MODIFYING INTESTINAL INTEGRITY AND MICROBIOME IN SEVERE MALNUTRITION WITH LEGUME-BASED FEEDS: refined feed and intervention study [MIMBLE 2.0 Study]

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ABSTRACT

Changes in intestinal mucosal integrity and gut microbial balance occur in severe malnutrition(1) resulting in treatment failure and adverse clinical outcomes(2, 3). Both gram-negative sepsis and diarrhoea are common co-morbidities and are key risk factors for poor outcome. Recent evidence demonstrates that the epithelial barrier can be supported by short chain fatty acids derived from microbiota fermentation of fermentable carbohydrates(4). Diarrhoea related to transient lactose intolerance due to loss of intestinal brush border lactase, has been proposed to be a major complicating factor in severe acute malnutrition (SAM)(5). We propose that nutritional flour comprising of a high fermentable carbohydrate content, added to a lactose-free version of standard F75/F100 nutritional feeds will enhance epithelial barrier function in malnourished children, and reduce incidence of and promote resolution of diarrhoea. To provide proof of this principal we will conduct a refinement of the MIMBLE pilot study. MIMBLE 2.0 will examine differing treatment responses to standard WHO recommended nutritional feeds (F75/F100) to chickpea flour (resistant starch source) containing, lactose-free alternatives. We will assess the effects on gut microbiota, host metabolism, gut inflammation (faecal calprotectin), and clinical outcomes.

We hypothesize that, if introduced early in the management of malnutrition, such lactose free, fermentable carbohydrate based feeds, could safely and cheaply improve global outcome by reducing lactose intolerance-related diarrhoea, improving mucosal integrity and enhancing immunity, and limiting the risk of systemic infection and associated broad spectrum antibiotic resistance.

BACKGROUND

Severe malnutrition and the Gut

Severe acute malnutrition (SAM) accounts for 1.5 million deaths worldwide annually(6). Undernutrition, highlighted in a Lancet series,(7) is a greatly neglected area of research despite contributing to ~60% of childhood deaths. Community-based care with ready-to-use therapeutic food for children with *uncomplicated* malnutrition has dramatically improved recovery rates; but little progress has been made for children hospitalised with SAM in whom mortality remains high.(8) Diarrhoea complicates 65% of these children and is associated with high morbidity and mortality (20%).(5, 8) SAM causes disruption of normal intestinal flora(9), increased turnover of vital nutrients,

disruption of gut barrier function, impaired mucosal immunity and increased risk of gram negative bacteraemia,(5) which together form a vicious cycle(10).

Lactose in SAM

The current recommended formulae by WHO, initially F75 followed by F100, are milk-based feeds and their major carbohydrate source are disaccharides (a mixture of maltodextrin, sucrose and lactose), which can cause osmotic diarrhoea(11) since they normally are hydrolysed by disaccharidases localized at the tip of small intestinal villi. Both lactose intolerance(12, 13) and intestinal atrophy have been demonstrated in children with SAM(14-16). Removing lactose and replacing with better tolerated simple carbohydrates that do not rely on brush border enzymatic activity for digestion and absorption, may be highly beneficial in preventing and ameliorating diarrhoea in SAM.

Benefits of non-digestible fermentable carbohydrates

Numerous studies have shown that intestinal mucosal integrity and gut microbial balance can be restored by inducing fermentation in the gastrointestinal tract(17). Fermentable carbohydrates are increasingly being investigated as potential adjuncts to improve the balance of normal gut flora and positively influence the immunological and metabolic function of the gut(18). Non-digestible fermentable oligosaccharides induce favourable colonic microbiota fermentation(19) leading to the generation of short-chain fatty acids which have a positive influence on gut integrity and nutritional health by improving energy yield, production of vitamins and the stimulation of gut homeostasis, including anti-pathogen activities(20, 21). Preliminary data collected from children with SAM by a multi-disciplinary team at Imperial College (SMIP study) involving specialists in international child health (Prof K Maitland), nutrition (Prof G Frost), metabonomics (Prof E Holmes) and colonic bacterial metataxonomics (Prof J Marchesi) suggest that there are changes in the inter-relationships of normal gut microflora, intestinal permeability, and markers of gut barrier dysfunction and immune dysregulation in SAM which effect clinical outcome (manuscript in preparation) and are therefore potential targets for intervention.

