Understanding Enteric Dysfunction as a Determinant of Post-Discharge Pediatric Outcomes in Kenya and Pakistan.

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Abstract

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Background: This dissertation aims to establish if enteric dysfunction appears to be an important interventional target for clinical trials hoping to reduce pediatric post-discharge mortality. We also aim to generate new knowledge regarding the pathophysiology of enteric dysfunction among acutely ill children, while validating previous observations from community cohorts. Finally, we will contribute to a better understanding of the lactulose-rhamnose ratio (LRR) test's utility and reproducibility as a clinical research tool.

Children aged 2-24 months without current diarrhea were recruited from Civil Hospital Karachi, Pakistan and Migori County Referral Hospital, Kenya. LRR tests were administered after children were clinically stable (oral feeds, not dehydrated, no oxygen needs). Similarly aged community children were pseudo-randomly selected from homes near those of the children being discharged, and were also tested using the LRR protocol. The distribution of LRR among the hospitalized and community children were compared, and potential confounders

adjusted for, in linear regression of log-transformed LRR. Post-discharge growth was considered a proxy for child health and risk of mortality. Growth data were available for the hospitalized children, and associations between their LRR at discharge and subsequent changes in height-for-age (HAZ) and weight-for-age z-score (WAZ) were estimated using linear mixed effect models. Blood plasma samples from both groups were also sent for proteomic analysis. Four plasma biomarkers of systemic inflammation (CD14, CRP, TNFα, IL-6) and one of intestinal enterocyte damage (IFABP) were compared between the hospitalized and community children to understand if their association with the LRR differed across groups. Finally, extreme gradient boosted models were built to predict LRR using the full proteomic dataset, and model explanation tools were used to understand influential variables in those models.

Results: 137 hospitalized and 84 community children had LRR results available. The median age was nine months in each group. Hospitalized children had lower median MUAC (12.4 vs 13.5cm) and higher HIV infection prevalence (5% vs 1%). The mean log LRR among children being discharged was 0.45 higher (95% CI: 0.05-0.86, p=0.028) than among their community peers. However, after adjusting for weight-for-height z-score, the difference in means was reduced to 0.25 higher (95% CI: -0.20, 0.69, p=0.274). This difference was not attenuated in a sensitivity analysis including only children without HIV infection. LRR at discharge was not associated with changes in WAZ or HAZ in any post-discharge time period. There was evidence that LRR had a different relationship to CRP (p=0.044), TNFα

(p=0.027), CD14 (p=0.078), and IL-6 (p=0.171) among the hospitalized as compared to the community children. Predictive models leveraging whole proteomic dataset identified a cluster of plasma proteins involved in leukocyte invasion and tissue repair pathways to be associated with increased enteric permeability. This cluster was correlated with WAZ and HAZ. The cluster was also linked to breastfeeding status and age through biomarkers of animal protein consumption and exposure to bacterial pathogens. Recent diarrhea was not correlated with any of the identified biomarkers of enteric permeability.

Conclusion: Children leaving hospital have greater enteric permeability as assessed by LRR than their community peers. However, among these hospitalized children, enteric permeability was not associated with systemic inflammation at the point of discharge and was not associated with subsequent growth. These findings cast doubt on the degree of benefit that children at hospital discharge are likely to receive from therapeutics targeting ED. Instead, these data suggest resources may be better spent on developing ED interventions for apparently healthy children in the community.

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Preface

This dissertation is in fulfilment of my PhD, using data from a sub-study I designed in collaboration with my Doctoral Committee. This project was funded through a Thrasher Early Career Award and the Childhood Acute Illness & Nutrition (CHAIN) Network. The design of this sub-study was informed by years of thought and discussion on the potential role of enteric dysfunction in post-discharge outcomes among investigators at UW, KEMRI and other institutions. I have endeavored to cite those preceding works in the following chapters, but I also owe much to the many conversations with friends and colleagues at those institutions which have colored the ideas presented in this dissertation.

I also want to acknowledge the work of the teams implementing the lactulose-rhamnose protocol which anchors this dissertation. It is a challenging protocol to implement, and our focal population – children leaving hospital – presented additional difficulties. Given these challenges, I am astonished by the consistency of our findings with previous work, which is entirely a testament to the meticulous work of the teams of Migori and Karachi who implemented this protocol.

My dissertation committee (Drs Judd Walson, Donna Denno, Stephen Hawes, Kristjana Ásbjörnsdóttir, Barbra Richardson, and Noah Simon), and colleagues in the CHAIN Network have been hugely influential in shaping the design and content of this dissertation and are acknowledged as authors on the individual papers presented in this manuscript. As a moderately dyslexic researcher, I should acknowledge that this dissertation is only coherent through a decade of dedicated teachers bribing me with biscuits to attend extra-english lessons (most notably Mrs Post of Horris Hill School), 25-years of Mrs. Pearl Tickell (mother) proofreading my major academic

outputs, approximately three days of Mrs. Mary-Anne Braymer (mother-in-lawyer) copy-editing this dissertation, and Dr. Emily Kalah Gade (PHD, wife, fellow dyslexic) being my template for excellence in academics, and for supporting me through assorted diatribes about academic norms. I can say with absolute clarity that I would not have begun my work at the University of Washington, nor written this dissertation, had I not benefited from the tutelage of these women. Despite their best efforts, I must ask readers to forgive the occasional errors in spelling, grammar, and formatting you may encounter in this dissertation.

Finally, 245 families chose to travel substantial distances or took time out of their child's already lengthy discharge process, to undergo this 3-hour test. I am pleased the data presented in this dissertation is already helping to prioritize clinical trials aiming to reduce post-discharge mortality, and that other research studies are capitalizing on the lactulose-rhamnose testing platform built by our work in Migori. Across these efforts, I am hopeful the time and data volunteered by these 245 families will contribute to lasting benefits for these communities.

Introduction

Child deaths have declined over the past two decades, but the pace of this decline was insufficient to meet the Millennium Development Goal 4 and is not on track to meet the United Nations Sustainable Development Goal child mortality target.[1–3] Mortality occurring in the 6-months following hospitalization accounts for 10-30% of childhood deaths in Low Middle Income Countries (LMICs).[(1-4)] In sub-Saharan Africa, children discharged from hospital are eight times more likely to die in the following 6-month period than peers from their community,[4] and this risk remains elevated for well over six months.[5–7] A similar distribution of post-discharge mortality has also been observed in Asia.[8] Evidence suggests this elevated mortality among apparently stabilized children is frequently preceded by declining nutritional status (characterized by wasting [weight-for-height/weight-for-age] or stunting [height-for-age]) and/or readmission to hospital.[6,9] Understanding the biological mechanisms driving high rates of declining nutritional status, readmission to hospital, and death may offer novel opportunities to introduce or optimize interventions aiming to reduce childhood mortality.

Enteric dysfunction (ED) is characterized by intestinal inflammation leading to malabsorption and impaired intestinal barrier function.[10–12] Among children in the community, environmental enteric dysfunction is increasingly understood to be a common condition in which chronic exposure to fecally contaminated environments incite an intestinal inflammatory response.[13] Environmental enteric dysfunction is thought to cause malabsorption and systemic inflammation, resulting in weakened host immunity, poor nutritional status, and suppressed neurocognitive development.[11,14] Hospitalized children may suffer a similar pathophysiologic process separate from, or in addition to, the environmentally-triggered gut dysfunction present in

the community. Children at admission to hospital are understood to have a higher prevalence of enteric pathogens than children in the community. In addition, hospitalized children in Malawi have evidenced very elevated fecal calprotectin levels, a biomarker of enteric inflammation.[15] This high pathogen prevalence and inflammation may be further compounded by hospital diarrhea-associated or asymptomatic pathogen acquisition during admission. The potential risk-factors associated with hospitalization may cause or exacerbate enteric inflammation leading to 1) reduced intestinal surface area and malabsorption, 2) increased enteric permeability, resulting in translocation of microbes and microbial products into the blood stream. Intestinal inflammation and translocation may, in turn, result in systemic inflammation. The ensuing malabsorption and systemic immune activation may simultaneously result in an inadequate nutritional supply and increased metabolic demand, leaving children vulnerable to undernutrition, recurrent infection, and death.[16,17]

Systemic inflammation may play a critical role in mediating the association between ED and negative outcomes in the community (Figure 1). In community cohorts, biological pathway analysis has linked subclinical enteric pathogen carriage to linear growth failure through increased enteric inflammation and systemic inflammation.[20] Decreased vaccine efficacy has also been linked to ED through activity of indoleamine pathway, a biomarker of systemic inflammation.[21] However, recent episodes of severe infectious disease may dominate the inflammatory profile of children being discharged from hospital. As a result, the relationship between biomarkers of ED and systemic inflammation in post-discharge children may differ from community populations.

Dual sugar testing has a long history of use as a functional measure of permeability, an important pathophysiologic facet of ED.[18].[18,19] Characterizing the impact of permeability, as measured by LRR (i.e., lactulose: rhamnose [LRR]), will offer vital insight into the poorly understood mechanisms of post-discharge mortality and may also illuminate modifiable risk factors which could be used to mitigate permeability during admission and at discharge. Therapeutic foods,[22] antibiotics,[23] and immunosuppressive agents [24] have all been considered as enteric health interventions which may allow the gut to recover from acute illness. A recent pilot trial in Kenya suggested that mesalazine can reduce markers of severe inflammation associated with ED.[(24)] However, prescribing immunosuppressive medications to vulnerable children may further increase their risk of adverse outcomes. Therefore, data demonstrating that ED is an important modifiable target driving failed recovery in the post-discharge period is needed to justify clinical trials in this high-risk population. Failure to observe an association between biomarkers of ED and post-discharge outcomes would suggest that ED-targeted interventions should be focused on children in the community, where ED appears to be a common cause of potentially preventable morbidity.

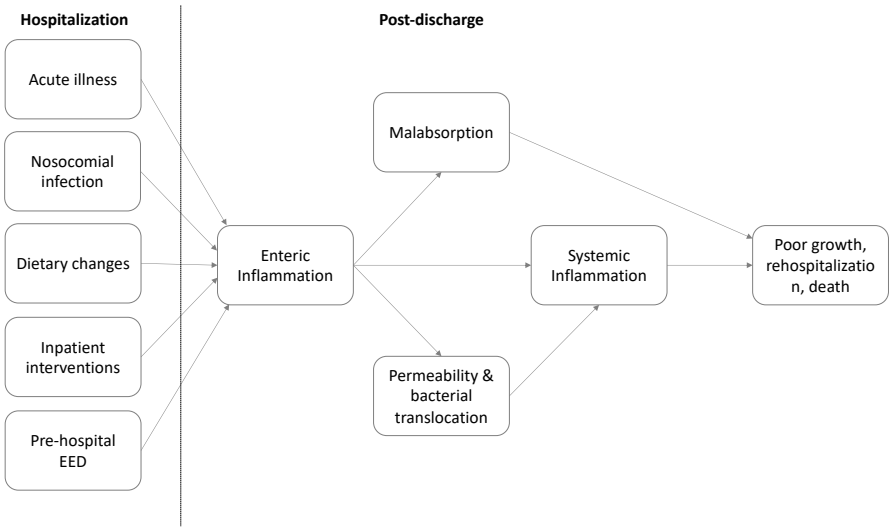

Figure 1. The proposed role of enteric dysfunction, characterized by malabsorption and increased permeability, in post-discharge mortality, rehospitalization, and poor nutritional status,.*Inpatient interventions, e.g. therapeutic foods, breast feeding promotion and antibiotics. Abbreviations: EED – environmental enteric dysfunction

This proposal leverages samples collected during a rigorous cohort study to better understand the causes of inpatient and post-discharge mortality and readmission. Our primary goal is to establish if enteric permeability appears to be an important interventional target for clinical trials aiming to reduce post-discharge mortality. We also hope to generate new knowledge regarding the pathophysiology of ED among acutely ill children, while validating previous observations from community cohorts. Finally, we will contribute to a better understanding of the LRR test's utility and reproducibility as a clinical research tool. The specific aims of this dissertation are:

Aim 1) To compare enteric permeability (lactulose: rhamnose ratio) among children being discharged from hospital to children in the community, and to determine the clinical, social, and host risk factors associated with increased enteric permeability among these two groups of children aged 2-23 months. ***Hypothesis 1a: Children at hospital discharge will have worse enteric permeability (higher LRR) than peers in the community. Hypothesis 1b: Modifiable risk factors, including length of hospital stay, use of antibiotics, use of therapeutic foods, and suboptimal breastfeeding status will be associated with LRR at hospital discharge.***

Aim 2) To test whether enteric permeability (lactulose: rhamnose ratio) at hospital discharge predicts growth in the 180 days following discharge among children aged 2-23 months, and whether the association between enteric permeability and biomarkers of systemic inflammation is the same among hospitalized and community children. ***Hypothesis 2a: Children with worse enteric permeability (higher LRR) at discharge will gain less HAZ and WAZ in the post-***

discharge period. Hypothesis 2b: Enteric permeability will be associated with biomarkers of systemic inflammation in both the hospitalized and community children.

Aim 3: To use machine learning techniques to establish the pattern of plasma protein expression associated with enteric permeability. ***Hypothesis 3: Model built using plasma proteins will be highly predictive of LRR values and will reflect our biologic understanding of ED.***

In the following chapters, each of these aims are presented as individual scientific papers. That format leads to some repetition in the introduction and methods of the papers, but I hope this allows readers to review each paper as a stand-alone manuscript.

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Chapter One

Title: Determinants of impaired enteric barrier function among children being discharged from hospital in Kenya and Pakistan

Authors: Kirkby D. Tickell, Donna M. Denno, Ali Saleem, Zaubina Kazi, Benson Singa, Catherine Achieng, Charles Mutinda, Barbra A. Richardson, Kristjana H. Ásbjörnsdóttir, Stephen E. Hawes, James Berkley, Judd L. Walson.

Background: In low- and middle-income countries (LMICs), acutely ill, undernourished children remain at high risk of mortality for months following discharge from hospital. Community-based studies suggest that enteric dysfunction (ED), including permeability and impaired absorption, is associated with poor outcomes. We used the lactulose rhamnose ratio (LRR) test, which provides a functional assessment of gut integrity, to determine if children at hospital discharge have worse enteric permeability than peers in the community.

Methods: Children aged 2-24 months without diarrhea in the prior 24 hours were recruited from Civil Hospital Karachi, Pakistan and Migori County Referral Hospital, Kenya. LRR tests were administered after children were clinically stable (oral feeds, not dehydrated, no oxygen needs). Similarly aged children were pseudo-randomly selected from homes near those of children being discharged and were

also tested. Urine samples were analyzed by high-performance chromatography mass spectroscopy. Crude LRR distributions of the hospitalized and community children were compared using the Mann-Whitney test. Potential confounders were evaluated and adjusted for in linear regression of log-transformed LRR. The median LRR of three nutritional groups, defined by the WHO definition of severe wasting, were compared to community median values. Finally, social, demographic, and clinical correlates of LRR scores were evaluated for both the hospitalized and community groups in linear regression models.

Results: 137 hospitalized and 84 community children had LRR results available. Median age was nine months in each group. Hospitalized children had lower median MUAC (12.4 vs 13.5cm) and higher HIV infection prevalence (5% vs 1%). The mean log LRR among children being discharged was 0.45 higher (95% CI: 0.05-0.86, p=0.028) than among their community peers. However, after adjusting for weight-for-height z-score, the difference in means was reduced to 0.25 higher (95% CI: -0.20, 0.69, p=0.274). No other potential confounders emerged as important. The median LRR among the community children was 0.27 (IQR: 0.17-0.46, p=0.02). In comparison to the median of this community group, hospitalized children with severe wasting had a median LRR of 0.40 (IQR: 0.28-1.00, p=0.018), those with moderate wasting had a median value of 0.34 (IQR: 0.22-0.87, p=0.088), and children without any wasting had median LRR of 0.32 (IQR: 0.21-0.82, p=0.090). In the correlates analysis, age and nutritional status emerged as important correlates of LRR in both the hospitalized and community groups.

Conclusion: Children at discharge from hospital in LMICs appear to have worse enteric function than community peers. This increased permeability appeared to be predominantly linked to the high prevalence of childhood malnutrition among acutely unwell children, although there was some evidence that hospitalized children without wasting also have increased permeability.

Introduction

Substantial reductions in under-five mortality are needed to achieve the UNDP's Sustainable Development Goals child health target.[1–3] Children who have recently been discharged from hospitals in low- and middle-income countries (LMIC) have high mortality rates over the subsequent six-months, despite their engagement with health services during inpatient management.[4–7] This suggests interventions given at the point of hospital discharge are an opportunity to target a highly vulnerable population of children.[8]

Enteric dysfunction (ED), characterized by intestinal inflammation, malabsorption, and impaired enteric barrier function is thought to be a common underlying contributor to childhood growth failure, severe illness and death in low-resource communities.[9–12] Many of the risk factors for ED, such as poor nutritional status and exposure to community acquired and nosocomial enteric pathogens, are common among children being discharged from hospital. The high prevalence of these factors suggests that children being discharged from hospital are at high risk of ED.

Dual sugar testing is a functional measure of enteric barrier integrity, in which metabolically inactive large (lactulose) and small (rhamnose or mannitol) sugars are administered in liquid suspension, and their urinary excretion is measured over the subsequent 1-5 hours.[13] The ratio of lactulose to the smaller sugar is thought to provide a standardized assessment of enteric barrier function, as lactulose should not cross an appropriately intact intestinal barrier, while rhamnose and mannitol are small enough to readily permeate a normal gut barrier. Faubion, *et al*. demonstrated that the enhanced sensitivity of high performance liquid chromatography-mass spectrometry (HPLC-MS) mass spectroscope laboratory techniques, as opposed to standard HPLC, can detect these sugars in one-hour post-dose urine collections and that rhamnose was detected less frequently compared to mannitol in pre-dose urine collections from children in Peru, Zambia, and the United States (US).[14] However, few studies to date have employed this relatively new timing protocol.

To evaluate whether hospital discharge may be a useful delivery point for ED focused interventions, we aimed to determine whether children being discharged from hospital have significant permeability, and if so, the factors associated with permeability among these children. Specifically, we compared LRR scores of children at hospital discharge to age-matched peers from their community, and we identified the factors associated with observed differences between these groups. We also assessed the performance of a 60 and 120-minute urine collection protocol for LRR testing in hospital and community settings in LMICs.

Methods

Parent study: Children enrolled in the Childhood Acute Illness and Nutrition (CHAIN) Cohort study at two sites, the Civil Hospital Karachi, Pakistan (March 2018 to September 2019) and the Migori County Referral Hospital, Kenya (December 2017 to October 2019), were eligible for inclusion in this sub-study. The full CHAIN protocol has been published previously.[15] Migori County Hospital is a rural district level referral facility in south-western Kenya. Migori County is highly malaria endemic and is among the highest HIV prevalence settings in Kenya. Approximately one-quarter of children under five in the county are stunted, but it has a relatively low prevalence of childhood wasting relative to national figures.[16] The Civil Hospital Karachi is a large urban teaching facility and national level pediatric referral facility. HIV and malaria infections are rare in Karachi, but over one-third of children under five are stunted and the prevalence of wasting is three times higher than in Migori.[17]

All children aged 2-23 months being admitted to these hospitals were eligible for inclusion in CHAIN, if their primary reason for admission was not a traumatic injury or a condition that the enrolling clinician believed would require surgery in the next six months.[15] CHAIN recruitment commenced in November, 2016, and enrollment into this sub-study was initiated in December, 2017. After CHAIN completed its primary cohort enrollment in January, 2019, an identical protocol was initiated that recruited children between one week and 6 months of age. Children from this young infants cohort were also eligible for the LRR test until the sub-study end date of October, 2019. Enrollment was stratified by child mid-upper arm circumference (MUAC), so that an approximate ratio of two children with very low MUAC (<11.5cm if older than 5 months, otherwise <11 cm) or bipedal edema, and two with moderately low MUAC (≥11.5cm but <12.5cm

if older than 5 months, otherwise ≥11 cm but <12.0cm) were recruited for each child with a normal MUAC (≥12.5cm if older than 5 months, otherwise ≥12.0cm). Detailed clinical, anthropometric, and sociodemographic data were collected at admission and discharge, with clinical observations and management recorded on standardized case report forms daily during admission. At discharge from hospital, approximately one-in-three CHAIN participants were matched on age group (<6 months, 6-11 months, 12-23 months) to a child from their community by pseudo-random selection (3rd house to the north of the enrolled child's house). Community reference children were not eligible for recruitment if they had a history of acute illness in the 14 days prior. Children in the hospitalized cohort had follow-up at 45, 90 and 180 days after discharge, to record vital status and anthropometric changes. Community children had no longitudinal follow-up.

The LRR sub-study: Children enrolled in the CHAIN hospitalized cohort became eligible for this sub-study when they were judged medically stable (no respiratory distress, no supplemental oxygen requirement, and tolerating oral feeds). Children with diarrhea on the day of the LRR test were excluded, as lactulose may exacerbate diarrhea. Parents of eligible children provided additional informed consent prior to inclusion in this sub-study. Community children were also included in this sub-study if they were from the same community as the hospitalized children enrolled in this LRR sub-study.

