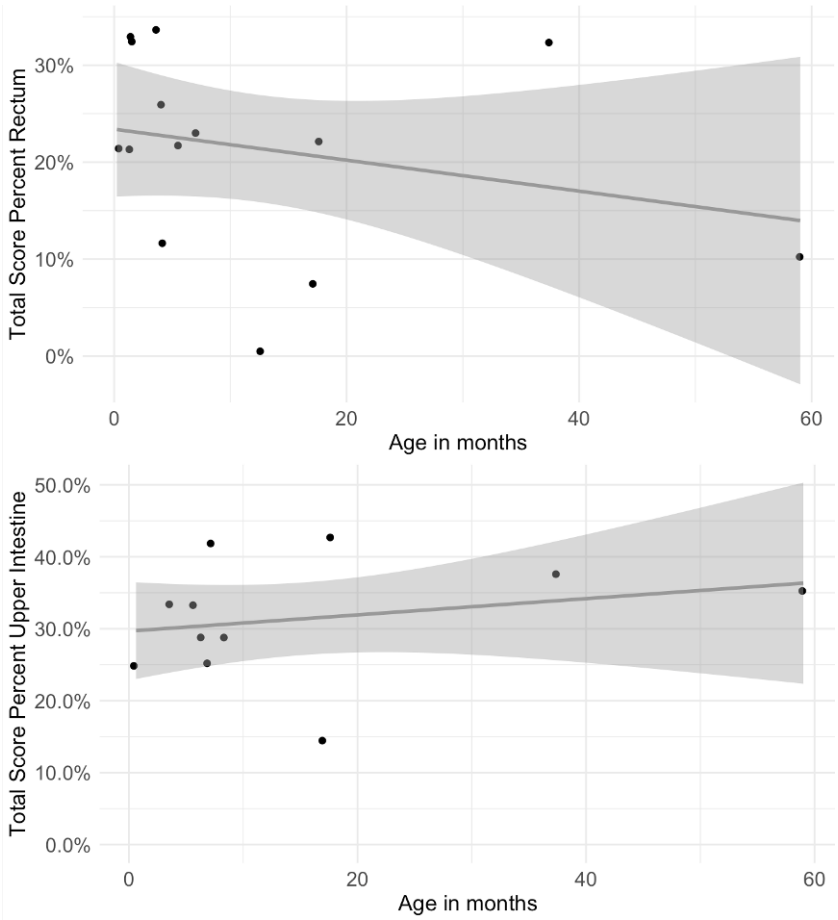

Figure 7: scatterplots demonstrating trend in age and total intestinal histopathologic score percent in the rectum and the upper intestine (D1, D2, D3/D4). The regression line is surrounded by a 95% confidence interval in gray.

	D1		D2		D3/D4		Rectum	
	n (%) or mean (SD)	p-value	n (%) or mean (SD)	p-value	n (%) or mean (SD)	p-value	n (%) or mean (SD)	p-value
Age (weeks)	48.2 (61.4)	0.95 +	54.1 (67)	0.27 +	50.3 (62)	0.06 +	47.0 (62)	0.01 + *
Female	14 (56)	0.24	12 (57)	1.0	14 (58)	0.70	14 (56)	0.25
SAM	18 (72)	0.14	14 (67)	0.88	17 (71)	1.0	19 (76)	0.63
Edematous SAM	4 (20)	1.0	3 (18)	0.44	4 (20)	0.08	4 (29)	0.17
Sepsis	6 (24)	0.42	4 (19)	0.51	5 (21)	0.88	7 (28)	0.26
HIV Infection	9 (56)	0.6	7 (50)	0.21	8 (50)	0.5	8 (53)	1.0
Acute Respiratory Infection	6 (24)	0.82	4 (19)	0.63	6 (25)	1.0	6 (24)	0.05
Gastroenteritis	5 (20)	0.67	5 (24)	0.30	5 (21)	0.44	4 (16)	0.35
Microbiome disrupting antibiotics	10 (43)	0.61	10 (53)	0.23	10 (45)	0.16	9 (39)	0.09
Time from death to initiation of endoscopy (hours)	10.0 (5)	0.35 +	11 (5)	0.9 +	10.3 (5)	0.83 +	9.6 (5)	0.90 +

Time body refrigerated	7.73 (5)	0.49 [+]	8.7 (5)	0.82 [+]	8.0 (5)	0.65 [+]	7.3 (5)	0.93 [+]
Contents in the stomach	15 (63)	0.80	13 (65)	1.0	14 (61)	0.74	15 (63)	0.67
Positive blood culture	16 (70)	0.61	14 (70)	0.76	15 (68)	1.0	16 (70)	0.46

Table 1: univariate linear regression or paired t-tests of the relationships between independent variables with tissue autolysis in the upper intestine and rectum.