Sources of non-digestible fermentable carbohydrate

Legumes, which contain a mixture of different types of non-digestible fermentable carbohydrates, have been demonstrated to augment microbial communities that enhance short chain fatty acid production(22). Furthermore, these legumes form part of the traditional East African diet consumed by children(23) and a number of current research programmes in East Africa are expanding legume

growth to improve the environmental impact of agriculture (nitrogen fixing)(24), meaning that this treatment will be both acceptable and readily available to local communities. One potential commonly consumed food with prebiotic effect is chickpea. Chickpea-based follow-on formulae have been explored as a potential prevention for undernutrition (25).

Summary & Importance

There are multiple reasons why nutritional support, at an early stage in the management of SAM, tailored towards prevention of diarrhoea and restoring gut mucosal integrity and manipulation of the gut microbiome could enhance resilience to secondary infection and morbidity during re-feeding. Legumes, through the support of microbiota networks to produce short chain fatty acids, offer a safe therapeutic adjuvant that is easily and cheaply sourced in East Africa. Additional removal of lactose from therapeutic feeds should reduce incidence of osmotic diarrhoea, and related morbidity.

The planned research will bring new knowledge to the field of severe malnutrition, thereby addressing a major treatment gap, which is a *high priority research issue and a significant barrier to progress in the reduction of mortality.* The study has the potential to suggest new therapies for enhancing recovery of gut mucosal function and resilience to infection through the modulation of the gut microbiome, thereby reducing mortality and morbidity in SAM. As the interventional feed from the original MIMBLE pilot is being refined in conjunction with a specialist food company (Campden BRI), this offers an opportunity to improve the food safety profile, product utility, and further optimise the feed by removing lactose.

DESIGN

OBJECTIVES

To generate additional proof supporting the hypothesis that non-digestible fermentable carbohydrate in the form of chickpea flour will have positive effects on intestinal mucosal integrity and permeability, gut inflammation, gut microbiome and clinical outcomes in children with severe acute malnutrition compared to children receiving standard nutritional feeds. Also to provide evidence that a lactose-free feed will reduce incidence/encourage resolution of diarrhoea, which is a major complicating factor in SAM.

OUTCOME

Primary

Co-primary outcome measures:

1. Change in MUAC at Day 90
2. Survival to Day 90

Secondary

1. Clinical:
    a. Weight gain (moderate to good: >5g/kg/day)
    b. Denovo development of diarrhoea (>3 loose stools/day)
    c. Time to diarrhoea resolution (if >3 loose stools/day)
    d. Time to bi-pedal oedema resolution (for those with Kwashiorkor)
2. Changes in intestinal biomarkers:
    a. Intestinal cell injury (faecal calprotectin)

Supportive biological and physiological data to support end points will include:

A. Anthropometric:
    a. Weight/height/BMI
B. Microbiota:
    a. % change in relative populations of gut microbiota
C. Metabolomics:
    a. Changes in generation of short chain fatty acids
    b. Changes in host and microbiota metabolic products
D. Lactose intolerance:
    a. Evidence of stool reducing substances at baseline

Study location

The study will take place in two locations. At the paediatric ward/nutrition unit at Mbale Regional Referral Hospital, Eastern Uganda and the general paediatric ward at Soroti Regional Referral Hospital (SRRH). The 17-bedded nutrition unit in Mbale admits 300 children each year with severe acute malnutrition, with a similar number being admitted to SRRH . Most of the clinical services provided by the paediatric departments are free of charge to the patients in accordance with the government policy of no cost sharing in public hospitals.

Study Population

160 children aged > 6 months to 5 years

Inclusion criteria:

1. Marasmus defined by mid-upper arm circumference (MUAC) <11.5cm
2. Kwashiorkor defined as symmetrical pitting oedema involving at least the feet irrespective of WHZ score or MUAC
3. Guardian or parent willing/able to provide consent

Exclusion criteria

Children with severe acute malnutrition with a very high risk of death due to a comorbidity eg malignant disease or terminal illness will be excluded.

Study Interventions

Children will be randomly allocated on a 1:1 basis to:

- Standard treatment with WHO feeds (F75/F100) – control arm
- Pre-packaged modified F75/F100 feeds which are lactose free include chickpea (gram) flour as a source of fermentable carbohydrate, altered to maintain equal energy provision as the control arm

Children will otherwise receive the standard inpatient hospital management as per national guidelines.

Sample Size

Mid-upper arm circumference (MUAC) has been selected as the primary criterion for nutritional recovery because it predicts mortality better and is less affected by oedema than other anthropometric measures and is also a good index of muscle mass. A recent trial of antimicrobial prophylaxis in Kenyan children admitted with severe malnutrition baseline mean MUAC was 10.6cm (SD 1.06) and at 90 days, 12.2cm (SD 1.35); a mean change of 1.6cm (SD 1.1) nutritional recovery at 90 days (26).