The LRR test was conducted in the morning and caregivers were asked to fast (food, drink, and breastmilk) their child for one hour. At the beginning of this hour, a urine bag was attached to obtain a pre-dose urine sample. A 10ml oral solution containing 1500mg lactulose and 300mg L-rhamnose was administered at the end of the fasting hour and a new urine bag attached. Urine

passed in the first 20 minutes after sugar administration was discarded, but all urine passed during the subsequent two hours was collected, with a change in the urine bag at the start of the second hour. The caregiver was encouraged to breastfeed or give water to the child after administration of the sugar solution. Any contamination of the urine bag with stool, urine spillage, or failure to pass urine in the two-hour post-administration period were recorded and judged as a test failure. Failed tests were repeated after 24 hours if the caregivers were willing to repeat the test.

Urine collection was divided into two periods, 20-80 minutes and 80-140 minutes post-administration. Urine from each time period was aliquoted into 100 μl cryovials and stored at -80^{o}c. These aliquots were shipped to the Mayo Clinic (Rochester, Minnesota) for HPLC-MS. Failure to detect rhamnose in post-administration samples was considered an additional reason for test failure. Percentage of lactulose and rhamnose recovery was calculated for each post-administration time period, as was LRR. The cumulative LRR encompassing both time periods was calculated by deriving a mean concentration of lactulose and rhamnose weighted to the volume of urine passed in each post-administration period. Formulae for calculation of all these variables are included in supplement materials.

Statistical methods: The median and range of LRR and percentage excretions were summarized for hospitalized and community children at both sites. The parent study oversampled children classified as wasted at admission to hospital, therefore in this analysis we also compared each stratum of WHO defined wasting within the hospitalized cohort to community children using the Wilcoxon rank-sum test. These WHO strata were defined as severe acute malnutrition (SAM, weight-for-height z-score <-3, mid-upper arm circumference <11.5 cm if older than 5 months of

age, or edema), moderate acute malnutrition (MAM, weight-for-height z-score between -3 and <-2, or mid-upper arm circumference ≥11.5cm and <12.5 cm if older than 5 months) and no acute malnutrition (NAM). To add further context, these LRR scores were compared to the 95th percentile value derived from Faubion, *et al's* data on healthy children in the US.[14]

To understand which sugar was driving differences in LRR, percentage lactulose and rhamnose recovery percentages were compared between the community and hospitalized groups. The median and range of lactulose and rhamnose concentrations in urine samples collected prior to sugar administration were also calculated by site and group. Pre-dose sugar excretion (binary: detected/not detected) patterns were explored and tested for association with participant group (hospitalized/community), site, age (6 months or older/less than 6 months) and breastfeeding status (currently receiving any breastmilk/not, and exclusively breastfed/not) using Chi-square tests.

To understand whether demographic and socioeconomic factors confound observed differences in LRR between the community and hospitalized children, we used linear regression to test the association between site and log-transformed LRR, and then ran site-adjusted models using the same outcome and including a random effect for site for other potential confounders. LRR was log transformed for these models as ratios generally perform better in models after this transformation.[13] Prior to log transformation, LRR scores equal to zero were replaced with an extremely small, non-zero value (0.0001). Each confounder was tested in a forward stepwise fashion, and retained in the model if it reduced or increased the association between hospitalization and the LRR value by greater than 10%. Potential confounders included age (continuous), sex, any current breastfeeding (binary), currently exclusively breastfeeding (binary), weight-for-height z-

score (continuous), weight-for-age z-score (continuous), height-for-age z-score (continuous), improved household sanitation (binary) and improved household water source (binary). HIV infection was too infrequent to include as a potential confounder. Therefore, a sensitivity analysis which excluded HIV infected children was also performed. All continuous variables were tested for non-linearity by including quadratic terms in the models.

Finally, we tested potential correlates of LRR in both the community and hospitalized groups. Again, the association between site and log-LRR was tested in a linear regression, and then site-adjusted models were built for the remaining variables. Among the community children, potential covariates included site, age (continuous), sex, any current breastfeeding, current exclusive breastfeeding, recent antibiotic use (binary), weight-for-height z-score (continuous), weight-for-age z-score (continuous), height-for-age z-score (continuous), HIV infection (binary), HIV exposure without seroconversion (binary), any recorded chronic condition (binary), biological mother as primary caregiver (binary), caregiver education (binary- primary or less), caregiver body mass index (BMI: ordinal, low, normal, high), household livestock ownership (binary), improved water source (binary), improved sanitation (binary), food insecurity (high, medium, low). The same variables were also tested among the hospitalized cohort, with the addition of factors related to the acute illness, including binary variables for LRTI, diarrhea, malaria, and systemic inflammatory response syndrome (SIRS) at admission, and length of hospitalization as a continuous variable.

Results

We conducted 245 LRR tests, 155 among children being discharged from hospital and 90 among community children (Figure 1). The test failure rate was slightly higher among hospitalized (n=18, 12%) compared to community children (n=6, 7%). Fourteen (58%) tests failed because no post-dose urine was collected and the caregiver refused a repeated test, a further nine (38%) tests were excluded because no rhamnose was detected in post-dose urine and a final test was excluded for implausibly high recovery of lactulose. Among the 137 hospitalized and 84 community children with complete and valid test results, those from the community were more likely than the hospitalized cohort to be exclusively breastfed and to have a primary caregiver who was overweight/obese, and who were less likely to be wasted, HIV infected, have a chronic condition, or come from a household with high food insecurity (Table 1). The median age of the hospitalized cohort was nine months (range 2-23), as was the community's (median 9 months, range 2-23).

Pre- and Post-dose Lactulose and Rhamnose: Seventy children passed urine prior to administration of the sugars. Lactulose was detected in eight (22%) pre-dose urines from community and ten (27%) hospitalized children, although this difference was not statistically significant. Lactulose concentrations in pre-dose samples were substantially lower than in post-dose samples. The pre-dose median lactulose concentration among samples with rhamnose detected was 1.6 ug/ml, whereas this measure was 7.7 ug/ml in the first hour of post-dose collection and 18.0 ug/ml in the second. Among the 18 children with pre-dose lactulose detections, eight were considered significant contaminations (>3x the lower limit of detection), of which six were major contaminations (>10x the lower limit of detection).[18] Pre-dose urine samples from the Karachi site were significantly more likely to have lactulose detected (12/31, 39%) compared than those from Migori (6/39, 15%, p=0.03). Children over 5-months of age (p=0.004) and those not

currently receiving any breast milk (p=0.006) had a significantly higher probability of lactulose detection in pre-dose samples. There was evidence that lactulose detection was less common among exclusively breastfeeding children (p=0.050).

Rhamnose was detected in pre-dose urines of six (16%) community and three (9%) of hospitalized children, but there was no association between its detection and site, age, or breastfeeding status. Rhamnose concentrations in pre-dose samples were also substantially lower than the concentrations in post-dose samples. The pre-dose median rhamnose concentration among samples with rhamnose detected was 2.4 ug/ml, whereas this measure was 22.5 ug/ml in the first hour of post-dose collection and 63.0 ug/ml in the second. Among the nine children with pre-dose rhamnose detected, eight were classified as significant contaminations (>3x the lower limit of detection), and two of these met the criteria major contamination (>10x the lower limit of detection).[18]

Among 221 children with successful tests, 135 (61%) passed urine in the first hour after sugar solution administration, and 172 (78%) passed urine in the second hour. In both the community and hospitalized groups fractional rhamnose and lactulose recovery were lower in the first hour compared to the second (Table 2). Rhamnose recovery appeared to vary widely across site and time period (supplementary table 1), with the community group having a significantly higher rhamnose recovery compared to the hospitalized group (0.59 [0.00, 3.31] vs 0.36 [0.00, 5.67], p=0.007). Conversely, the distribution of lactulose recovery was narrower across the different settings, and while there did appear to be greater lactulose recovery in community, it was not

significantly different to the hospitalized population (0.03 [0.00, 1.11] vs 0.02 [0.00, 1.37], p= 0.100).

Community vs Hospital: Pooling data from both sites and using the cumulative post-dose LRR, crude LRR was significantly higher among hospitalized (median: 0.35, IQR: 0.21, 0.88) compared to community children (median: 0.27, IQR: 0.17-0.46, p=0.02, Table 2). After stratification of the hospitalized cohort by acute nutritional status, the LRR of all three nutritional strata appeared to be higher than that of the community. Compared to this community group, the hospitalized children with WHO defined severe wasting had a median LRR of 0.40 (IQR: 0.28-1.00, p=0.018), those with moderate wasting had a median of 0.34 (IQR: 0.22-0.87, p=0.088), while those with no wasting had a median LRR of 0.32 (IQR: 0.21-0.82, p=0.090). Forty (29%) hospitalized and ten (12%) community children had cumulative LRRs above the 95th percentile value previously observed among a group of healthy children from the US.[14] Only three (6%) community children in Migori had an LRR greater than US 95th percentile value, whereas seven (22%) community children in Karachi were above this reference value. Among hospitalized children, 30 (28%) children in Migori and 10 (34%) in Karachi exceeded this cut-off.

To better understand the difference between community and hospitalized children, models using the statistically optimal log transformed LRR were built. In univariate linear regression with a random effect for site, the hospitalized children had mean log LRR 0.45 (95% CI: 0.05-0.86, p=0.028) higher than the community population. After adjustment for weight-for-height, the mean difference in log LRR was 0.25 (95% CI: -0.20, 0.69, p=0.274). Age, sex, breast feeding status, weight-for-age, height-for-age, improved toilet type and improved water source did not further

confound the relationship between hospitalization and higher LRR scores. Exclusion of children with HIV infection did not meaningfully alter the univariate or multivariable result.

Factors associated with LRR: Among hospitalized children, the mean log LRR was 1.9 (95%CI: 0.5-3.2, p=0.007) higher among children with a recent antibiotic prescription (n=132) in comparison to those without (n=5). All five of hospitalized children who were not prescribed antibiotics were from the Migori site, were not severely malnourished and typically had admissions lasting 48-72 hours. Three of these children were admitted for the management of diarrhea. Age was also a correlate of LRR among the hospitalized children, with each additional month of age being associated with a 0.2 (95%CI: 0.0, 0.4, p=0.033) increase in LRR. Age had a non-linear association with LRR, and therefore a quadratic model was used for this estimate. Conversely, one standard deviation increase in height-for-age z-score was associated with a -0.1 (95%CI: -0.2, -0.0, p=0.070) lower mean log LRR score. No other child, caregiver or environmental correlates were associated with LRR.

In the community group, children with a chronic condition (n= 3) had mean log LRR -3.3 (95%CI: -4.9, -1.7, p<0.001) lower than children without one of these conditions (n=81). All three of these children had hemoglobinopathies. Similarly, a one standard deviation increase in weight-for-height z-score among the community children was associated with a -0.3 (95%CI: -0.5, -0.1, p=0.018) lower mean log LRR score. The other factors were not associated with LRR in the community group.

Discussion

Children being discharged from hospital had a higher degree of enteric permeability than their peers in the community, but our data suggest that much of this association was confounded by the high prevalence of wasting among hospitalized children. Nutritional status has been associated with enteropathy in multiple previous studies across diverse settings, including both community and hospitalized children,[11,19,20] and wasting is a known risk factor for poor post-discharge outcomes even in settings where adherence to the WHO management guidelines is high.[5,6,21,22] It is possible that the relationship between poor anthropometric measurements at discharge and adverse post-discharge outcomes, may be mediated by enteric barrier dysfunction, which suggests it may be a viable target for interventions aiming to improve the health of malnourished children recovering from acute illness.

Despite the importance of poor nutritional status in the association between hospitalization and elevated LRR scores, we also found some evidence that hospitalized children without wasting had higher LRR scores than their community peers. Our data did not indicate that enteric permeability was associated with any of the common acute syndromes, such as pneumonia and malaria, but it is possible that pre-illness enteric leakiness may predispose children to become severely unwell when exposed to a broad range of disease challenges. We were surprised that recently resolved diarrhea among the children being discharged from hospital was not associated with increased enteric permeability. This variable may have been too broadly defined to capture the relationship between symptomatic enteric infection and enteric permeability, as it collapses both children with a primary diagnosis of gastroenteritis and those with diarrhea as a symptom of another primary presenting complaint. This variable will also have captured multiple viral, protozoal and bacterial pathogens which may have different effects on enteric permeability.[12] Given that children in LMIC

settings are estimated to have 3.8 episodes of diarrhea in their first two years of life,[23] a richer understanding of the etiologies and severities of diarrhea that are associated with increased enteric permeability may aid in efforts to reduce enteric barrier dysfunction among these children.

Receipt of antibiotics among hospitalized children, and a reported diagnosis of a chronic conditions among the community participants, were associated with higher LRR scores in our data. In both cases, this association was driven by a very small number of children with notably low LRR scores. Almost all children in our hospitalized cohort received antibiotics, and the few that did not were typically short admissions for intravenous rehydration. Given that there was no association between antibiotics and LRR in the community, it seems likely that observed relationship among hospitalized children was confounded by the indication for antibiotics. In other words, this association may have been attributable to the relatively rare presenting compliant of severe dehydration requiring admission, but without any sign suggesting another complicating co-morbidity. Similarly, for community children with chronic conditions, which were largely sickle cell and thalassemia, it seems unlikely that these diseases are causally associated with better enteric permeability. However, there may have been a selection bias among the families of these children, whereby only higher socioeconomic families or those who were very engaged with their medical providers were interested in consenting to our study, or perhaps reporting bias which could have influenced a caregiver's willingness to disclose their child's condition.

Across both the hospitalized and community groups, there was a consistent difference in the LRR between urban Karachi and rural Migori. Many authors have suggested that some degree of ED is nearly ubiquitous in LMIC communities,[24] but in this study, the range of LRR values among the

Migori community children were very similar to those found in a US-based cohort.[14] Our community groups were similar in age to the US cohort, and like the US cohort, did not have a history of recent acute illness. If acute illnesses are a key driver of ED in the Migori community and are more prevalent in the general population of Migori than the reference population in the US, this exclusion criterion would bias the Migori community estimate by making it more comparable to the US norm than a representative sample would have been. Alternatively, the rural Migori population, which does have a lower prevalence of stunting than Karachi, may truly have a healthier enteric barrier than we expected.

We observed a range of LRR scores in the both hospitalized and community groups that were similar to a recently published study from Zambia and Peru that implemented a similar protocol among a community-based cohort,[14] including minimal detection of rhamnose in urine collected prior to administration of the sugar solution. Due to difficulties in obtaining urine in the two-hour window among hospitalized children, we repeated several tests 24-hours after the initial attempted test. Urinary clearance of lactulose and rhamnose can persist up to 24-hours after sugar administration,[25,26] which suggests that some of the pre-administration detections may be attributable to the repeated tests, although sugar concentrations of pre-dose samples were minimal. We detected lactulose more often than rhamnose in pre-dose samples. We also noted that lactulose was more likely to be detected in the pre-dose urine of older children and children who were not breastfeeding. While this finding may be also be an artifact of repeated tests, older children or those who were not breastfeeding may have been less likely to void in the post-dose collection period compared to younger or breastfed children. However, lactulose is used to treat constipation, and it is also used by the food industry as an inexpensive, stable and safe prebiotic in infant and

toddler food products, suggesting there may be a plausible relationship between age and breast feeding status and detections of pre-dose lactulose,[27,28] although the extent to which these lactulose containing products are accessible to families in Migori and Karachi is unclear.

This study had several limitations. Children with acute illness were excluded from the community group, and therefore we were unable to quantify the extent to which acute illnesses accounted for the difference between community and hospitalized children. Multiple studies have linked enteric pathogens to increased enteric inflammation and permeability, suggesting that more detailed analysis of enteric pathogen carriage may further illuminate the associations between hospitalization and high LRR scores. The LRR is a heterogenous test and this may have led to falsely dismissing some determinants of LRR, particularly when stratifying by participant group. The LRR test also often has test failure in the 10-15% range,[13,14] and this may introduce some selection bias into the results. Our test failure rates were comparable to other LRR test studies, and only minimally different between the hospitalized and community children, but some degree of bias may still be present in our findings.

Conclusion:

Enteric permeability is increased among many children being discharged from hospital. In comparison with their peers in the community, hospitalized children are more likely to be undernourished, which may partially explain the difference in enteric function between these populations. Children with wasting and stunting are known to be at a high risk of mortality in the three months following presentation.[21,29] It is possible that poor enteric barrier function may contribute to the sustained risk of morbidity and mortality these children are subject to in the post-

discharge period, and it may be an important interventional target to prevent adverse outcomes among these children.

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Figure 1: Study flow chart

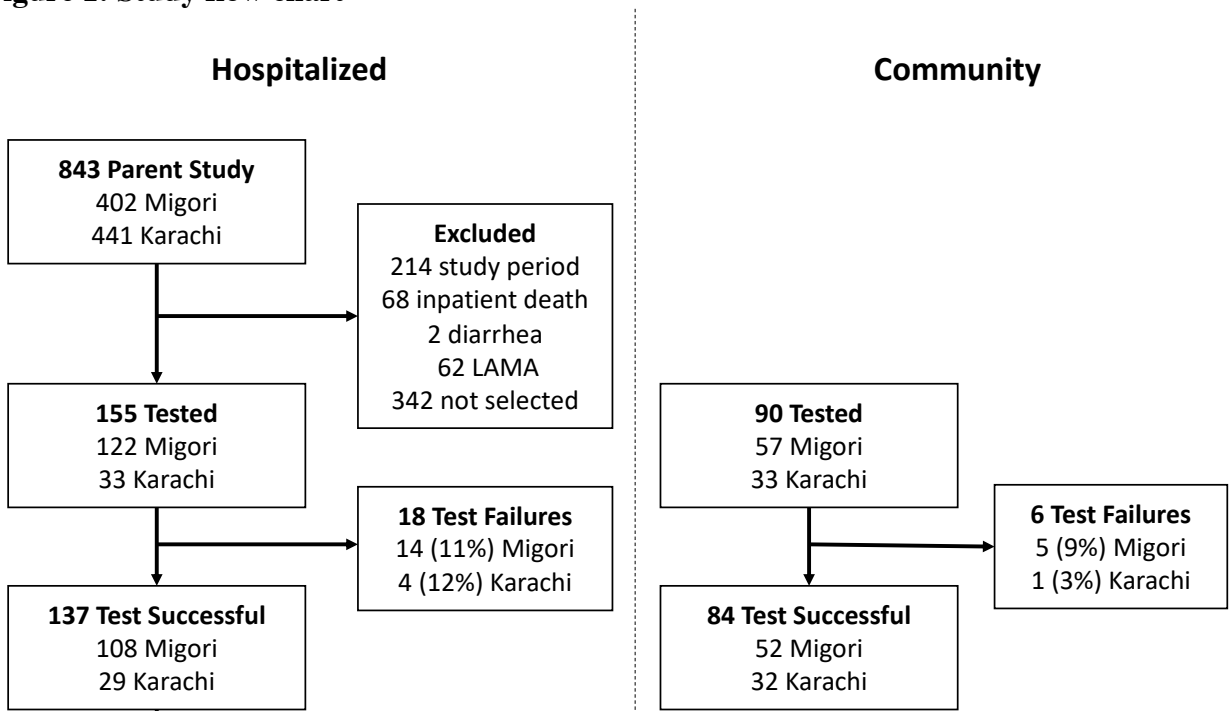


Table 1: Participant Characteristics

	Hospital Discharge N= 137		Community N= 84	
	n	(%)	n	(%)
Child				
Hospital				
Migori	108	79	52	62
Karachi	29	21	32	38
Age in months				
<6	39	28	20	24
6-11	43	31	28	33
12-23	55	40	36	43
Sex (male)	90	66	49	58
Currently breast-feeding	90	66	65	77
Currently exclusively breastfed	35	26	51	61
Length of Stay[1]				
<48hrs	25	18	--	
48hrs-5 days	57	42	--	
>5 days	55	40	--	
Recent Antibiotics	132	96	14	17
Stunted	20	15	15	18
Wasted[1]	31	23	9	11
Discharge diagnosis				
Diarrhea[2]	55	40	--	
Malaria[3]	29	21	1	1
LRTI[2]	64	47	--	
SIRS at admission[2,4]	44	32	--	
HIV status				
Unexposed	114	83	70	83
Exposed, uninfected	16	12	13	15
Infected	7	5	1	1
Chronic Condition[5]	13	9	3	4
Caregiver				
Biological mother is primary caregiver	126	92	79	94
Caregiver education[4]				
None	17	12	16	19
Primary	79	58	44	52
Secondary	35	26	22	26
Body Mass Index				
Underweight (<20)	19	14	5	6
Normal (20-25)	92	67	51	61
Overweight (>25)	23	17	26	31
Household				
Livestock ownership[4]	80	58	47	56
Improved water source[4]	93	68	60	71
Improved toilet[4]	67	49	44	52
Food insecurity				
Low	51	37	31	37
Moderate	57	42	42	50
High	29	21	11	13

[1]WHO definition of moderate/severe wasting: WHZ<-2, edema, MUAC<12.5cm. [2]Not assessed for children in the community. [3]Malaria RDT positive. [4]Missing data: SIRS-5 hospitalized;

Caregiver education – 6 hospitalized, 2 community; Livestock – 5 hospitalized; Water source – 6 hospitalized; Toilet type – 5 hospitalized. [5]Chronic conditions as reported by caregivers: 5 sickle cell disease, 11 thalassemia.
Abbreviations: LRTI: Lower Respiratory Tract Infection, SIRS: Systemic Inflammatory Response Syndrome.