Abbreviations: SAM: severe acute malnutrition, HIV: human immunodeficiency virus, D1: first portion of the duodenum, D2: second portion of duodenum, D3: third portion of duodenum, D4: 4th portion of the duodenum, SD: standard deviation

*denotes statistical significance based on a=0.05

[+]p-value from univariate linear regression, all others from paired t-test

UPPER INTESTINE					
Criteria		**Mean Score**	**Median Score**	**Score Range**	**# Observations**
Epithelial Neutrophils		0	0	0 – 0	52
Eosinophil Infiltration		0	0	0 – 0	52
Chronic Inflammation [1]		1.78	2	0 – 3	27
	D1	1.53	2	0 - 3	15
	D2	2.10	2	1 – 3	12
	D3/D4	1.92	2	1 – 3	11
Intraepithelial Lymphocytes		0.55	0	0 – 2	9
	D1	0.2	0	0 – 1	5
	D2	1	1	1 – 1	1
	D3/D4	1	1	0 – 12	3
Villus Architecture		1.75	2	1 – 2	4
	D1	2	2	2 – 2	3
	D2	-	-	-	0
	D3/D4	2	2	2 – 2	1
Brunner glands		1.2	1	0 – 3	36
	D1	1.14	0.5	0 – 3	14
	D2	1.5	2	0 – 3	11
	D3/D4	0.91	1	0 – 3	11
Foveolar Metaplasia		0	0	0 – 0	4
Goblet cell density (out of 4)		2.3	2	1 – 4	12
	D1	2.2	2	1 - 4	5
	D2	2.33	2	1 – 4	3
	D3/D4	2.5	2.5	1 – 4	4
Paneth cell density		2.2	2	1 - 3	13
	D1	2	2	1 – 3	3
	D2	2	2	1 – 3	3
	D3D4	2.3	3	1 - 3	7
Enterocyte Injury		1	1	0 – 2	7
	D1	0.5	0.5	0 – 1	1
	D2	1	1	1 – 1	1
	D3D4	2	2	2 – 2	2
Epithelial Detachment		1.7	2	0 – 3	10
	D1	1.6	2	0 – 3	5
	D2	1.5	1.5	0 – 3	2
	D3D4	2	2	1 – 3	2
LOWER INTESTINE					
Criteria		**Mean Score**	**Median Score**	**Score Range**	**# Observations**
Chronic Inflammation [2]		1.96	2	0 – 3	23

	Colon	1.4	1	0 – 3	5
	Rectum	2.11	2	0 – 3	18
Lamina Propria Neutrophils		0	0	0 – 0	23
Epithelial Neutrophils		0	0	0 – 0	23
Ulceration		0	0	0 – 0	3

Table 2: frequency and description of each criterion used in the intestinal disease scoring.

Abbreviations: D1: first portion of the duodenum, D2: second portion of duodenum, D3: third portion of duodenum. D4: fourth portion of the duodenum

[1]As defined by presence of epithelial monocytes

[2]As defined by presence of lymphocytes or monocytes in epithelia or lamina propria

	D1		**D2**		**D3/D4**		**Rectum**	
	n (%) or mean (SD)	**p-value**	**n (%) or mean (SD)**	**p-value**	**n (%) or mean (SD)**	**p-value**	**n (%) or mean (SD)**	**p-value**
Age (weeks)	61.3 (68)	0.74	61.9 (75)	0.83	62.3 (71)	0.10	53.5 (73)	0.38
Female	10 (53)	0.73	8 (50)	0.61	8 (47)	0.75	8 (44)	0.46
SAM	13 (68)	0.59	10 (63)	0.68	11 (65)	0.82	15 (83)	0.82
Edematous SAM	3 (30)	0.35	1 (14)	0.33	2 (25)	0.67	2 (20)	0.88
Sepsis	5 (26)	0.39	4 (25)	0.29	4 (24)	0.91	6 (33)	0.33
HIV Infection	6 (55)	0.87	5 (56)	0.95	4 (44)	0.94	3 (38)	0.39
Liver steatosis	12 (67)	0.55	10 (63)	0.97	11 (69)	0.30	9 (56)	0.06
Gastroenteritis	4 (21)	0.69	4 (25)	0.48	3 (18)	0.95	2 (11)	0.40
Positive postmortem blood culture	12 (71)	0.86	10 (67)	0.26	11 (73)	0.71	11 (70)	0.43
Anemia	9 (90)	0.67	7 (88)	0.59	8 (89)	0.44	6 (67)	0.45

Table 3: univariate linear regression of the relationships between independent variables with disease severity in the upper intestine and rectum.

Abbreviations: SAM: severe acute malnutrition, HIV: human immunodeficiency virus, D1: first portion of the duodenum, D2: second portion of duodenum, D3: third portion of duodenum, D4: 4th portion of the duodenum, SD: standard deviation

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