The overall sample size will be 160 children which will include 80 in each study arm. From the original MIMBLE trial, safety in the study arms were comparable to the standard intervention. As a Phase I study, our pilot is designed to provide adequate data of a proof of principal that the modified nutritional feed provides clinical, physiological and biological evidence of benefit to the child in terms of nutritional rehabilitation and the predicted effect on biological markers of gut-barrier dysfunction and on the intestinal microbiota. Additionally, it will compare the safety and tolerability of the modified nutritional feed against the current standard of care. The data will thus inform the design

and effect size for future pragmatic trials. Formal sample sizes were not calculated; we aim to study 160 in total which is realistic given the timeframe and funding available for the study.

Procedures

Screening procedure

A dedicated trial research nurses will be employed to conduct the study. Eligible children will be identified by the nurse and clinician on duty and registered in the eligibility screening log. A member of the trial team will then perform a structured clinical assessment of:

- WHO criteria to confirm severe acute malnutrition
- Exclusion criteria

Details of those fulfilling the entry criteria will be entered onto a screening form, while reasons for non-eligibility will be added to the eligibility screening log. It is anticipated that this process will take approximately 10 minutes**.**

Consent

Prospective written, informed consent will be sought from parents or guardians of children. Parents or guardians will be given an information sheet in their usual language containing details of this pilot study. The sheet will be read aloud to those who are unable to read. Parents and guardians will be encouraged to ask questions about the trial prior to signing the consent form. For parents who are unable to sign the consent form, a thumbprint will be required and a witness will be required to sign the consent.

Randomisation

Randomisation will be in permuted blocks. Cards with treatment allocation (standard or altered standard with chickpea flour) will be prepared in Kilifi and sent to Mbale. They will be kept in numbered, sealed opaque envelopes, each signed across the seal. The cards will be numbered consecutively and will be opened in numerical order.

Laboratory investigations

Following consent and randomisation, routine admission blood samples will be taken, in accordance to routine clinical practice, for the following investigations: full blood count, clinical chemistry, lactate, glucose, blood culture, malaria status and HIV status. HIV testing is conducted with parental or guardian consent. Pre- and post-test counselling will be done in accordance to routine practice. The total admission blood sampling volume equates to 10mls, the majority of which is for clinical purposes and only includes an extra 3mls for plasma storage. We will measure haemoglobin using bedside

haemocue at 24 and 48 hours as part of routine monitoring. Haemoglobin estimation will be done immediately for patients with de novo signs of deterioration. Routine bloods will also be taken on day 7 and day 28 and day 90 which again will include an extra 3mls for plasma storage. Plasma will be used for short chain fatty acid and metabolite analysis.

At admission, day 7, day 28 and day 90 stool and urine samples will be taken and be stored. The total volume of urine and stool collected across the study will be 30mls and 30g respectively. Stored samples will be used for metabolite analysis (urine and stool), faecal calprotectin (stool), reducing substances (stool) and DNA extraction for 16s rRNA metataxonomics (stool).

All stored samples will be sent to Imperial College, London in order to assess the following:

- **Intestinal cell injury** by faecal calprotectin assays.
- **Changes in gut microbiota** by 16S analysis for species related to gut barrier function e.g. *Bifidobacterium* and *Lactobacillus*.
- **Examination of changes in host and bacterial metabolic products** by Nuclear Magnetic Resonance Spectroscopy and GC-MS.

CLINIAL MONITORING AND OTHER ASSESSMENTS

Clinical monitoring

Clinical monitoring will be in line with standard care at a minimum of twice daily temperature, blood pressure and pulse oximetry.

Anthropometric indices

Children will have admission height/length measured and daily weight and MUAC measurements until discharge performed at the same time each day. Additionally, at approximately this time. These measurements will be repeated on day 28 and day 90.

Stool assessment

Frequency and consistency of stool will be assessed daily until discharge and at day 28 and day 90.

Dietary recall

Children and/or parents/guardians will be asked to complete a 24-hour multi-pass dietary recall via a structured interview to assess the nutritional intake of children on day 28 and day 90 as most children will have progressed onto normal diet with additional RUTF. Intake of standard and modified feeds will be recorded daily during the inpatient episode.

Day 28 and Day 90 Follow-up

Children will be invited to return to the hospital for a follow-up visit on day 28 and day 90 as a day case which will last approximately 2 hours. This will include a clinical review, anthropometric measurements, and the study investigations outlined above.