Table 2: Median lactulose rhamnose values for the children leaving the hospital and their community peers across the test time periods.

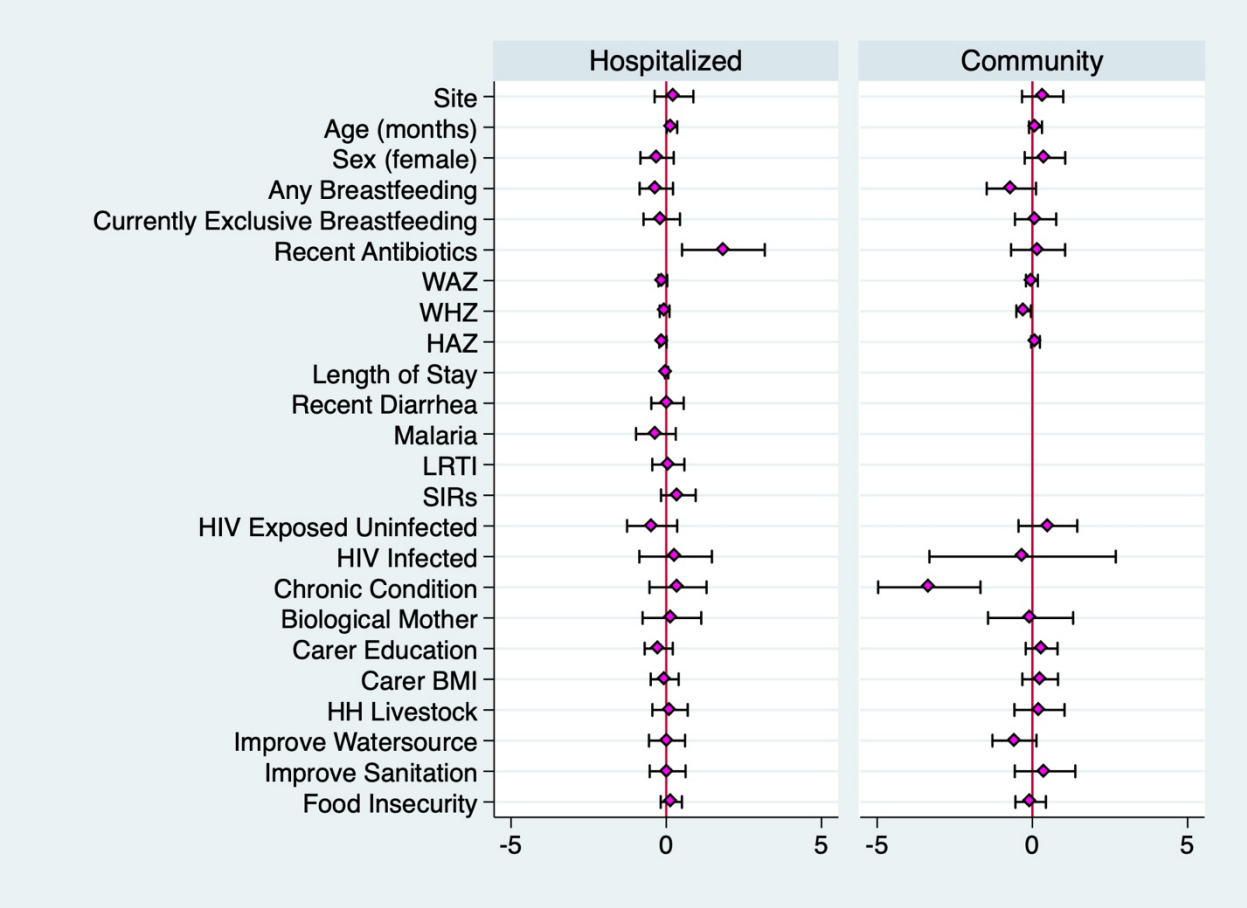
	Hospitalized Median (Range)		**Community** Median (Range)	
Pre-dose	n= 33		n= 37	
Rhamnose (ug/mL)	0.00	(0.00, 2.40)	0.00	(0.00, 7.90)
Lactulose (ug/mL)	0.00	(0.00, 51.00)	0.00	(0.00, 4.70)
Post-dose (1st hour)	n=84		n=51	
Rhamnose recovery (%)[1]	0.10	(0.00, 1.39)	0.22	(0.00, 2.33)
Lactulose recovery (%)	0.01	(0.00, 1.37)	0.01	(0.00, 1.11)
LRR	0.40	(0.00, 8.33)	0.25	(0.00, 4.75)
Post-dose (2nd hour)	n=100		n=72	
Rhamnose recovery (%)[1]	0.38	(0.00, 5.76)	0.51	(0.00, 3.31)
Lactulose recovery (%)	0.02	(0.00, 0.90)	0.03	(0.00, 0.29)
LRR	0.30	(0.04, 4.55)	0.26	(0.00, 2.81)
Post-dose (Cumulative)[2]	n=137		n=84	
Rhamnose recovery (%)	0.36	(0.00, 5.76)	0.59	(0.00, 4.02)
Lactulose recovery (%)	0.02	(0.00, 1.37)	0.03	(0.00, 1.11)
LRR	0.35	(0.00, 8.33)	0.27	(0.00, 4.75)
Above USA 95th percentile	29%	-- --	12%	-- --

[1]Lowest rhamnose values are greater than 0, but rounded down.

[2]Cumulative fractional rhamnose and lactulose are calculated by adding the fractional recovery from both time periods if a child passed urine in both periods. The cumulative LR ratio is the mean concentration of lactulose recovered in both periods, weighted by the volume of urine recovered in that period, over a similarly weighted mean of concentration of rhamnose.

Abbreviations: LRR – lactulose-rhamnose ratio.

Figure 2: Determinants of LRR among hospitalized and community children.



Estimates and confidence interval values are given in supplemental table 2.
Site: Karachi (1) vs Migori (0)

Supplementary Table 1: Median LR values

	Migori[1]				Karachi[1]			
	Hospitalized		**Community**		**Hospitalized**		**Community**	
	Median	(Range)	Median	(Range)	Median	(Range)	Median	(Range)
Pre-dose	n= 17		n=22		n= 16		n=15	
Rhamnose (ug/mL)	0.00	(0.00, 1.50)	0.00	(0.00, 7.20)	0.00	(0.00, 2.40)	0.00	(0.00, 7.90)
Lactulose (ug/mL)	0.00	(0.00, 51.00)	0.00	(0.00, 1.90)	0.00	(0.00, 10.00)	0.00	(0.00, 4.70)
Post-dose (1st hour)	n=59		n=31		n=25		n=20	
Rhamnose recovery (%)	0.15	(0.00, 1.39)	0.16	(0.00, 1.85)	0.08	(0.00, 0.83)	0.32	(0.00, 2.33)
Lactulose recovery (%)	0.01	(0.00, 1.37)	0.01	(0.00, 1.11)	0.01	(0.00, 0.08)	0.01	(0.00, 0.10)
LR ratio	0.38	(0.00, 6.13)	0.25	(0.00, 4.75)	0.54	(0.07, 8.33)	0.23	(0.09, 1.80)
Post-dose (2nd hour)	n=79		n=44		n=21		n=28	
Rhamnose recovery (%)	0.37	(0.00, 5.76)	0.56	(0.00, 3.31)	0.44	(0.03, 1.55)	0.35	(0.00, 1.37)
Lactulose recovery (%)	0.02	(0.00, 0.90)	0.03	(0.00, 0.29)	0.03	(0.00, 0.28)	0.02	(0.00, 0.14)
LR ratio	0.30	(0.00, 4.55)	0.23	(0.05, 0.56)	0.27	(0.07, 3.88)	0.36	(0.00, 2.81)
Post-dose (Cumulative)[2]	n=108		n=52		n=29		n=32	
Rhamnose recovery (%)	0.32	(0.00, 5.76)	0.71	(0.00, 4.02)	0.46	(0.00, 1.83)	0.40	(0.00, 2.84)
Lactulose recovery (%)	0.02	(0.00, 1.37)	0.03	(0.00, 1.11)	0.03	(0.00, 0.31)	0.03	(0.00, 0.23)
LR ratio	0.34	(0.00, 6.13)	0.24	(0.00, 4.75)	0.41	(0.07, 8.33)	0.39	(0.00, 2.81)
Above USA 95th percentile	28%	-- --	6%	-- --	34%	-- --	22%	-- --

[1]Note: in smaller n cells, such as the Karachi results in this table, the median does not adequately describe the distribution of values, and so it cannot be assumed that changes in median lactulose and rhamnose reflect the changes median LR ratio.

[2]Cumulative fractional rhamnose and lactulose are calculated by addition of the fractional recovery from both time periods if a child passed urine in both periods. The cumulative LR ratio is the mean concentration of lactulose recovered in both periods, weighted by the volume of urine recovered in that period, over a similarly weighted mean of concentration of rhamnose.

Supplementary formulae for calculation of sugar recovery and LRR:

Lactulose or rhamnose recovery in each timepoint:

$$Sugar\ recovery\ in\ any\ timepoint = {[Sugar] * volume\ of\ urine}\Big/{Dose\ of\ Sugar\ administered}$$

Overall:

$$Sugar\ recovery\ overall\ = Sugar\ recovery_{t1} + \ Sugar\ recovery_{t2} +$$

Lactulose:Rhamnose ratio for each time period

$$LRR_{tx} = {[Lactulose]_{tx}}\Big/{[Rhamnose]_{tx}}$$

Overall:

$$LRR_{overall} = \frac{\left([L]_{t1} \times {Vol.urine_{t1}}/{total\ urine}\right) + \left([L]_{t2} \times {Vol.urine_{t2}}/{total\ urine}\right)}{\left([R]_{t1} \times {Vol.urine_{t1}}/{total\ urine}\right) + \left([R]_{t2} \times {Vol.urine_{t2}}/{total\ urine}\right)}$$

Supplementary Table 2: mean difference in log LRR.

	Hospital		Community	
	Coef	**(95% CI)**	**Coef**	**(95% CI)**
CHILD				
Site[1]	0.2	(-0.4, 0.8)	0.3	(-0.3, 1.0)
Age	0.2	(0.0, 0.4)	0.1	(-0.1, 0.3)
Sex	-0.4	(-0.9, 0.2)	0.4	(-0.2, 1.1)
Any current breastfeeding	-0.4	(-0.9, 0.1)	-0.7	(-1.5, 0.1)
Current Exclusive breastfeeding	-0.2	(-0.8, 0.4)	0.1	(-0.6, 0.8)
Length of stay	0.0	(-0.1, 0.1)		
Recent antibiotics	1.9	(0.5, 3.2)	0.2	(-0.7, 1.0)
HAZ	-0.1	(-0.2, 0.0)	0.1	(-0.0, 0.2)
WAZ	-0.1	(-0.3, 0.0)	0.0	(-0.2, 0.2)
WHZ	-0.1	(-0.2, 0.1)	-0.3	(-0.5, -0.1)
Diagnosis				
Diarrhea	0.1	(-0.4, 0.6)		
Malaria	-0.4	(-1.0, 0.3)		
LRTI	0.0	(-0.5, 0.5)		
SIRS at admission	0.3	(-0.2, 0.9)		
HIV				
Exposed uninfected	-0.2	(-1.0, 0.6)	0.5	(-0.4, 1.4)
Infected	0.3	(-0.9, 1.4)	-0.3	(-3.3, 2.6)
Chronic conditions[2]	0.4	(-0.6, 1.3)	-3.3	(-4.9, -1.7)
CAREGIVER				
Biological mother	0.2	(-0.7, 1.2)	-0.1	(-1.4, 1.3)
Education[3]	-0.3	(-0.8, 0.1)	0.3	(-0.2, 0.8)
Body mass index[4]	-0.1	(-0.6, 0.3)	0.3	(-0.3, 0.8)
HOUSEHOLD				
Livestock ownership	0.0	(-0.6, 0.6)	0.2	(-0.6, 1.0)
Improved water source	0.1	(-0.5, 0.7)	-0.6	(-1.3, 0.1)
Improved toilet type	0.1	(-0.5, 0.7)	0.4	(-0.6, 1.4)
Food insecurity	0.2	(-0.2, 0.5)	-0.1	(-0.5, 0.4)

[1]Karachi (1) vs Migori (0) in a linear regression model, all other models are linear regression adjusted for site. [2]Including sickle cell, thalassemia, and congenital heart disease. [3]Educations coding: 0 Less than primary, 1 primary, 2 some secondary or more. [4]BMI coding: -1 underweight, 0 normal weight, 1 obese.

Chapter Two

Title: Enteric barrier function, systemic inflammation, and post-discharge growth among children in Kenya and Pakistan

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Summary:

Background: Children recently discharged from hospital are at higher risk of adverse health outcomes, including growth failure and mortality, than similarly aged children in the community. Both of these outcomes may be mediated through systemic inflammation. This analysis assesses whether enteric permeability may be an underlying cause of the adverse events these children experience, by testing its association with post-discharge growth and systemic inflammation.

Methods: Children aged 2-23 months being discharged from Civil Hospital Karachi (Pakistan) and Migori County Referral Hospital (Kenya) underwent the lactulose rhamnose ratio (LRR) test to assess enteric permeability. Similarly aged children from the communities served by these two hospitals also took the test. Post-discharge growth data were available for the hospitalized children, and

associations between their LRR score at discharge and subsequent changes in height-for-age (HAZ) and weight-for-age z-score (WAZ) were estimated using linear mixed effect models. Blood plasma samples from both groups (children at discharge from hospital and their community peers) were sent for analysis of four biomarkers of systemic inflammation (CD14, CRP, TNFα, IL-6) and one biomarker of intestinal enterocyte damage (IFABP). Linear regression was used to test if the association between LRR and these biomarkers was consistent across the hospitalized and community groups.

Results: LRR results from 137 children being discharged from hospital and 84 community participants were included in this analysis. LRR at discharge was not associated with changes in WAZ or HAZ in any post-discharge time period. The mean concentration of CRP, TNFα, IL-6 and IFABP were all higher among children at hospital discharge compared to the community children. There was some evidence that LRR had a different relationship to CRP (p=0.044), TNFα (p=0.027), CD14 (p=0.078), and IL-6 (p=0.171) among the hospitalized as compared to the community children. Among the community group, effect estimates suggested that increased LRR was associated with a 0.26 (95%CI: 0.04, 0.48) standard deviation increase in TNFα, and non-significantly associated with a 0.18 (95%CI: -0.04, 0.41) standard deviation increase in CD14, a 0.19 (95%CI: -0.03, 0.42) standard deviation increase in IL-6. Conversely, in the hospital group there was no relationship between LRR and TNFα (-0.01 standard deviations, 95%CI: -0.17, 0.15), CD14 (-0.03 standard deviations, 95%CI: -0.20, 0.13), or IL-

6 (0.02 standard deviations, 95%CI: -0.14, 0.19). LRR was not associated with CRP in the community (0.10 standard deviations, 95%CI: -0.13, 0.32), but higher LRR had a non-significant association with a 0.16 standard deviations lower CRP among the hospitalized children (95%CI: -0.32, 0.01). There was no evidence that the relationship between LRR and IFABP was different in the two groups. Pooling these groups, we found a non-significant association between higher LRR and elevated I-FABP (0.15 standard deviations, 95%CI: 0.00, 0.3, p=0.054).

Conclusions: These findings support prior data indicating enteric permeability is associated with enteric damage and systemic inflammation among relatively healthy children in community settings. However, they also suggest enteric permeability may not be an important determinant of systemic inflammation or post-discharge growth failure among children recovering from acute illness.

Introduction

Children under five years of age discharged from hospitals following admission for acute illness in low-and-middle income countries remain at high risk of poor health outcomes for the subsequent 6-12 months.[1–3] These children are eight times more likely to die during this period than their peers in the community.[2] There is also a high incidence of both ponderal and linear growth failure in the post-discharge period, measured by trajectories in weight for age and height for age respectively.[4,5] Growth failure is associated with life-long increased risk of morbidity, including poor cognitive development and non-communicable diseases, as well as mortality.[6,7]

Enteric dysfunction (ED) is increasingly understood as an important determinant of child growth[7–9] and is characterized by blunting of intestinal villus and loss of enteric barrier function. ED may be caused by multiple etiologies including frequent exposure to a contaminated environment (termed environmental enteric dysfunction), or specific diseases such as HIV, wasting, zinc deficiency, celiac disease, or inflammatory bowel disease.[9–11] The relationship between ED and adverse child outcomes is thought to be mediated through both chronic systemic inflammation and reduced absorption (Figure 1).[8,12] Gut permeability can facilitate translocation of pathogens and/or pathogenic products, potentially leading to systemic inflammation, immune activation, and growth failure.[7,13] While this mechanism is increasingly well understood among asymptomatic children in the community, little is known about the role of enteric permeability in recovery from acute illness. The first paper in this series showed children recovering from acute illness to have significantly worse nutritional status at discharge from hospital than children in the community, a high prevalence of poor nutritional status, and a high prevalence of enteric permeability as measured by LRR. As a result, gut leakiness may also be an important determinant of growth failure in the post-discharge period. In addition, healthcare visits, including hospitalization, may be effective timepoints to deliver ED targeted therapeutics to children at high risk who have access to the healthcare system.

We evaluated the association of enteric permeability, as measured by the lactulose rhamnose ratio (LRR) test, with ponderal and linear growth among children being discharged from hospital after recovery from acute illness. We also assessed the association between four plasma biomarkers of systemic inflammation and one plasma biomarker of intestinal enterocyte damage on growth in

the post-discharge period. Finally, we compared children leaving hospital to age matched children in the community, to better understand if LRR had the same relationship with these plasma biomarkers in the two groups.

Methods

Parent study enrollment and follow-up

Children enrolled in the Childhood Acute Illness and Nutrition (CHAIN) Cohort study at the Civil Hospital Karachi (March 2018 to September 2019) and the Migori County Referral Hospital (December 2017 to October 2019) sites were eligible for inclusion in this sub-study. The full protocol of this study has been published previously.[14] These two sites represent distinct geographic, social, cultural, and epidemiological settings. Migori County Referral Hospital is a rural district level referral facility in south-western Kenya. Migori County is highly malaria endemic and has an HIV prevalence among the highest in Kenya. In addition, approximately one-quarter of children under five years of age are stunted in Migori.[15] Civil Hospital Karachi is a large urban teaching facility and national level pediatric referral facility in Pakistan. HIV and malaria infections are rare in Karachi but over one-third of children under five are stunted and the prevalence of wasting is three times higher than Migori.[16]

All children aged 2-23 months being admitted to these hospitals were eligible for inclusion in the CHAIN parent study if their primary reason for admission was not a traumatic injury or a condition that the enrolling clinician believed would require surgery in the next six months.[14] Enrollment was stratified by child mid-upper arm circumference (MUAC), so that approximately two children with very low MUAC (<11.5cm if older than 5 months, otherwise <11 cm) or bipedal edema, and

two with moderately low MUAC (≥11.5cm but <12.5cm if older than 5 months, otherwise ≥11 cm but <12.0cm) were recruited for each child with a normal MUAC (≥12.5cm if older than 5 months, otherwise ≥12.0cm). Detailed clinical, anthropometric, and sociodemographic data were collected at admission and discharge in addition to blood samples. Clinical observations and management were recorded on standardized case report forms daily during admission. Home environment characteristics were assessed at home visits at discharge. Children were followed-up at 45, 90 and 180 days after discharge to record vital status and anthropometric changes.

For one-third of those discharged home, community reference participants were recruited from households near the hospitalized children's homes using a pseudo-random selection method (3rd house to the north of the enrolled child's house). Community children in the same age bracket as the index hospitalized child (<6 months, 6-11 months, 12-23 months) were recruited if they had no history of acute illness in the 14 days prior and if their caregiver consented to participation. If the selected household did not have an eligible child, the next household continuing north would be selected. Community participants were invited to the clinic for the evaluation, including demographics, medical history and examination, socioeconomic assessment, anthropometry and blood sample collection, using the same methods as in the hospitalized children.

Sub-study enrollment

Children enrolled in the CHAIN hospitalized cohort became eligible for this sub-study when they were judged medically stable (no respiratory distress, not requiring supplemental oxygen, and nutritional intake was by oral route). The first three eligible children each week were selected for participation. This enrollment cap facilitated accurate implementation of the LRR test. Additional

informed consent was obtained prior to inclusion in this sub-study. Both hospitalized and community children with diarrhea on the day of the LRR test were excluded, as lactulose may exacerbate diarrhea.

The lactulose-rhamnose ratio test

The LRR test was conducted in the morning and caregivers were asked to fast (food, drink, and breastmilk) their child for one hour. At the beginning of this hour, an adhesive urine bag was attached, to obtain a pre-dose urine sample. A 10ml oral solution containing 1500mg lactulose and 300mg L-rhamnose was administered at the end of the fasting hour and a new urine bag attached. Urine passed in the first 20 minutes after sugar administration was discarded and all urine passed during the subsequent two hours was collected. The caregiver was encouraged to breast feed or give water to the child after administration of the sugar solution. Contamination of the urine bag with stool, urine leakage, or failure to void in the two-hour post-administration period were recorded as a test failure. Failed tests were repeated after 24 hours if the caregivers were willing.

Urine samples from each time period (20-80 minutes and 80-140 minutes post-sugar administration) were aliquoted into 100 μl cryovials and stored at -80^{o}c. These aliquots were shipped to the Mayo Clinic (Rochester, Minnesota) for high performance liquid chromatography mass spectrometry. Percentage of lactulose and rhamnose recovery was calculated for each post-administration time period, as was LRR. The cumulative LRR encompassing both time periods was calculated by deriving a mean concentration of lactulose and rhamnose weighted to the volume of urine passed in each post-administration period. In keeping with previous LRR analyses, failure to detect rhamnose in the post-administration sample was classified as a test failure.[17]

Plasma biomarkers

Blood samples, collected at discharge for the hospitalized children or enrollment for the community group, were processed in site laboratories within one hour of collection. Samples were spun in a refrigerated centrifuge into plasma and serum, stored and shipped at -80^{o}c. Resources permitted analysis of 175 plasma samples; all deaths and a random subset of the hospitalized and community groups were selected for this analysis. Overall, samples from 111 hospitalized and 64 community children participating in the LRR sub-study underwent plasma proteomic analysis. The proteomic approach was implemented by Somalogic, Inc using the SomaScan technology, and we selected five-biomarkers that have been previously associated with both poor growth and intestinal permeability, CD-14, CRP, IL-6 and TNFα (inflammatory) and IFABP (gut injury).[13]

Statistical methods

Children with discharge LRR results who survived to day 180 without being lost to follow-up were included in the growth analysis. Community children had no follow-up visits and so were not included in the growth analysis. The children's height and weight at each timepoint was converted to age standardized z-scores using WHO norms.[18] Change in height for-age z-score (ΔHAZ) and weight-for-age z-score (ΔWAZ) were chosen as the primary outcomes of interest. Weight-for-height z-score has been previously noted to compound measurement error and be statistically inferior to WAZ and HAZ.[19] MUAC is highly correlated with WAZ, and was therefore also not assessed.

Graphical comparisons of HAZ and WAZ across LRR scores were completed before running the primary models of LRR association with ΔHAZ and ΔWAZ. To compare HAZ and WAZ graphically across LRR scores, children were categorized using the 95th centile value of LRR scores (LRR >0.7) that was observed from among similar aged children in the USA.[17] The mean HAZ and WAZ at each of the four time points (discharge, day 45, day 90, day 180) was first calculated using the data collected on participants who attended at each timepoint. Then, to account for missing data and to understand the accuracy of the growth modelling, linear mixed effect models with random effects for patient, and including multiple imputation of missing data, were used to estimate the mean and 95% confidence intervals for HAZ and WAZ at each timepoint in the groups of children with LRR above and below the 95th centile value for LRR among similar aged children in the USA. Finally, these linear mixed effect models were adjusted for *a priori* identified potential confounders (listed below). All three approaches – the raw data, imputed models, and imputed-adjusted models -- were displayed graphically.

The primary growth models in this paper use linear mixed effect models with a random effect for patient to estimate the association between the continuous LRR score and ΔHAZ or ΔWAZ between the timepoints. The LRR scores were not normally distributed, so the natural log of LRR was used.[20] Some children missed one or more of their post-discharge follow-up visits, leading to missing anthropometric data. After exclusion of children who died and were lost to follow-up, all missing data was assumed to be missing at random and imputed using Markov chain Monte Carlo models. All imputations for HAZ, ΔHAZ, WAZ, ΔWAZ were based on site, age, sex, recent diarrhea, timepoint, and the baseline value for that anthropometric measure (i.e., HAZ or WAZ at discharge).

Crude models included continuous log transformed LRR, timepoint as a dummy variable, and interaction terms between timepoint and LRR to allow for different effects of LRR at discharge across the timepoints. Adjusted models included the crude model variables, and *a priori* identified confounders: patient age in months, sex, site of recruitment, recent diarrhea, HAZ at discharge, and WAZ at discharge. Finally, to understand if recent diarrhea history may be an effect modifier, this variable was removed from the adjusted model, the data were stratified into children who had diarrhea reported at admission to hospital, or on any day during their hospitalization, and those who did not, and the adjusted model was fit to both groups. To assess linearity, quadratic terms for LRR and age were tested for an association with the outcomes and found to be non-significant.

Several plasma biomarkers of systemic inflammation (CD-14, CRP, IL-6, TNFα), and a marker of intestinal damage (I-FABP), have been previously associated with enteric dysfunction.[13] We compared the association between LRR and these biomarkers in the hospitalized cohort to the associations observed among the community reference group, by including an interaction term between LRR and recruitment group (community vs hospital) in linear regression. The concentrations of these biomarkers were drawn from the aptamer-based proteomic dataset described above. These units are not readily convertible to clinical measures; consequently, all observed values were standardized by subtracting the mean biomarker concentration and dividing by the standard deviation of the whole sample. As with the primary models, the natural log of LRR was used as the primary predictor. These models were adjusted for patient age in months, sex, site of recruitment, baseline HAZ, and baseline WAZ. Diarrhea status was not included as children with acute illness in the last 14 days were excluded from the community group. Finally, we

assessed the relationship between the plasma biomarkers and post-discharge growth in crude linear mixed effect models, with interaction terms for timepoint. Due to the small sample size of this last analysis, we did not adjust these models for confounders.

All analyses were conducted in Stata version 14.0. Ethical approval for this study was obtained from the Aga Khan University, the Kenya Medical Research Institute, the Oxford University, and the University of Washington.

Results

At hospital discharge, 155 children were eligible for LRR testing and 137 (88%) tests were successfully conducted (Figure 2). Among the 18 unsuccessful tests, nine were due to failure to detect rhamnose in collected urine. A further eight were due to the child not voiding in the post-administration period and caregivers not consenting to a repeat test. One test was excluded due to an implausibly high lactulose reading (>13 standard deviations above the mean). Among the community children, 90 children were eligible for LRR testing, and 84 (93%) had successful tests. All the failed tests among community children were caused by the child not passing urine in the post-administration period and caregiver declining repeated testing.

The median age of hospitalized children was nine months (IQR: 5-14, Table 1), and median length of hospitalization was 4 days (IQR: 3-8). A history of diarrhea during the admission was reported in 55 (40%) children. Twenty-nine (21%) children had WHO defined moderate wasting (WHZ<-2 but ≥ -3, or MUAC <12.5 and 11.5 cm if >6months old), and an additional 30 (21%) were severely wasted WHZ< -3, or MUAC < 11.5 cm if >6months old, or edema). During follow-up,

five children died (4%) and five (4%) children were rehospitalized (one of whom died during rehospitalization). One (1%) child was lost-to-follow-up.

Lactulose-rhamnose ratio and outcomes among hospitalized children

The median LRR of included, hospitalized children was 0.34 (IQR 0.21-0.88). There was no difference in the LRR between the five (4%) children who died during follow-up (median: 0.34, IQR: 0.25-0.38) and those who survived (median: 0.36, IQR 0.20-0.96, p=0.510). There was also no evidence of higher LRR among children who were rehospitalized (median: 0.38, IQR 0.31-0.67) in comparison to those who survived without rehospitalization (median: 0.34, IQR: 0.20-0.96, p=0.620).

Among the 132 survivors, 426 (81%) of the 528 possible height assessments were collected during follow-up. Children with an LRR above the 95th centile value from the reference population had lower HAZ scores at discharge (-1.87 [IQR: -3.00, -0.78] vs -1.27 [IQR: -2.28, -0.11], p: 0.07, Figure 3). The mean HAZ of both groups of children declined substantially in the first 45 days after hospitalization and then continued to decline at slower rate for the duration of follow-up. These was no evidence of a difference in the HAZ trajectory between children above and below the LRR cutoff in the raw data, crude-imputed model, or adjusted-imputed model. This result was supported by linear mixed effect models of the continuous LRR on ΔHAZ, which also found no evidence of an association (Table 2). We repeated the ΔHAZ model in subpopulations of children with and without a history of recent of diarrhea and did not find evidence that either subpopulation differed from the primary results.

Of the 528 possible weight assessments, 427 (81%) were collected. Children with an LRR below the reference cutoff also had lower WAZ scores at discharge (-1.95 [IQR: -3.62, -0.71] vs -1.60 [IQR-2.57, -0.72], p: 0.277). Among children below the reference cutoff value, WAZ slowly increased across the follow-up period, with the largest gains between day 90 and 180. Children above the reference cutoff also had a net WAZ increase during follow-up but appeared to have greater gains between discharge and day 90. However, there was no significant association noted between the continuous LRR at discharge and ΔWAZ between timepoints. Again, we repeated the ΔWAZ model in subpopulations of children with and without a history of recent of diarrhea and found no evidence of a difference between these groups and the overall study population.

Lactulose-rhamnose ratio and plasma biomarkers

Among the 111 hospitalized and 64 community children in this sub-study selected for proteomics analysis, 91 (82%) in the hospitalized group and all 64 (100%) in the community had plasma collected at the correct timepoint and available for analysis. The demographic, child, caregiver, and household characteristics of these two groups are given in Appendix 1. The mean levels of CRP were 0.50 (95% CI: 0.18, 0.81; p=0.002) standard deviations higher among children leaving hospital in comparison to their peers in the community. Similarly, TNFα, IL-6, I-FABP were 0.67 (95% CI: 0.36, 0.97; p<0.001), 0.33 (95% CI: 0.01, 0.65; p=0.043), and 0.35 (95% CI: 0.04, 0.67; p=0.029) standard deviations higher among the children leaving hospital, respectively. There was also some evidence that CD14 (SD: 0.29; 95% CI: -0.03, 0.61; p=0.078) may be elevated among these children.

The association between LRR and CRP (p=0.044), CD14 (p=0.078), TNFα (p=0.027) all showed some evidence of a difference between the community and hospitalized groups (Table 3). Among the community group, a higher LRR was significantly associated with a 0.26 (95%CI: 0.04, 0.48, p=0.020) standard deviation increase in TNFα, but no association was found among the hospitalized cohort (coef: -0.01 standard deviation, 95%CI: -0.17, 0.15, p=0.927). LRR was estimated to be non-significantly associated with a 0.18 (95%CI: -0.04, 0.41, p=0.110) standard deviation increase in CD14 in the community group, but again, there was no evidence of an association among the hospitalized children (coef: -0.03 standard deviation, 95%CI: -0.20, 0.13, p=0.670). There may have been some evidence of an interaction between LRR and IL-6 (p=0.171). In the community group, a one unit increase in the LRR was associated with a non-significant 0.19 (95%CI: -0.03, 0.42, p=0.093) standard deviation increase in IL-6, but no evidence of an association seen among the hospitalized children (coef: 0.02 standard deviation, 95%CI: -0.14, 0.19, p=0.778). Conversely, there was no evidence that CRP was associated with the LRR in the community group (coef: 0.10 standard deviation, 95%CI: -0.13, 0.32, p=0.403), but there may have been some evidence that higher LRR was associated with lower CRP among the hospitalized children (coef: -0.16 standard deviation, 95%CI: -0.32, 0.01, p=0.061).

There was no evidence to suggest that the relationship between LRR and I-FABP was different among hospitalized and community children (p=0.595). Because there was no evidence of an interaction, a model without an interaction term was run for I-FABP, which found a non-significant association between higher LRR and elevated I-FABP (coef: 0.15 SD, 95%CI: 0.00, 0.3, p=0.054).

Plasma biomarkers and post-discharge growth

Of the hospitalized cohort, 87 children had both plasma biomarker and growth data available (four of the children who had plasma results passed away prior to growth data being collected). Among this subset of children, there was a trend toward a negative association between CD14, IL-6, TNFα, and I-FABP with linear-growth in the first 45-days following discharge (Figure 4). However, none of these trends were statistically significant, with only TNFα showing a borderline association (Appendix table 2, p=0.078). There was also a trend toward an association between CD14 and IL-6 and increased weight gain in the first 45 days, but again none of these trends achieved statistical significance. There was no association between biomarkers collected at discharge and either linear or ponderal growth in the 45-to-90 or 90-to-180-day periods.

Discussion

Children hospitalized in low-resources settings are particularly vulnerable to growth failure in the 6 months following discharge.[4,5] The mechanisms underlying this vulnerability are not well understood. Multiple community-based studies in LMICs have suggested that enteric dysfunction may cause malabsorption and chronic systemic inflammation which can lead to childhood growth failure.[8,21,22] Our analysis also found evidence to support a link between enteric permeability and systemic inflammation among children in the community, but we found no such relationship among hospitalized children. We also found no evidence that permeability at the point of discharge was associated with post-discharge growth, but among a modestly sized subset of hospitalized children with both plasma biomarker and growth data we did find trends suggesting systemic inflammation was associated with patterns of growth in the first 45 days following discharge.

These findings suggest that enteric permeability could be an important determinant of systemic inflammation and poor outcomes among children in the community, but it may have less bearing on the health of children recovering from an acute illness in whom systemic inflammation is likely to be driven by other mechanisms.

Chronic systemic inflammation, particularly as measured by TNFα, and IL-6, may be caused by either chronic enteric inflammation or translocation of antigens from the gut lumen into the circulation, and could play a key role in childhood growth failure among community children without acute illnesses. These biomarkers are known to mediate the association between chronic localized inflammation and poor childhood growth,[23,24] and the MAL-ED cohort also concluded there was good evidence to suggest systemic inflammation is linked to childhood growth.[8] Acute phase reactions also prompt expression of TNFα and IL-6, and in the context of a severe illness, it seems likely enteric permeability is not a primary driver of these cytokines. Our observation that permeability has a tangibly different relationship with biomarkers of chronic systemic inflammation among community children compared to hospitalized children may explain why we did not find permeability to have an effect on post-discharge growth.

Two recent studies assessing growth among children recovering from acute illness observed children appear to gain WAZ but not HAZ during the first 30 to 45 days.[4,5] Between discharge and 45-day follow-up, our study population also gained WAZ but lost HAZ, and our analysis of plasma biomarkers among the hospitalized children suggested TNFα and IL-6 may have been associated with HAZ declines, but increased WAZ in the first 45-days of the post-discharge period. This supports other studies suggesting that TNFα and IL-6 may be mediators or biomarkers of the

systemic inflammatory mechanisms that instigate childhood growth failure.[12,13] Further investigation is needed to validate this hypothesis and to see if it may also apply to other adverse health outcomes in the post-discharge period.

The association between LRR and I-FABP was not different among the community and hospitalized children, suggesting that the relationship between permeability and enterocyte damage was generalizable across the two groups. When combining these groups, we found a borderline association between LRR and I-FABP, lending support to other studies that have also found other biomarkers of ED to be associated with this plasma protein.[25,26] Enterocyte damage may be associated with malabsorption, reduced vaccine responsiveness, and it has been suggested that it may facilitate enteric-source post-discharge sepsis.[9,27] These mechanisms may operate independently of the permeability-systemic inflammation relationship and may warrant further investigation among children recovering from acute illness. It is also possible that permeability is a fluctuating condition such that LRR at discharge does not reflect permeability status for the duration of the follow-up period, and repeated testing during follow-up may have yielded a more accurate and nuanced picture of gut leakiness following acute illness. The multiple mechanisms that could link ED to child health outcomes independent of permeability, indicate that our findings should not be used to dismiss ED entirely as a post-discharge interventional target. Nevertheless, the lack of permeability association with systemic inflammatory markers at discharge should temper our enthusiasm for permeability (and perhaps ED) related interventions aimed at improving early post-discharge outcomes.

This study combined highly standardized LRR procedures, rich medical and social phenotyping of the tested children, and quantitative plasma proteomics. However, our approach did have several limitations, most notably, we only assessed LRR and plasma biomarkers at discharge. It is possible that the importance of permeability increases as the child progresses through the post-discharge period and the influence of the acute illness that caused the hospitalization wanes. However, the majority of adverse outcomes in the post-discharge period occur within the first 30 days,[28] including linear growth failure and death. The LRR is a heterogenous test, influenced by gastric emptying, intestinal motility, and the frequency of urinary voiding,[20] which is likely to introduce a degree of non-differential misclassification in our results. However, the medians and ranges of our LRR results; the association with known risk factors for enteric permeability, including diarrhea, age, and nutritional status observed in previous analyses; and LRR's relationship to systemic inflammation in the community group indicate LRR performed similarly in our study to previous work conducted by other researchers. The LRR, and closely related lactulose-mannitol ratio, are challenging tests to implement, but they remain the only widely implemented method of assessing enteric permeability. This analysis also included a relatively small number of children, and therefore was unable to draw meaningful conclusions about the association between LRR and death and/or hospital readmission. Finally, we also conducted quite a few hypothesis tests without adjusting for multiple comparisons, which should urge caution in interpreting the p-values presented in our result section. It is likely the analysis of LRR associations with plasma biomarkers would be most affected by this issue, and therefore warrants validation in other data.

Conclusion:

We found no evidence to support an association between LRR at discharge from hospital and linear and ponderal growth in the post-discharge period. Among children recovering from acute illness, LRR was not associated with biomarkers of systemic inflammation that have been previously associated with childhood growth. However, in a group of similarly aged, relatively healthy children in the community, LRR was associated with these plasma biomarkers. This data suggests we cannot readily generalize the findings of community cohorts to children recovering from acute illness, and further indicates interventions aiming to reduce enteric permeability may not prove effective in reducing adverse child health outcomes in the early post-discharge period.

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Figure 1: Proposed mechanisms linking enteric dysfunction to childhood growth

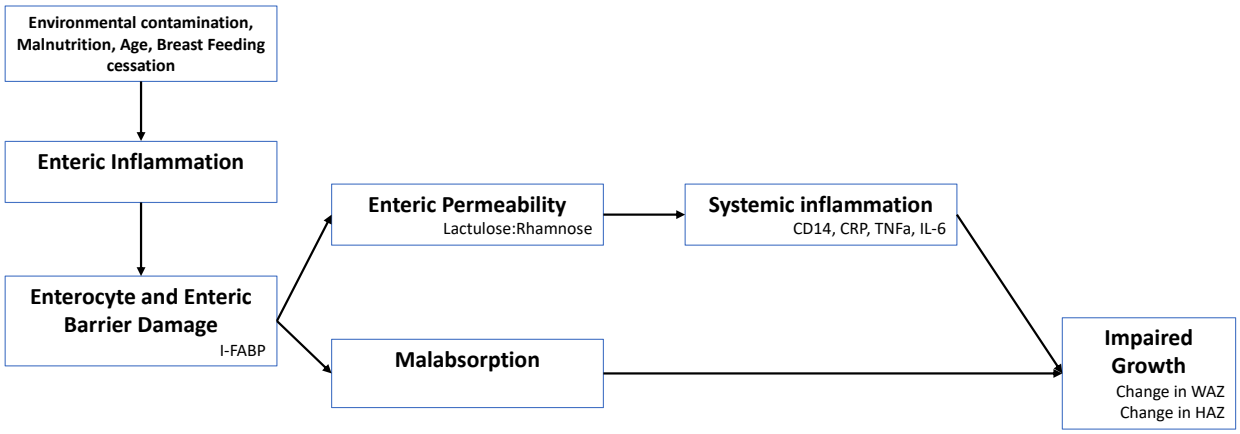
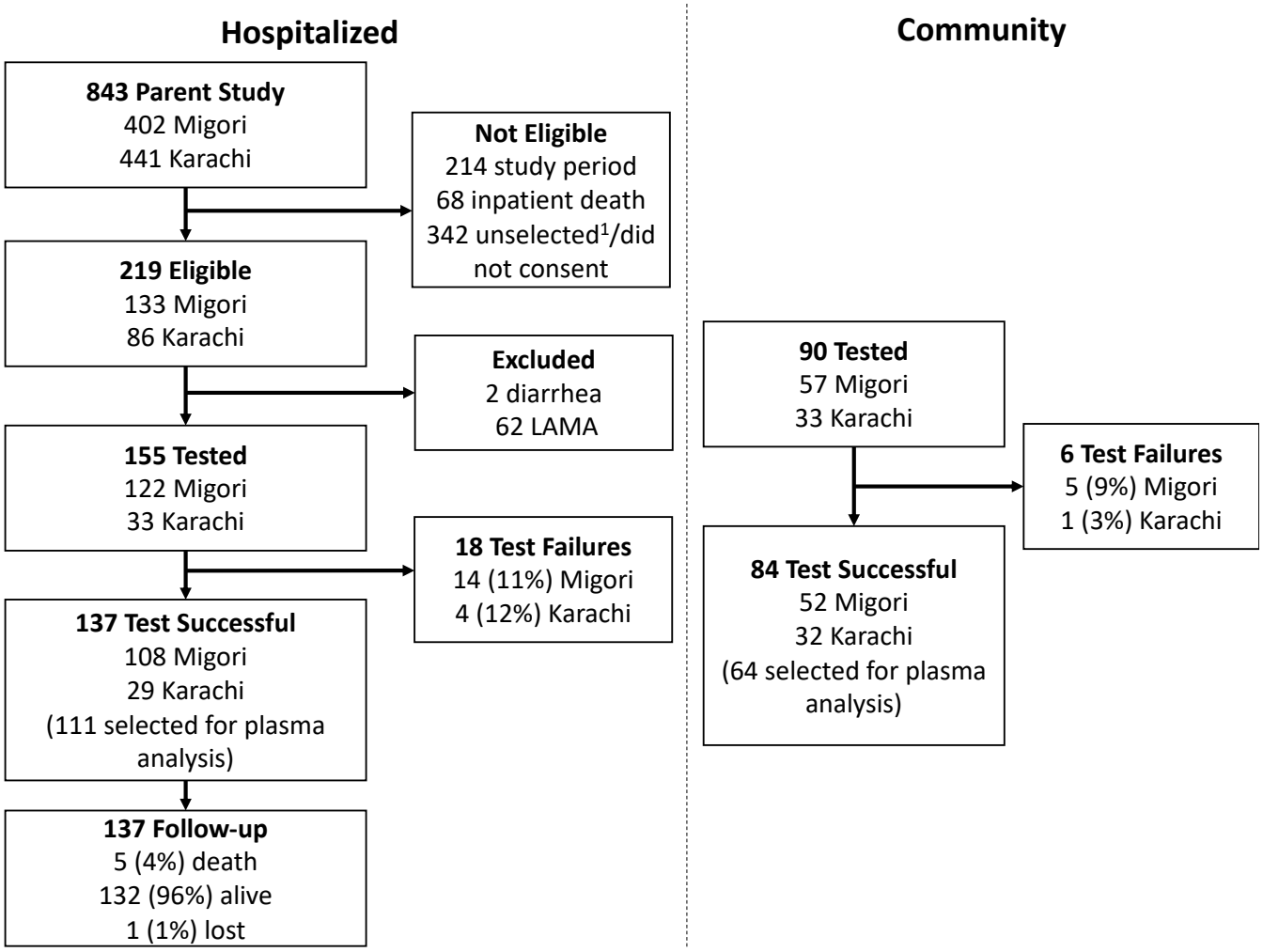


Figure 2: Flow of recruitment into the study.



[1]Unselected children are children who were not among the first three eligible children identified each week but were otherwise eligible.

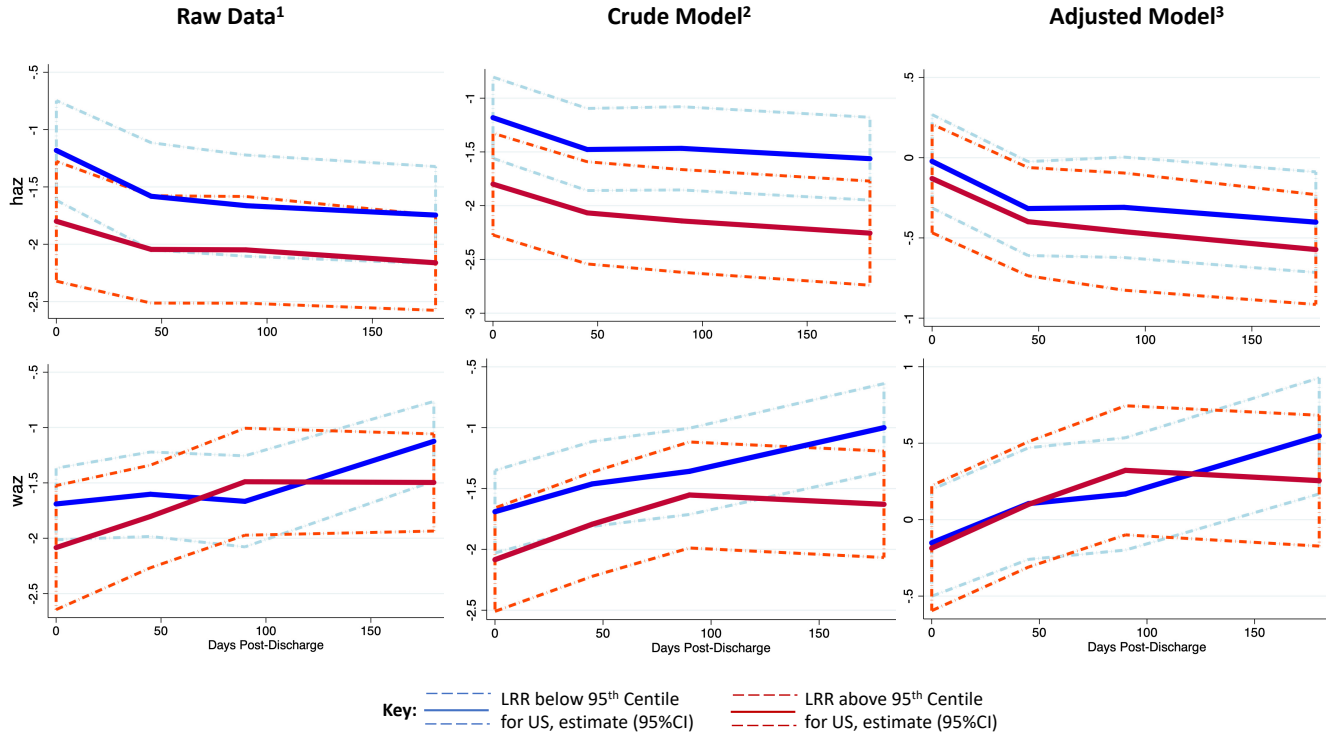
Table 1: Characteristics of hospitalized children aged 2-24 months recruited into the lactulose rhamnose study cohort.

	Migori N= 108		Karachi N= 29		Total N= 137	
	n	(%)	n	(%)	n	(%)
Child						
Age in months						
<6	27	25	12	41	39	27
6-11	33	31	10	34	43	31
≥12	48	44	7	24	55	40
Sex (male)	72	67	18	62	90	66
Currently breast-feeding	72	67	18	62	90	66
Currently exclusively breastfed	29	27	6	21	35	26
Length of Stay[1]						
<48hrs	16	15	9	31	25	18
48hrs-5 days	47	44	10	34	57	42
>5 days	45	45	10	38	55	40
Recent Antibiotics	103	95	29	100	132	96
Stunted	32	30	14	48	46	34
Wasted[2]	44	41	15	52	59	43
Discharge diagnosis						
Diarrhea[1]	45	42	10	34	55	40
Malaria[3]	29	27	0	0	29	21
LRTI[1]	47	44	17	59	64	47
SIRS at admission[1,4]	37	35	7	28	44	32
HIV status						
Exposed, uninfected	16	15	0	0	16	12
Infected	7	6	0	0	7	5
Caregiver						
Biological mother is primary caregiver	98	91	28	97	126	92
Caregiver education[4]						
None	5	5	12	50	17	12
At least some primary	72	67	7	29	79	58
At least some secondary	30	28	5	21	35	26
Body Mass Index						
Underweight (<20)	16	15	3	10	19	14
Normal (20-25)	74	69	18	62	92	67
Overweight (>25)	15	14	8	28	23	17
Household						
Livestock ownership[4]	74	69	6	25	80	58
Improved water source[4]	77	72	16	67	93	68
Improved toilet[4]	44	41	23	96	67	49
Food insecurity						
Low	40	37	11	38	51	37
Moderate	42	39	15	52	57	42
High	26	24	3	10	29	21

[1]Not assessed for children in the community. [2]Defined by WHO criteria (WHZ<-2, or MUAC <12.5 if >6months old, or edema). [3]Malaria RDT positive. [4]Missing data: SIRS-5; Caregiver education – 6; Livestock – 5; Water source – 6; Toilet type – 5.

Abbreviations: LRTI: Lower Respiratory Tract Infection, SIRS: Systemic Inflammatory Response Syndrome.

Figure 3: Growth patterns in the post-discharge period, stratified by lactulose-rhamnose ratio results above or below the 95th centile value for this test in a US population of similarly age children.



[1]Mean and 95% confident intervals of raw data without missing data imputation. [2]Linear mixed effect model with random effect for individual and missing data imputation. [3]Linear mixed effect model with random effect for individual and missing data imputation and adjusted for age, sex, site, baseline HAZ, baseline WAZ and recent diarrhea.

Table 2: Difference in growth (change in anthropometry) between time periods associated with a log increase in LRR score.

	Day 45		Day 90		Day 180	
	Coef	**(95% CI)**	**Coef**	**(95% CI)**	**Coef**	**(95% CI)**
Difference in change in HAZ						
Crude model	**0.03**	(-0.03, 0.10)	**0.01**	(-0.06, 0.08)	**0.01**	(-0.06, 0.07)
Adjusted[1]	**0.00**	(-0.03, 0.03)	**0.01**	(-0.01, 0.04)	**-0.01**	(-0.04, 0.02)
No Diarrhea	**-0.01**	(-0.05, 0.03)	**0.02**	(-0.02, 0.05)	**-0.02**	(-0.06, 0.01)
Diarrhea only	**0.02**	(-0.04, 0.07)	**0.02**	(-0.02, 0.06)	**0.00**	(-0.04, 0.04)
Difference in change in WAZ						
Crude	**0.03**	(-0.05, 0.11)	**0.01**	(-0.07, 0.10)	**-0.01**	(-0.08, 0.07)
Adjusted[1]	**0.03**	(-0.04, 0.10)	**0.01**	(-0.07, 0.10)	**0.00**	(-0.08, 0.07)
No Diarrhea	**0.03**	(-0.06, 0.12)	**0.01**	(-0.07, 0.09)	**0.00**	(-0.09, 0.09)
Diarrhea only	**0.01**	(-0.13, 0.14)	**0.02**	(-0.16, 0.20)	**0.00**	(-0.14, 0.13)

[1]Adjusted for patient age in months, sex, site of recruitment, recent diarrhea history, HAZ at discharge, and WAZ at discharge.

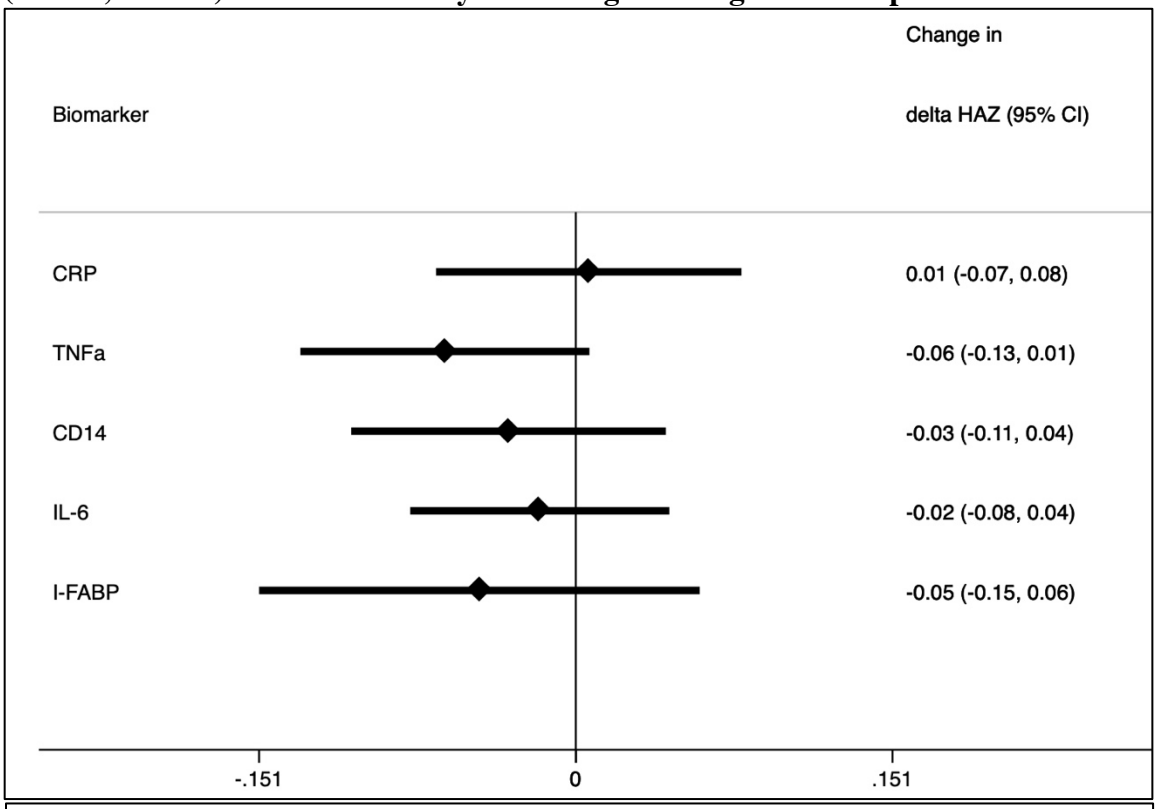
Table 3: The association between lactulose rhamnose ratio and plasma biomarkers of systemic inflammation and intestinal damage.

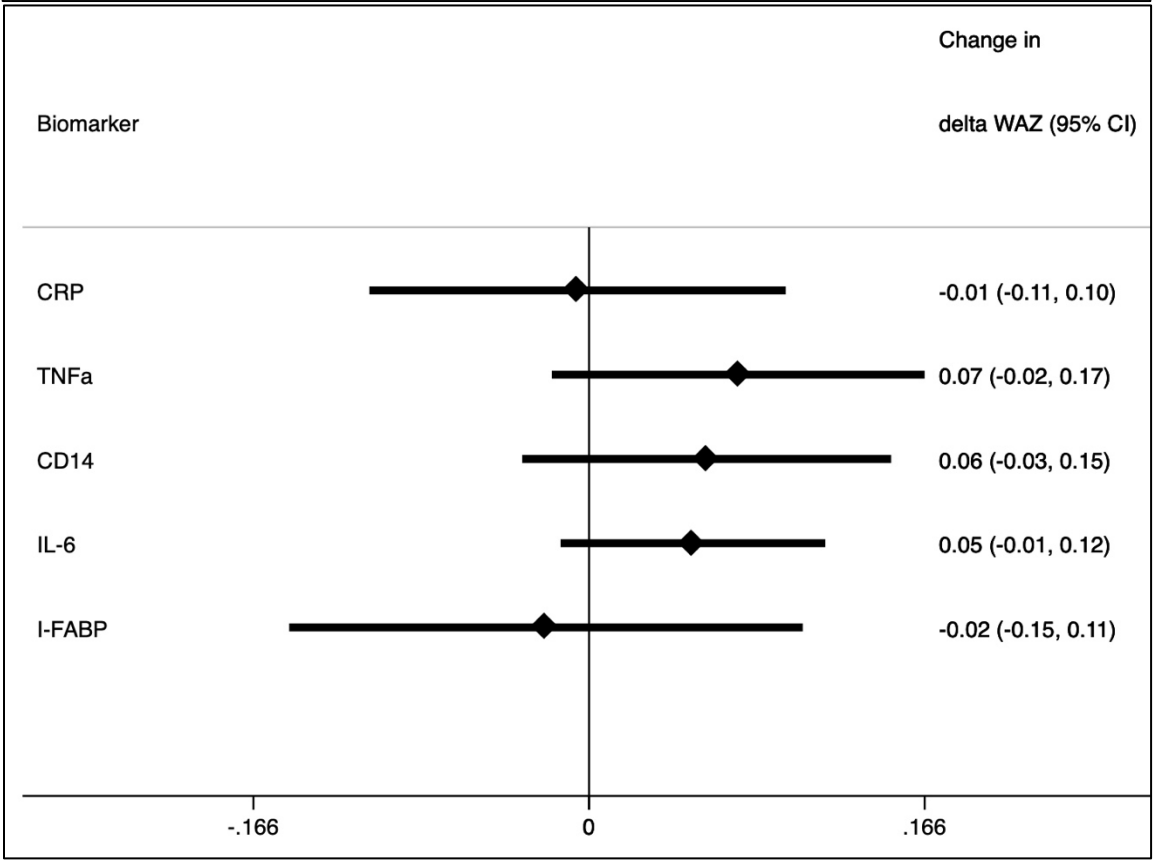
	Biomarkers of Systemic Inflammation[1]								**Intestinal damage (I-FABP)**	
	CD14		**CRP**		**IL-6**		**TNFa**			
	Coef	(95% CI)	Coef	(95% CI)	Coef	(95% CI)	Coef	(95% CI)	Coef	(95% CI)
Community	0.18	(-0.04, 0.41)	0.10	(-0.13, 0.32)	0.19	(-0.03, 0.42)	0.26*	(0.04, 0.48)	0.15	(-0.07, 0.37)
Hospital	-0.03	(-0.20, 0.13)	-0.16	(-0.32, 0.01)	0.02	(-0.14, 0.19)	-0.01	(-0.17, 0.15)	0.03	(-0.01, 0.07)

***<0.05**

[1]Adjusted for patient age in months, sex, site of recruitment, recent diarrhea history, HAZ at discharge, and WAZ at discharge. Units are standard deviations, (e.g., 0.10 = one tenth of a standard deviation change)

Figure 4: Relationship between plasma biomarkers and post-discharge growth (ΔHAZ, ΔWAZ) in the first 45 days following discharge from hospital.





Supplementary Appendix

Table 1: Characteristic of hospitalized and community children who had plasma proteomic and lactulose rhamnose results available.

	Hospital (N: 91)		Community (N: 64)	
	N	**(%)**	**N**	**(%)**
CHILD				
Site				
Migori	75	(82)	37	(58)
Karachi	16	(18)	27	(42)
Age (months)				
<6	26	(29)	16	(25)
6-12	29	(32)	19	(30)
≥12	36	(40)	29	(45)
Sex (male)	57	(63)	37	(58)
Current breastfeeding	58	(64)	49	(77)
Current exclusive breastfeeding	25	(27)	38	(59)
Length of stay				
<48 hours	13	(14)	--	--
2-5 days	36	(40)	--	--
>5days	42	(52)	--	--
Recent antibiotics	88	(97)	10	(16)
Stunted	32	(35)	19	(30)
Wasted	47	(52)	7	(11)
Admission diagnosis				
Diarrhea	37	(41)	--	--
Malaria	19	(21)	1	(2)
LRTI	46	(51)	--	--
SIRS	29	(32)	--	--
HIV				
Unexposed	73	(80)	56	(88)
Expose uninfected	11	(12)	7	(11)
Infected	7	(8)	1	(2)
Chronic illness	7	(8)	2	(3)
CAREGIVER				
Biological mother	85	(93)	59	(92)
Education				
None	10	(11)	15	(24)
Primary	54	(59)	32	(52)
Secondary	27	(30)	15	(24)
BMI				

Underweight	10 (11)	1 (2)
Normal	67 (74)	41 (64)
Overweight	12 (13)	21 (33)
SES		
Livestock owned	51 (56)	34 (53)
Improved water source	64 (70)	49 (77)
Improved sanitation	42 (46)	35 (55)
Food Insecurity		
Low	31 (34)	26 (41)
Moderate	39 (43)	31 (48)
High	21 (23)	7 (11)

Table 2: Difference in growth (change in anthropometry) between time periods associated with a log increase of the plasma biomarkers.

	Day 45	Day 90	Day 180
	Coef (95% CI)	**Coef (95% CI)**	**Coef (95% CI)**
Difference in change in HAZ			
CD14	**-0.03** (-0.11, 0.04)	**-0.01** (-0.07, 0.05)	**0.00** (-0.04, 0.05)
CRP	**0.01** (-0.07, 0.08)	**-0.01** (-0.07, 0.05)	**0.02** (-0.03, 0.07)
IL-6	**-0.02** (-0.08, 0.04)	**-0.01** (-0.06, 0.04)	**0.00** (-0.05, 0.05)
TNFa	**-0.06*** (-0.13, 0.01)	**-0.01** (-0.07, 0.05)	**0.00** (-0.05, 0.05)
I-FABP	**-0.05** (-0.15, 0.06)	**-0.01** (-0.06, 0.05)	**0.00** (-0.05, 0.05)
Difference in change in WAZ			
CD14	**0.06** (-0.03, 0.15)	**0.05** (-0.06, 0.15)	**0.08** (-0.03, 0.19)
CRP	**-0.01** (-0.11, 0.10)	**0.06** (-0.05, 0.17)	**-0.01** (-0.14, 0.13)
IL-6	**0.05** (-0.01, 0.12)	**0.02** (-0.05, 0.09)	**0.01** (-0.06, 0.07)
TNFa	**0.07** (-0.02, 0.17)	**0.06** (-0.05, 0.17)	**0.03** (-0.07, 0.13)
I-FABP	**-0.02** (-0.15, 0.11)	**0.00** (-0.17, 0.18)	**0.01** (-0.18, 0.19)

*$p<0.05$

Chapter Three

Title: Plasma proteomic signatures of enteric permeability

Authors: Kirkby D. Tickell, Donna M Denno, Ali Saleem, Zaubina Kazi, Benson Singa, Catherine Achieng, Charles Mutinda, Barbra A. Richardson, Kristjana H. Ásbjörnsdóttir, Stephen E. Hawes, James Berkley, Judd L. Walson.

Summary:

Background: Enteropathies are a major cause of childhood morbidity and growth failure in low-and-middle-income countries, but relatively little is known about the mechanisms that link these enteric diseases to their systemic consequences.

Methods: Plasma samples from 155 children who had undergone the lactulose rhamnose ratio (LRR) gut permeability test were sent for aptamer based proteomic analysis to establish the systemic signature of enteric permeability. These children were aged 2-23 months, and were either being discharge from Civil Hospital Karachi (Pakistan) or Migori County Referral Hospital (Kenya), or were healthy children living in the same communities as the hospitalized cohort. Data were split into training and test sets. An extreme gradient boosted model with five-fold cross validation was built in the training set using 7,500 plasma proteins as predictors of the LRR. The tuned model was applied to the test set and the root-mean squared error (RMSE) calculated. Shapely additive values identified the variables that were

most influential in the model, and the top twenty of these variables were matched to biological functions listed in the UniProt database. Finally, the correlation between these twenty variables and known risk factors for increased enteric permeability were calculated.

Results: The final tuned model had a relatively modest predictive performance (RMSE: 1.0) but did out-perform random chance (RMSE: 1.4) in the held-out test set. The common biological functions of the most influential proteins in this model fell into four categories: leukocyte invasion, tissue repair, exposure to bacterial pathogens, and homeostatic hormonal function, including renin and glucagon. Correlation network analysis of the selected plasma proteins and common risk factors for increased enteric permeability, found multiple associations between the biomarkers of leukocyte invasion and tissue repair. This leukocyte invasion/tissue repair cluster was correlated with weight-for-age and height-for-age. The cluster was also linked to breastfeeding status and age through biomarkers of animal protein consumption and exposure to bacterial pathogens. Recent diarrhea was not correlated with any of the identified biomarkers of enteric permeability.

Conclusions: A cluster of plasma proteins involved in leukocyte infiltration, tissue repair, and the host response to bacterial pathogens were associated with enteric permeability and many of the known risk factors for enteropathy among young children in low- and middle-income countries. Acute diarrhea was not associated with this cluster of proteins, challenging the assumption that diarrhea shares a

common underlying mechanism with other etiologies of enteric permeability, such as environmental enteric dysfunction.

Introduction

Globally, undernutrition is thought to be an underlying factor in up to 45% of all deaths before five years of age.[1] In addition, undernutrition is associated with increased risk of multiple lifelong morbidities and decreased educational attainment.[1–3] A combination of social, clinical and health system vulnerabilities likely contribute to the risk of developing undernutrition and to the associated morbidity and mortality. Enteric dysfunction may be a predominant driver of such risk in many low and middle-income (LMIC) settings.[3,4] The most prevalent risk factors for enteric dysfunction in LMIC settings are persistent exposure to fecal pathogens, recent diarrhea, early cessation of breastfeeding, micronutrient deficiencies, poorly controlled HIV, wasting and stunting.[4,5] Many of these etiologies of enteric dysfunction appear to converge on a shared pathophysiologic pathway in which enteric inflammation leads to increased enteric permeability and decreased surface area, which may lead to malabsorption. This increased enteric permeability is thought to allow microbes or microbial products to translocate into the systemic circulation. Intestinal inflammation and translocated microbial products can ultimately result in systemic inflammation and immune activation.[6,7] However, available studies have not consistently demonstrated a clear association between enteric inflammation and markers of decreased permeability, increased translocation or systemic inflammation, suggesting that this pathway has not yet been completely elucidated.[6]

Enteric permeability can be dynamically assessed using dual sugar tests,[8,9] such as the lactulose-rhamnose ratio (LRR). In addition, advances in both proteomic analysis of tissue samples and computational approaches to analyzing "omics" datasets suggest it is now possible to gain deeper mechanistic insights into the underlying biology of enteropathy-associated gut permeability. A deeper understanding of the mechanistic pathways driving enteropathy related risk among undernourished children may inform the development of novel interventions to reduce risk among highly vulnerable children in these settings.

This analysis combines clinical data from the Childhood Acute Illness and Nutrition (CHAIN) Cohort with both LRR test results collected on a subset of included children and a proteomic panel of 7,500 plasma biomarkers covering a broad swath of biological processes. We use supervised machine learning to identify plasma proteins from this biomarker panel which were associated with the LRR score, and then we describe the correlation between the identified plasma proteins and known clinical and demographic risk factors for increased enteric permeability.

Methods

Parent study enrollment and follow-up

The CHAIN Cohort was a prospective cohort study in which acutely ill children aged 2–23 months across nine sites in six countries in Africa and South Asia were systematically enrolled at admission to hospital. Details of this study have been published previously.[10] Children were enrolled across a range of rural and urban environments and differing malaria and HIV endemicities: Bangladesh, Dhaka Hospital and Matlab Hospital: Burkina Faso, Banfora Referral Hospital; Kenya, Kilifi County Hospital, Mbagathi Sub-County Hospital-Nairobi and Migori

County Referral Hospital; Malawi, Queen Elizabeth Central Hospital-Blantyre; Pakistan, Civil Hospital-Karachi; and Uganda, Mulago Hospital-Kampala.

Enrollment in CHAIN was stratified by child mid-upper arm circumference (MUAC), so that approximately two children with very low MUAC (<11.5cm if older than 5 months, otherwise <11 cm) or bipedal edema, and two with moderately low MUAC (≥11.5cm but <12.5cm if older than 5 months, otherwise ≥11 cm but <12.0cm) were recruited for each child with a normal MUAC (≥12.5cm if older than 5 months, otherwise ≥12.0cm). Detailed clinical, anthropometric, and sociodemographic data were collected at admission and discharge in addition to blood samples. Clinical observations and management were recorded on standardized case report forms daily during admission. Home environment characteristics were assessed at home visits at discharge.

The CHAIN study also recruited community reference participants from households near the hospitalized children's homes, using a pseudo-random selection method (3rd house to the north of the enrolled child's house). Community children were recruited in the same age bracket as the index hospitalized child (<6 months, 6-11 months, 12-23 months) if they had no history of acute illness in the 14 days prior and if their caregiver consented to participation. A community recruitment was attempted for every hospitalized child who was discharged. Demographics, medical history and examination, and anthropometry data and blood sample collection were obtained using the same methods as in the hospitalized children.

Sub-study enrollment

Children enrolled in CHAIN at the Civil Hospital Karachi and the Migori County Referral Hospital sites, including the hospitalized and community groups, were eligible for inclusion in this sub-study when they were judged medically stable (no respiratory distress, not requiring supplemental oxygen, and nutritional intake was by oral route). The first three eligible children each week were selected for participation. This enrollment cap facilitated accurate implementation of the LRR test. Additional informed consent was obtained prior to inclusion in this sub-study. Both hospitalized and community children with diarrhea on the day of the LRR test were excluded, as lactulose may exacerbate diarrhea.

The lactulose-rhamnose ratio test

The LRR test was conducted in the morning and caregivers were asked to fast (food, drink, and breastmilk) their child for one hour. At the beginning of this hour, an adhesive urine bag was attached, to obtain a pre-dose urine sample. A 10ml oral solution containing 1500mg lactulose and 300mg L-rhamnose was administered at the end of the fasting hour and a new urine bag attached. Urine passed in the first 20 minutes after sugar administration was discarded and all urine passed during the subsequent two hours was collected. The caregiver was encouraged to breastfeed or give water to the child after administration of the sugar solution. Any stool contamination of the urine bag, urine leakage, or failure to void in the two-hour post-administration period were considered a test failure. Failed tests were repeated after 24-hours if the caregivers were willing.

Urine samples from each time period (20-80 minutes and 80-140 minutes post-sugar administration) were aliquoted into 100 μl cryovials and stored at -80^{o}c within 1 hour of collection. These aliquots were shipped to the Mayo Clinic (Rochester, Minnesota) for high performance

liquid chromatography mass spectrometry. Percentage of lactulose and rhamnose recovery was calculated for each post-administration time period, as was LRR. The cumulative LRR encompassing both time periods was calculated by deriving a mean concentration of lactulose and rhamnose weighted to the volume of urine passed in each post-administration period. In keeping with previous LRR analyses, failure to detect rhamnose in the post-administration sample was classified as a test failure.[11]

Plasma biomarkers

Blood samples, collected at discharge for the hospitalized children or at enrollment for the community group, were processed in site laboratories within one hour of collection. Samples were spun in a refrigerated centrifuge into plasma and serum, stored and shipped at -80^{o}c. We analyzed proteomics samples from a subset of children who participated in the LRR sub-study and who died post-discharge or were from a random subset of the surviving hospitalized children and the community participants, resulting in 111 hospitalized and 64 community children included in the plasma proteomic analysis. The proteomic approach was implemented by Somalogic, Inc using an aptamer-based technology, which assesses the concentration of 7,500 proteins.[12,13] The names and known functions of these proteins are linked to the UniProt database to aid interpretation of the data.[14]

Statistical analysis

The LRR was used as the outcome of interest for the predictive modelling, and the full panel of proteins was available to the models as potential predictors. All data were standardized, including the outcome, by subtracting the mean value of that variable from each observation and then

dividing by the standard deviation (i.e., z-scores were created). The aptamer-based proteomics do not naturally fall into clinically interpretable units, making standardization the most interpretable way to represent these variables in models (i.e., one unit = one standard deviation change). Missing values in the potential predictors were imputed using K-nearest neighbor method.[15] We split available data into training (75%) and test sets (25%). An extreme gradient boosted (XGBoost) model was tuned in the training set using 10-fold cross validation and then the final tuned model was applied to the test set, and the root-mean square error (RMSE) calculated. XGBoost modelling was chosen as this approach is highly flexible, but also contains a penalization term that aims to minimize overfitting which makes it suitable for a wide variety of predictive challenges.[15] Graphical representations of the tuning process are given in Appendix 1.[15] For comparison, a normally distributed random prediction of the LRR was generated and the RMSE calculated. To understand if an alternative model choice would have yielded better predictive performance, we conducted sensitivity analyses using ElasticNet and simulated annealing stepwise linear models were built in the training set and then applied in the test set.

To better understand the variables contributing to the model's prediction, we analyzed the variable importance, i.e., those variables which most informed the model prediction. Heuristically, we then took the 20 most influential variables and identified known biological functions per the UniProt database.[14] To understand the relationships between these predictors we built Pearson's correlation coefficient matrices and displayed these heatmaps and network diagrams. Finally, we added known risk factors for increased enteric permeability -- WAZ, HAZ, age, any current breast feeding, and history of recent diarrhea -- to these correlation matrices. Correlation coefficients

≥0.7 were described as strong associations, 0.50-0.69 were moderate correlations, 0.30-0.49 were weakly correlated, and <0.30 were considered not correlated.[16]

Results

Among the 245 children with LRR results available, 155 (63%) were selected for plasma proteomics analysis; 112 (72%) of these children were from Migori and 43 (28%) were from Karachi (Table 1). Among the included children, 91 (58.7%) were recruited at hospital discharge, and 64 (41.3%) were community participants. Median age was 9 months (IQR 5-16), 51 (32.9%) children were stunted, 54 (34.8%) were wasted, and 28 (18.1%) were both stunted and wasted. Only eight children (5.2%) were HIV infected, but a further 18 (11.6%) were HIV exposed but uninfected.

The untransformed median LRR was 0.32 (IQR: 0.18-0.63), and after standardization was -0.17 (standard deviation 1.0). The tuned predictive model achieved a RMSE of 0.71 in the training data, and 1.03 in the test set, which was better than the normally distributed random prediction (which had an RMSE of 1.26 and 1.41 in the respective datasets). The twenty variables that were most influential to the model are displayed in Figure 1. For all the selected proteins, increased concentrations were associated with an increased LRR, except for two unnamed proteins (seq.20932.10 and seq.23307.7), where lower concentrations were associated with increased LRR. Many of the variables displayed clear cutpoints, suggesting these proteins may only be associated with increased LRR when above a certain threshold. Sensitivity analyses using ElasticNet (RMSE: training 0.98, test 1.00) and stepwise linear model building (RMSE: training 0.96, test 1.03) did not yield substantively different predictive performances.

Functions of the top twenty protein predictors

Fourteen of the top 20 proteins have recorded known functions (Figure 2), with each protein participating in a mean of 16.5 recognized biological processes. The most common functions were related leukocyte infiltration through the vascular endothelium into peripheral tissues, including biomarkers of leukocyte migration, endothelial cell growth and migration, platelet degranulation and aggregation, and phosphatidylinositol 3-kinase signaling. Two of the selected proteins (regenerative islet-derived protein 3-alpha, P-selectin) are thought to be specific mediators of the host response to bacterial infections (appendix 2), while a third is a modulator of immune activation (tyrosine-protein kinase). Among the biomarkers of leukocyte infiltration, P-selectin and tyrosine protein kinase are thought to specifically relate to neutrophil and monocyte activity, while junctional adhesion molecule-like protein is primarily a bi-product of lymphocyte migration. The other biomarkers of immunological activity are not specific to particular cell lines.

A second group functions encapsulated enhanced cell-cell adhesion and extracellular matrix construction, including cell proliferation, cell differentiation, heterophilic cell-cell adhesion, extracellular matrix organization, and wound healing. Nine of the selected proteins participate in these cell adhesion, cell proliferation or extracellular matrix construction pathways. Among these nine proteins, three of the cell-adhesion proteins (junctional adhesion molecule-like protein, fibroblast growth factor-2, P-selectin) are thought to aid leukocytes in adhering to the vascular endothelium and migrating into peripheral tissues. A further two markers of cell adherence also participate in leukocyte migration, although they also play a role in broader cell-cell (endothelial cell-selective adhesion molecule) or cell-matrix binding (integrin alpha-IIb: beta-3 complex).

Three of the selected proteins are part of the hormonal regulation of nutrients (protein FAM3D, glucagon) and blood pressure/fluid regulation (renin). FAM3D promotes glucagon secretion and suppression expression of insulin, while glucagon itself is a catabolic hormone responsible for increasing blood glucose and blood lipid concentration. Renin maintains blood pressure through vasoconstriction, sodium reabsorption, and thirst. Beta-Ala-His dipeptidase preferentially hydrolyses carnosine, an exclusively animal source protein. Finally, there were several non-specific cellular signal transduction pathways, including integrins, ERK, and MAPK activity.

Correlation between identified proteins and known risk factors of enteric permeability

The cell adhesion, proliferation, and migration proteins shared many strong, moderate, and weak correlations with the extracellular matrix repair and leukocyte infiltration proteins (Figure 3). In correlation network mapping, these proteins formed a highly interwoven cluster (Figure 4). This cluster of junctional adhesion molecule-like protein, procollagen-lysine2-oxoglutarate 5-dioxygenase 3, teratocarcinoma-derived growth factor 1, endothelial cell-selective adhesion molecule, Ly6/PLAUR domain-containing protein 3, fibroblast growth factor 2, integrin alpha-IIb: beta-3 complex, tyrosine-protein kinase and P-selectin is also referred to as the leukocyte infiltration/tissue repair cluster. P-selectin, a mediator of the response to bacterial infection, had a particularly strong association with endothelial cell-selective adhesion molecule (corr: 0.80), and moderate correlations with integrin alpha-IIb: beta-3 complex (corr: 0.67), tyrosine-protein kinase (corr 0.51), and fibroblast growth factor 2 (corr: 50). Regenerative Islet-derived protein 3-alpha, another biomarker of bacterial infection, was also weakly correlated with this cluster through junctional adhesion molecule-like protein (corr: 0.46).

The hormonal biomarkers glucagon, renin and FAM3D were correlated with each other, and there was some evidence that higher levels of these hormones were correlated with increased expression of proteins in the leukocyte infiltration/tissue repair cluster. For example, FAM3D was correlated with teratocarcinoma-derived growth factor 1 (corr: 0.43), fibroblast growth factor 2 (corr: 39), junctional adhesion molecule-like protein (corr: 0.38) and endothelial cell-selective adhesion molecule (corr: 0.37), while higher renin was associated with higher procollagen-lysine2-oxoglutarate 5-dioxygenase 3 (corr: 0.45) and teratocarcinoma-derived growth factor 1 (corr: 0.38).

To better understand how known clinical and demographic risk factors for enteric permeability may link to the plasma protein pattern identified, we included WAZ, HAZ, currently breastfeeding, age, and recent diarrhea in our correlation matrices. HAZ and WAZ were strongly associated with each other (corr: 0.80). Higher WAZ had correlation with decreased detection of several of the plasma proteins in the leukocyte infiltration/tissue repair cluster, including procollagen-lysine 2-oxoglutarate 5-dioxygenase 3 (corr: -0.46), junction adhesion molecule like (corr: -0.39). Higher WAZ was also correlated with lower levels of the one bacterial response biomarker, p-selectin (corr: -0.31), renin (corr: -0.36), and glucagon (corr: -0.3).

Age and current breastfeeding had inverse correlations with each other (corr: -0.44), and older age was further associated with higher Beta-Ala-His dipeptidase (corr: 0.44), the biomarker of animal source protein consumption. Higher Beta-Ala-His dipeptidase was in turn associated with higher levels of both biomarkers of bacterial defense, regenerative islet-derived protein 3-alpha (0.38)

and p-selectin (corr: 0.37). Increased age was also moderately associated with increased regenerative islet-derived protein 3-alpha (corr: 0.38). Finally, recent history of diarrhea was not found to be correlated with any of the plasma proteins identified by our model.

Discussion

We found enteric permeability to be associated with an intercorrelated group of plasma proteins important in leukocyte migration, including some specific biomarkers of lymphocyte, neutrophil, and monocyte activation. Our model suggested this association may have been driven by abnormally high values of these biomarkers, with low-to-average concentrations not being associated with enteric permeability. The identified plasma proteins also included biomarkers of tissue repair, with some specifically associated with fibroblast growth and extracellular matrix deposition. There is an interesting similarity between these systemic markers of leukocyte infiltration and tissue repair, and factors used to define the histopathological severity enteropathy in environmental enteric dysfunction.[17] This histopathological score includes lymphocyte, monocyte, neutrophil infiltration into the gut wall to measure the severity of EED, and also classes replacement of enterocytes with fibrino-inflammatory infiltrate and/or fibroblast proliferation as the most severe grade of enterocyte damage associated with EED. The interesting similarity between histopathological definitions of severe EED, and the plasma proteomic correlates of enteric permeability, may be an indication of the degree of enteropathy required to leave a systemic signature.

Many enteropathies are characterized by leukocyte invasion into the intestinal wall, which is thought to exacerbate enteric permeability.[8] In low-and-middle income countries, enteric

pathogens are thought to be a major driver of this leukocyte invasion among young children, and we found two biomarkers of bacterial infection to be correlated with both the LRR and the cluster of leukocyte migration/tissue repair proteins.[7,8] These findings support the hypothesis that exposure to enteric pathogens may drive enteric inflammation and subsequent disruption in permeability, but they do not rule out other etiologies of leukocyte invasion, including microbiome dysbiosis, micronutrient deficiencies, or other sources of inflammatory antigens. Age and current breastfeeding were correlated with each other and had associations with Beta-Ala-His dipeptidase, a biomarker of animal source protein consumption, in addition to associations with the two biomarkers of bacterial infection. These children were 2 to 23 months of age, a period of childhood when many children transition from exclusive breast feeding to family foods, often including animal source proteins. This transition also signifies the waning of passive immunity provided by breast milk, and an increased exposure to enteric pathogens. While we cannot draw causal conclusions from this analysis, our findings suggest that during weaning, the increased exposure to enteric pathogens and the body's response to these pathogens, may lead to increased enteric permeability.

WAZ and HAZ were highly correlated, and higher values of both had strong inverse-associations with several of the plasma proteins in the leukocyte infiltration/tissue repair cluster. Childhood wasting and stunting are also known to be associated with several potential etiologies of increased enteric permeability, including increased pathogen exposure, specific micronutrient deficiencies, and untreated HIV infection.[8,18,19] However, the malabsorption and systemic inflammation enteropathy mechanisms may also be a cause of childhood undernutrition.[3] The bidirectional association between enteropathy and undernutrition makes the correlations between these factors

difficult to interpret. It also complicates the relationship between the hormonal factors, glucagon and renin, and the cluster of leukocyte infiltration/tissue repair. These factors were strongly associated with nutritional status, and nutritional status was associated with the leukocyte infiltration/tissue repair cluster. This pattern suggests the observed association between and these hormones and leukocyte infiltration/tissue repair cluster may have been confounded by undernutrition. Causal modelling efforts, ideally using longitudinal data, are required to further disaggregate the direction and causal nature of the correlations we observed in this study.

Acute diarrhea is a thought to be a risk factor for increased enteric permeability.[20–22] However, in this analysis, we did not find any strong correlations between recently resolved diarrhea and the leukocyte infiltration and tissue repair complex. This may indicate that these biomarkers do not capture the mechanism linking diarrhea to enteric permeability. Fecal leukocytes are typically not a feature of viral acute diarrhea, while invasive bacterial or protozoal infections more often lead to fecal leukocyte detection.[23] There may be substantial heterogeneity in the leukocyte migration during acute diarrhea between different etiologies, and the syndromic diagnosis of acute diarrhea may not be specific enough to capture this nuance. Other studies, including the MAL-ED study, have demonstrated associations between the burden of asymptomatic enteric pathogen infection and impaired growth that appear to be mediated by enteric and systemic inflammation.[24] Notably however, MAL-ED also did not find an association between episodes of acute diarrhea and childhood growth.[25] Collectively, these data suggest that the pathological mechanisms linking acute diarrhea and asymptomatic enteric pathogen infection to growth may be subtly different. Alternatively, biomarkers of leukocyte invasion may reflect a more chronic process that may be difficult to capture in the setting of acute diarrhea before such proteins have been fully expressed.

This analysis leverages rigorously conducted dual sugar testing and a state-of-the-art plasma proteomics analysis to generate novel hypotheses about the biological mechanisms underpinning enteric permeability. However, the analytic methods applied are designed for hypothesis generation and not causal modelling. Our data captured a highly heterogenous population, with and without acute illnesses, and from two different countries. While this diverse population may serve to strengthen the generalizability of the machine learning models, it can also complicate interpretation. Despite the model's observed associations between the proteomic biomarkers and the LRR test, none of the models explored in our primary or sensitivity analyses had particularly good predictive performance in our test set. The assay performance of the LRR test is highly variable, influenced by factors such as gastric emptying, intestinal motility, and frequency of urinary voiding. This suggests that LRR is a weak target for machine learning applied to plasma proteomics data, because much of this heterogeneity is unlikely to be addressed through the available data. Finally, many of the proteins our model associated with the LRR have unknown functions, and even among those with known association to particular processes, there is still room for additional biological processes currently not known.

Conclusion:

We identified a cluster of proteins associated with leukocyte infiltration and tissue repair that were associated with both measures of enteric permeability and many known risk factors for enteropathy among young children in LMICs. This cluster of proteins also included biomarkers of bacterial infection. However, acute diarrhea was not associated with this cluster of proteins, challenging the

assumption that diarrhea shares a common underlying mechanism with other etiologies of enteric permeability, such as environmental enteric dysfunction.

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Table 1: Characteristics of participants include in the machine learning analysis.

	N=155	(%)
CHILD		
Site		
Migori	112	(72.3)
Karachi	43	(27.7)
Group		
Hospitalized cohort	91	(58.7)
Community participant	64	(41.3)
Age		
< 6 months	42	(27.1)
6-12 months	48	(31.0)
≥ 12 months	65	(41.9)
Male	94	(60.6)
Breast Feeding		
Any current	107	(69.0)
Currently exclusive	63	(40.6)
Recent antibiotics	98	(63.2)
Recent diarrhea	37	(23.9)
Stunted	51	(32.9)
Wasted[1]	54	(34.8)
HIV status		
Unexposed	129	(83.2)
Exposed uninfected	18	(11.6)
Infected	8	(5.2)
CAREGIVER		
Education[1]		
None	25	(16.1)
≤ Primary	86	(55.5)
> Primary	42	(27.1)
Body Mass Index		
Underweight	11	(7.1)
Normal	108	(69.7)
Overweight	33	(21.3)
HOUSEHOLD		
Improved water source[1]	113	(72.9)
Improved toilet[1]	77	(49.7)
Food insecurity		
Low	57	(36.8)
Moderate	70	(45.2)
High	28	(18.1)

[1]Defined by WHO criteria (WHZ<-2, or MUAC <12.5 if >6months old, or edema). [1]Missing data: Caregiver education – 8 community; Water source – 6; Toilet type – 5.

Figure 1: The 20 variables most influential variables in our model are displayed in descending order. Each participant has one dot on each variable line, this dot is colored by the value of that variable - purple for a high value, orange for a low value. The dots are positioned along the x-axis according to contribution of that variable to the child's predicted LRR, left-side indicating the variable lowered the predicted LRR and the right-side increased the LRR.

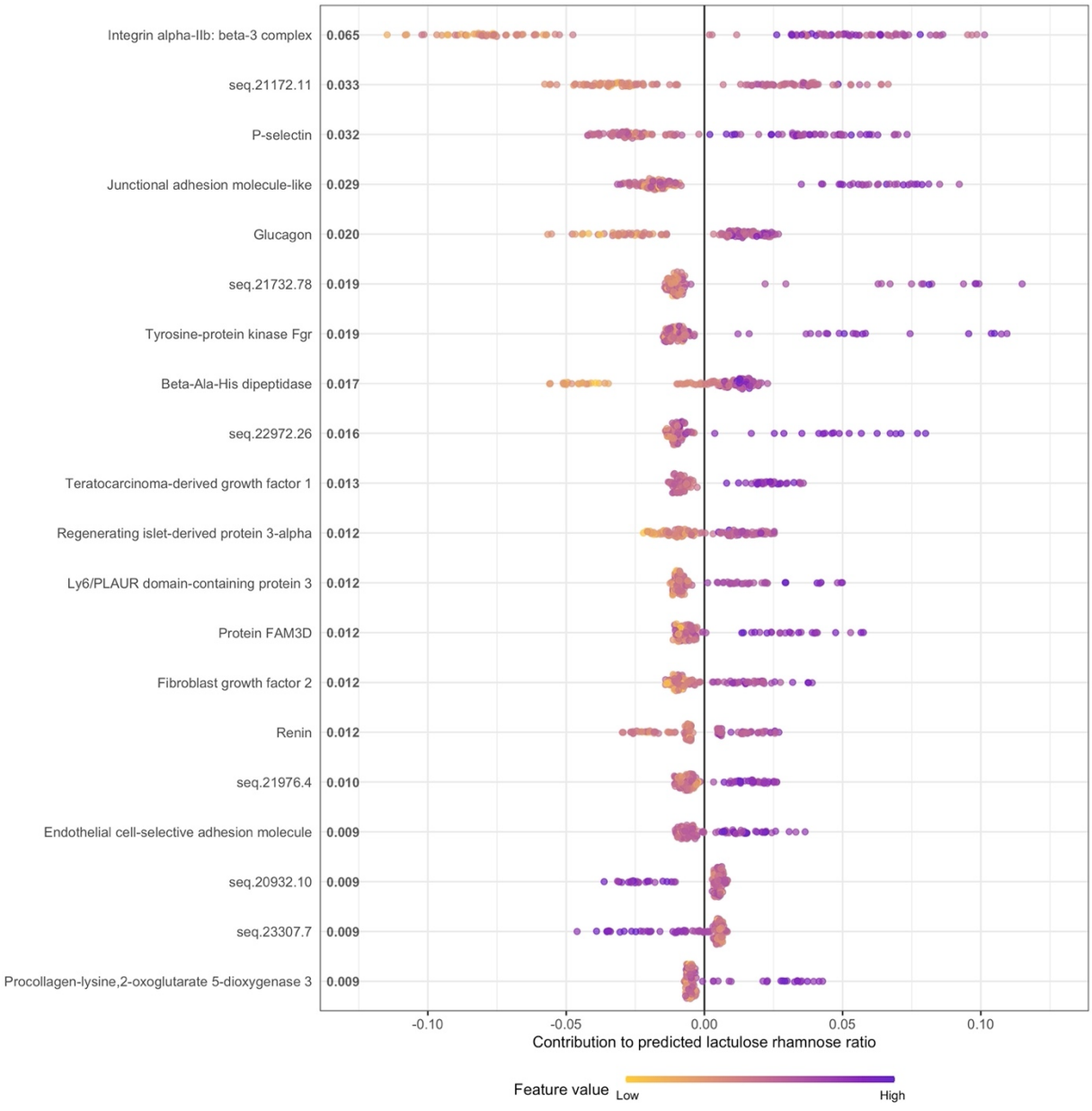


Figure 2: The biological processes list in UniProtKB database associated with the top-twenty proteomic predictors of lactulose-rhamnose ratio. Frequency indicates the number of proteins of that include the named biological processes.

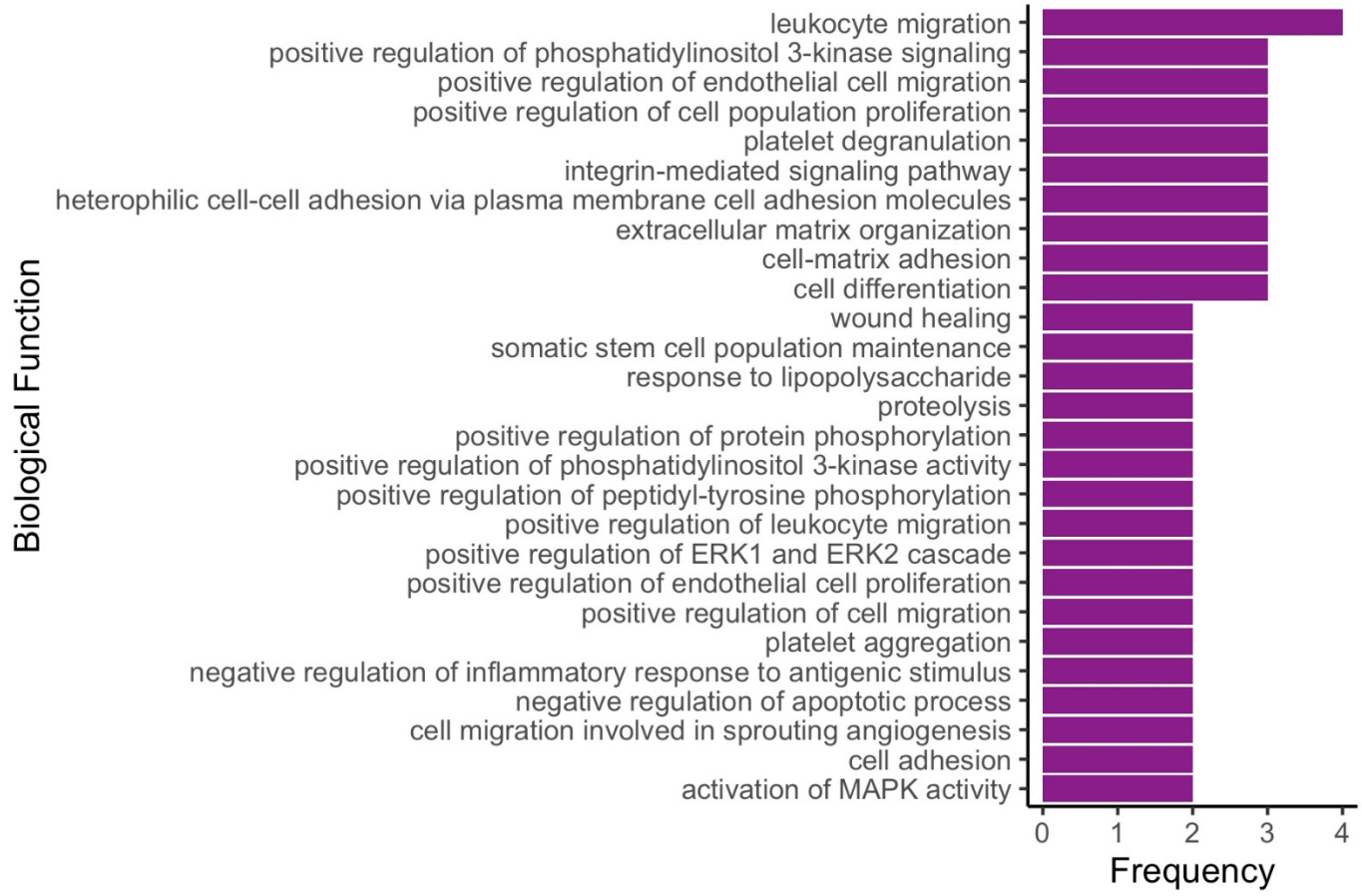


Figure 3: Correlation between the top-twenty proteomic predictors of lactulose-rhamnose ratio and known risk factors of enteric permeability.

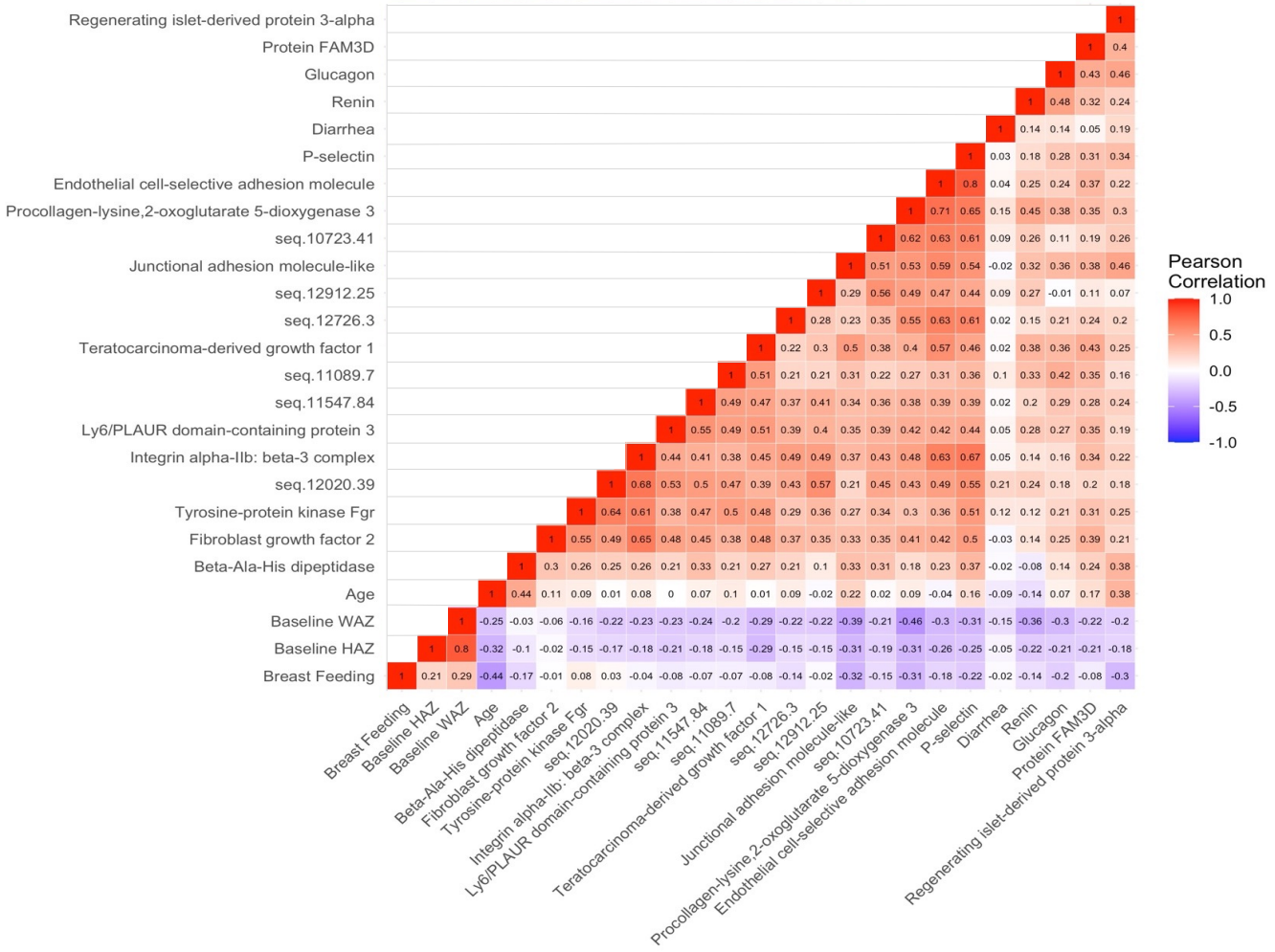
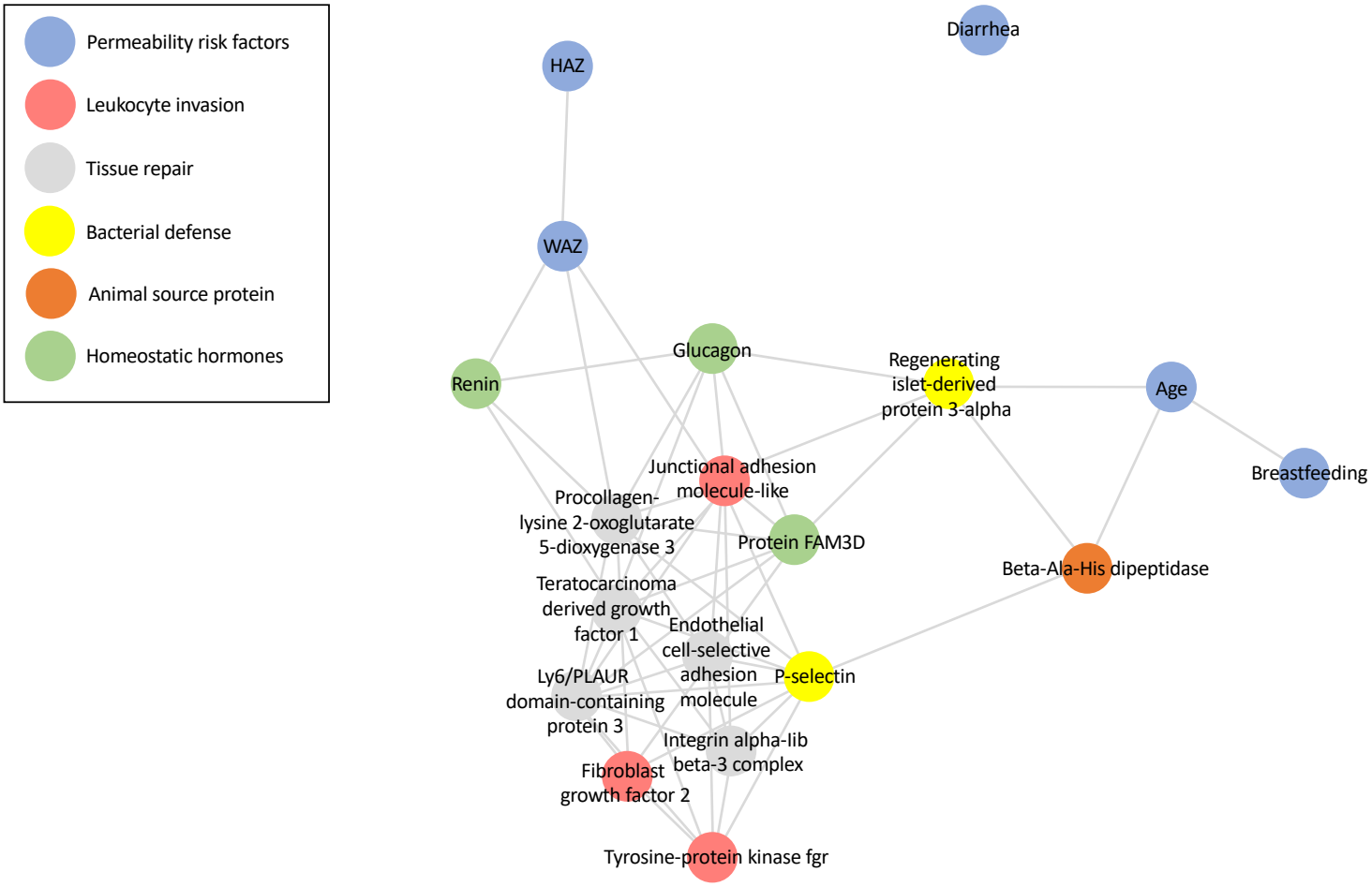
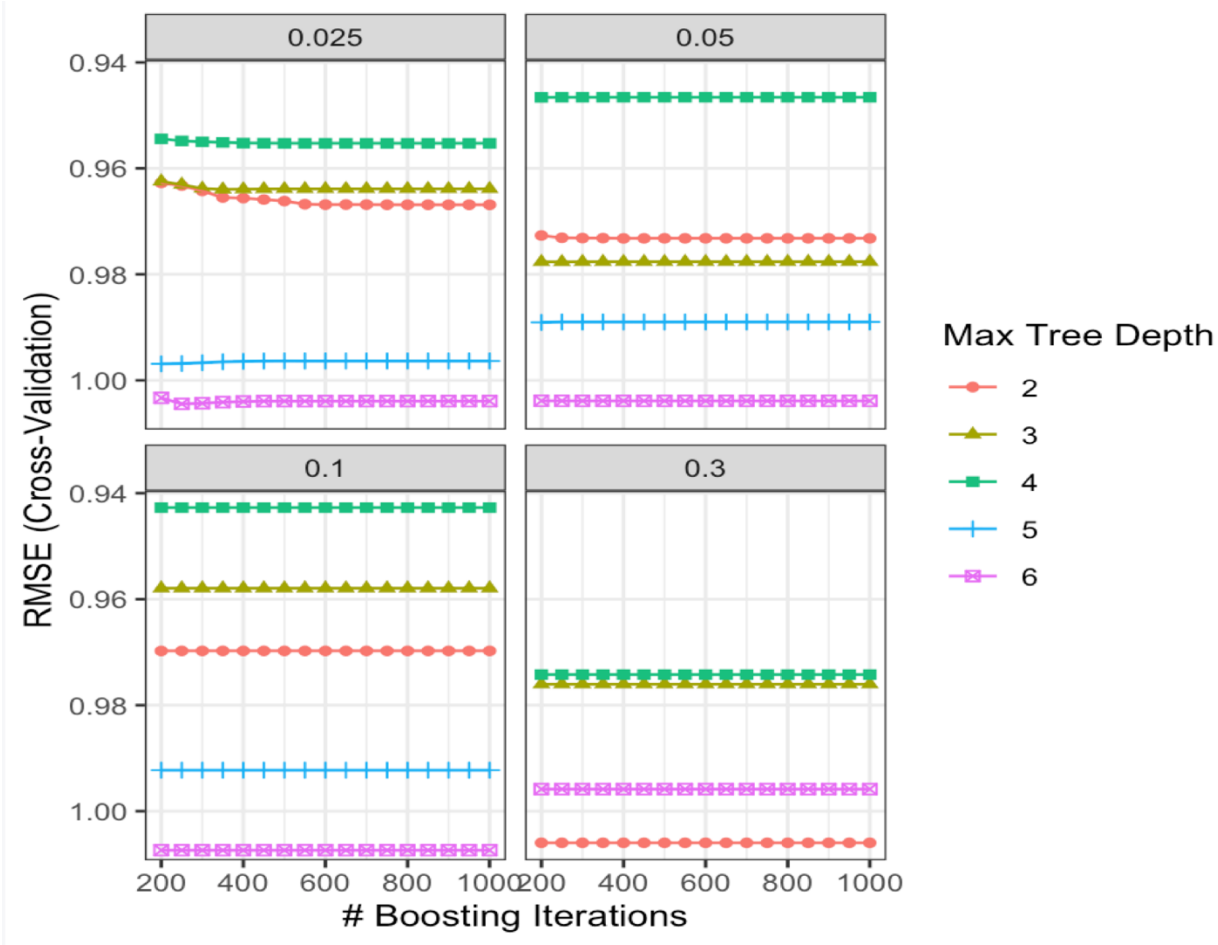
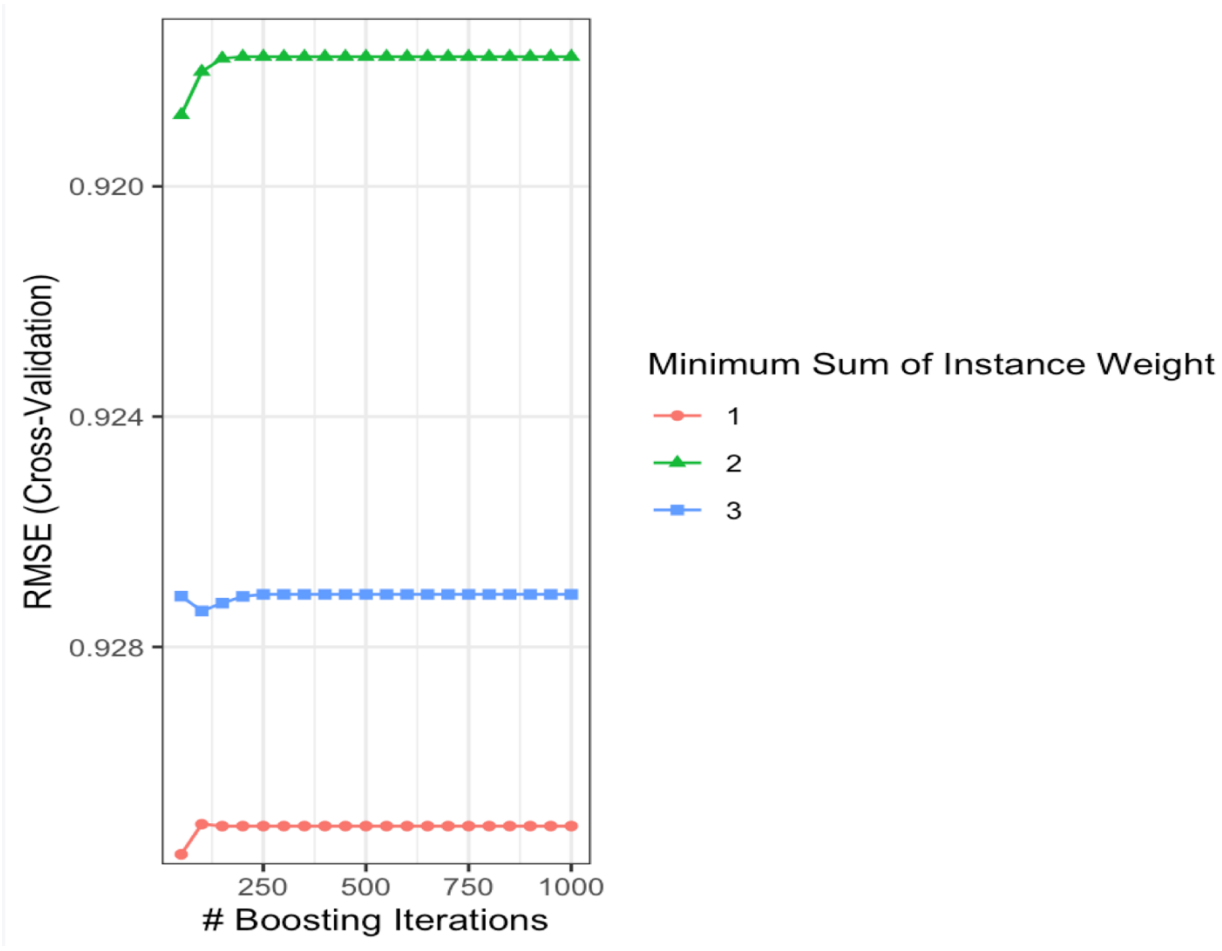


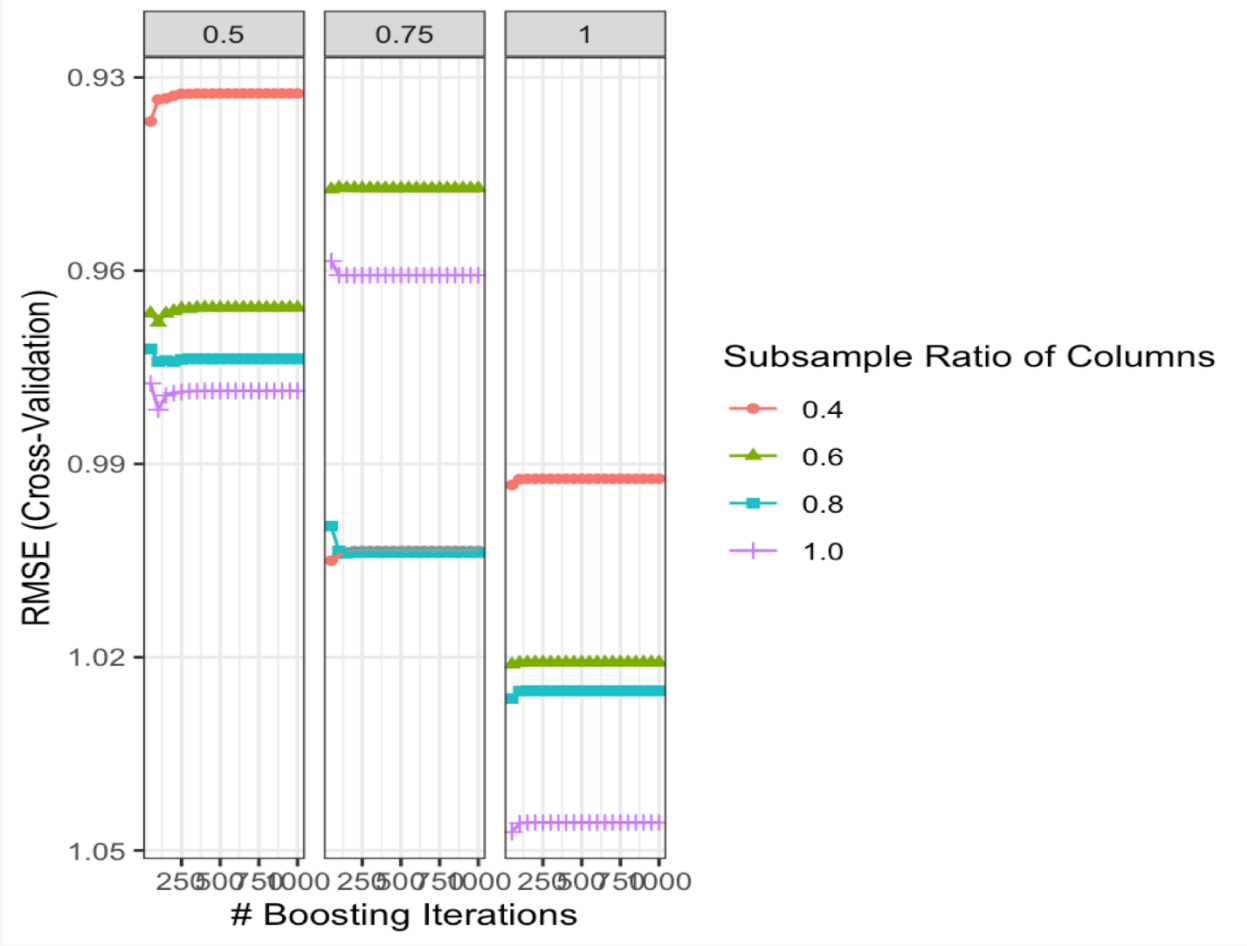
Figure 4: Correlation of named plasma proteins associated with lactulose-rhamnose ratios and previously documented determinants of enteric permeability.

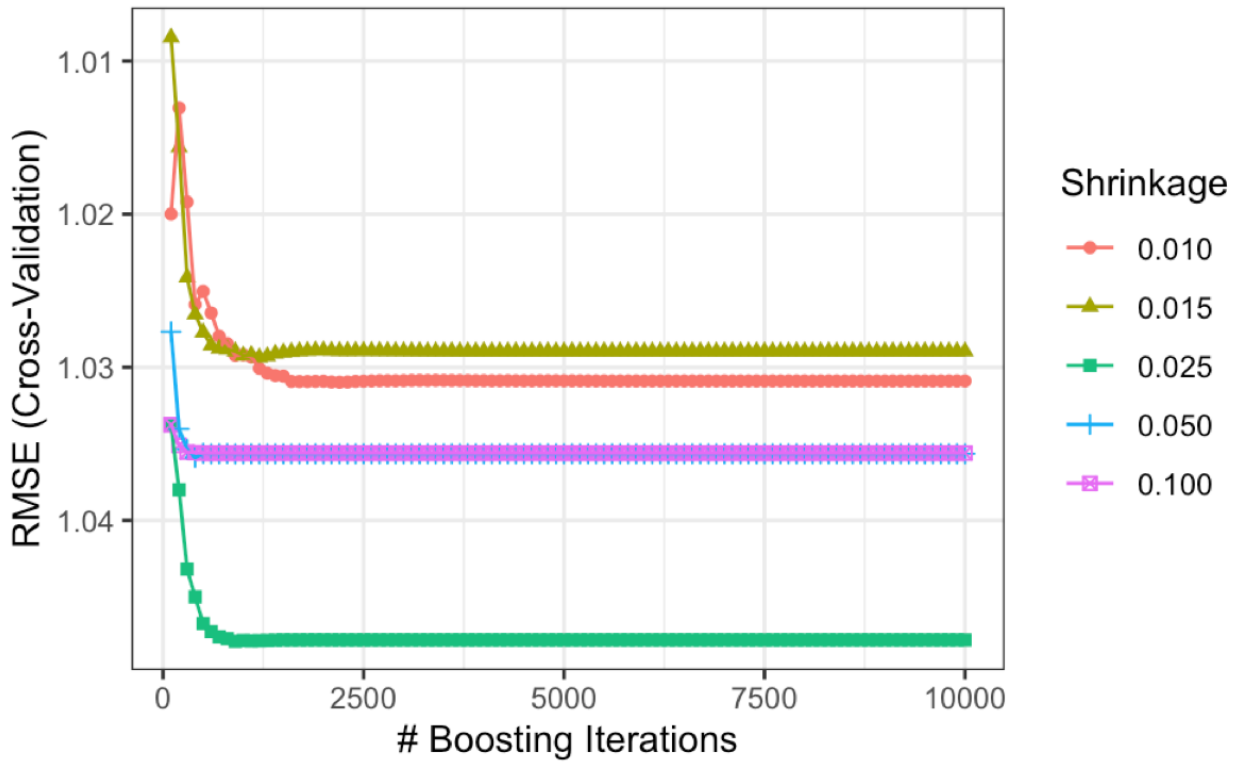


Appendix 1: Model tunning




RMSE (Cross-Validation)
0.920
0.924
0.928
250
500
750
1000
Boosting Iterations
Minimum Sum of Instance Weight
1
2
3


0.5
0.75
1
0.93
0.96
0.99
1.02
1.05
RMSE (Cross-Validation)
Boosting Iterations
Subsample Ratio of Columns
0.4
0.6
0.8
1.0


RMSE (Cross-Validation)
1.01
1.02
1.03
1.04
0
2500
5000
7500
10000
Boosting Iterations
Shrinkage
0.010
0.015
0.025
0.050
0.100

Appendix 2: Common functions of the proteins in the top predictors of lactulose-rhamnose ratio as listed in UniProtKB database.

Protein	Select UniProtKB Biological Function[1]													
	Cell adhesion	Cell growth/ migration	Extra cellular matrix	Leukocyte migration	Platelet aggre-gation	Platelet degran-ulation	G- bacterium defense	Gluconeo-genesis	Raising blood pressure	Antigen response	G+ bacterium defense	Bone formation	Innate immunity	Animal protein hydrolysis
Integrin alpha-IIb: beta-3 complex	■		■	■	■	■								
P-selectin[2]	■			■	■	■	■							
Junctional adhesion molecule-like[2]	■			■										
Glucagon								■		■				
Tyrosine-protein kinase fgr				■						■	■	■	■	
Beta-Ala-His dipeptidase[3]														■
Teratocarcinoma-derived growth factor 1		■												
Regenerating islet-derived protein 3-alpha[2]	■	■									■		■	
Ly6/PLAUR domain-containing protein 3	■	■	■											
Protein FAM3D								■						
Fibroblast growth factor 2		■	■	■										
Renin									■					
Endothelial cell-selective adhesion molecule	■			■										
Procollagen-lysine2-oxoglutarate 5-dioxygenase 3			■											

[1]Some functions omitted if not relevant to this analysis or condensed into these larger headings.
[2]P-selectin, Regenerating islet-derived protein 3-alpha, and Junctional adhesion molecule-like are mediators of cell-cell adhesion but primarily as part of leukocytes adherence and migrating through endothelial and interstitial tissues.
[3]Beta-Ala-His dipeptidase preferentially hydrolyses carnosine, an animal source protein.
Abbreviations: G+: Gram Positive, G-: Gram Negative, Misc: Miscellanous – this protein is generic marker of protein synthesis.

Conclusion

Data from multiple community cohorts suggest ED is an important cause of impaired growth and morbidity among children living in LMIC settings.[1–3] In many of these studies, the impact of ED appears to be mediated by systemic inflammation. This dissertation aimed to establish if ED, measured by enteric permeability, could also be a major determinant of growth failure during the post-discharge period, and if it could be an important interventional target for improving outcomes during the recovery from severe childhood illnesses. Our analyses show children at hospital discharge have worse enteric permeability than community peers, and that this relationship was strongly confounded by poor nutritional status, most prominently childhood wasting. However, we found absolutely no evidence to suggest that enteric permeability among hospitalized children was associated with poor post-discharge growth. Further, we found the relationship between enteric permeability and systemic inflammation to differ between hospitalized and community peers, with the effect estimates suggesting a much stronger association among the community group. Children admitted to health facilities with acute illness have multiple reasons for the elevated systemic inflammation, and in the context of severe childhood illnesses, it appears that enteric permeability may not be a major determinant of systemic inflammation.

Systemic inflammation is not the only mechanism that could link ED to poor outcomes during childhood, so we cannot entirely dismiss ED as an important determinant of post-discharge outcomes. Malabsorption and enteric source sepsis are both thought to be associated with ED, and could still link ED to adverse outcomes.[4–7] In addition, ED may become more important in later phases of the post-discharge period, as the children's acute phase response to the severe illness begins to wanes. These alternative mechanisms should be further explored to better capture the full effects of ED during recovery from acute illness.

While our results cast some doubt over the link between enteric permeability and post-discharge outcomes, we did find evidence that enteric permeability may be associated with systemic inflammation in the community group. These data support the findings of recent community cohorts that suggest ED is an important underlying determinant of health among children without acute illnesses.[2,8–10] Our data contribute to these findings and suggest management strategies targeting ED may be better suited to children in the community, rather than children being discharged from hospital. Child health platforms, including the Community Management of Acute Malnutrition (CMAM) and six or nine-month child health visits, may be valuable touch points to deliver such interventions. Many studies have found a strong association between nutritional status and enteric permeability, particularly wasting, and it has been suggested that ED may be a determinant of slowed recovery during CMAM management.[11–16] This suggests that the CMAM platform may be well suited to delivering interventions to children at high risk of ED. Our machine learning analysis also noted a correlation between breastfeeding and consumption of family foods, with plasma biomarkers of enteric permeability. Multiple previous studies have found suboptimal breastfeeding to be associated with higher enteric permeability.[17–20] Weaning often begins around six-months of age, after which time there are two routine health visits in ED endemic settings: first, to deliver vitamin A at 6-months and second, the measles, mumps and rubella vaccine around 9-months. These platforms may be useful for implementing novel interventions.

Our third paper presented a hypothesis-free effort to understand the proteomic signatures of enteric permeability in blood plasma. We found plasma proteins to be weak predictors of enteric permeability, as measured by the lactulose-rhamnose ratio (LRR). This finding may a degree of misclassification in LRR's ability to accurately measure enteric permeability, or it may indicate that only very severe enteric permeability has systemic ramifications. However, our machine learning model did identify several biomarkers closely linked to the proposed etiology and pathology of environmental enteric dysfunction, a common etiology of ED – including biomarkers of leukocyte invasion, tissue repair, and response to bacterial infections.[4,21,22] As noted above, there were also strong correlations between known risk factors for environmental enteric dysfunction: including age, suboptimal breastfeeding, and nutritional status and the plasma proteins our model found to be associated with enteric permeability.

Our study had several strengths, including a highly standardized protocol of LRR testing implemented in two different settings, rigorously collected inpatient and post-discharge data, and state-of the art proteomic analysis of blood plasma. However, we also noted several limitations that span our three analyses. Foremost among these is the observational nature of our data. We are working to integrate assessments of ED into a recently funded clinical trial (the LactoLyze study: NICHD R01HD103642), which will test two breast-milk derived proteins (lactoferrin and lysozyme) as a method of treatment and secondary prevention for children with wasting and medically attended diarrhea. While enteropathy is not the primary target of these interventions, by reducing the burden of enteric pathogens, we may be able to correlate changes in gut health with the subsequent incidence of childhood morbidity and mortality. Our findings are also limited by a degree of misclassification that is inherent to the LRR test,[23] which suggests it will be important to validate our findings using other approaches to measuring enteropathy in these populations. Accordingly, we have been working closely with the International Center for Diarrheal Disease Research, Bangladesh (icddr,b) and the Center for Microbiological Research at the Kenya Medical Research Institute (KEMRI) on a case-cohort analysis of fecal biomarkers of enteric inflammation in the CHAIN Cohort. This fecal biomarker work offers an important opportunity to validate our findings.

In conclusion, children leaving hospital have greater enteric permeability than their community peers. However, among these hospitalized children, enteric permeability was not associated with systemic inflammation at the point of discharge and was not associated with subsequent growth. These data cast doubt on the degree of benefit that children at hospital discharge are likely to receive from therapeutics targeting ED. Instead, these data suggest resources may be better spent on developing ED interventions for comparatively healthy children in the community.

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