Standard Case Management- in-hospital

All study patients will be otherwise managed and clinically monitored as per national guidelines; World Health Organisation Guideline- Updates on the management of severe acute malnutrition in infants and children (2013). This includes management of dehydration, hypothermia, hypoglycaemia, dermatosis, treatment of infection, eye care, anaemia and vitamin and mineral supplementation. The criteria to transition from F75 to F100 will be as per national guidelines. The discharge criteria from hospital will also be as per national guidelines.

Assessment & Investigation Flow Chart

Time	**Adm**	**Day 1**	**Day 7**	**Day 28**	**Day 90**	**Discharge**
Consent + patient information leaflet	X					
Anthropometric (weight, MUAC)	Daily as inpatient			X	X	X
Height	X			X	X	X
Clinical observations	Daily as inpatient			X	X	X
Fluid balance	Daily as inpatient					X
Stool Assessment	Daily as inpatient			X	X	X
24-Dietary recall	X			X	X	
Length of stay						X
Laboratory investigations						
Haematology	X	X	X	X	X	
Biochemistry	X	X	X	X	X	
Glucose	X	X				
Lactate	X					
Malaria test	X					
HIV testing	X					
Urine dipstick (Multi-stick)	X					
Stool culture/microscopy	X					
Stored samples						
For Metabolomics and SCFA (NMR, GC-MS)						
Plasma		X	X	X	X	
Urine		X	X	X	X	
Stool		X	X	X	X	
For Metataxonomics (16s rRNA)						
Stool		X	X	X	X	
For Intestinal Cell Injury (Faecal Calprotectin)						

Stool		X	X	X	X	

SAFETY REPORTING

All relevant adverse events will be reported in the case report form (CRF) and serious adverse event (SAE) forms. The reporting procedure will be captured within a dedicated safety reporting SOP. At each clinical review the child will be assessed for adverse events, both expected and unexpected. Non-serious and expected events will be routinely captured in the CRF.

Serious adverse events (SAEs)

Serious adverse events will be reported immediately to the on-site PI so that co-PIs may be notified of the event. Serious adverse events include:

1. Any untoward medical occurrence or effect that is: **1]** fatal, **2]** life threatening, **3]** permanently or temporarily disabling or incapacitating, **4]** causes prolongation of hospital stay (*if in the clinician's judgment the adverse event causes the child to stay in hospital longer than would have been necessary if the adverse event had not occurred*.) or **5]** any other event that investigator considers serious, having a real, not hypothetical, risk of one of the previous outcomes.
2. Allergic reactions will automatically be considered as SAEs in this pilot study.

A dedicated SAE form will be completed including details of the nature of event, date of onset, severity, corrective therapies given, outcome and causality (i.e. unrelated, unlikely, possible, probably, definitely). This data will be reviewed as part of the study monitoring process.

PARAMETER	GRADE 1 MILD	GRADE 2 MODERATE	GRADE 3 SEVERE	GRADE 4 LIFE-THREATENING/FATAL
ESTIMATING SEVERITY GRADE				
Clinical adverse event NOT identified elsewhere in the grading table	Symptoms causing no or minimal disturbance with current illness	Symptoms causing greater than minimal disturbance with current illness	Symptoms requiring corrective medical treatment	Symptoms requiring medical treatment to prevent permanent impairment, persistent disability, or death
ALLERGIC REACTION				
Acute systemic allergic reaction	Local urticaria or flushing with no medical intervention indicated	Allergic reaction: Fever, chills, flushing, limited pruritic rash, nausea and vomiting without generalised angiooedema or brochospasm	Grade 2 reaction PLUS Generalized urticaria OR angioedema with medical intervention indicated OR Symptomatic mild bronchospasm	Grade 3 reaction PLUS Acute anaphylaxis OR Life-threatening bronchospasm OR laryngeal edema

TRIAL MONITORING

This pilot study will be monitored locally, by designated GCP trained and experienced monitors, through assessment of any SAE forms and accumulation of data collected.

DATA MANAGEMENT and STORAGE

Patient admission data collection forms and observation charts will be kept in a locked cupboard. Designated members of the research team will transcribe relevant data from the source documents to the CRF. Data entry will be done through OpenClinica, an FDA-approved, web-based application, to a relational PostgreSQL database. Security is enforced through authentication of users by use of encrypted passwords. Different access level accounts authorize users on actions they may perform on the database.

Analysis Plan

Data will be analysed on an intention-to-treat basis. Differences in outcome measures between the three treatment groups will be compared with ANOVA or linear regression as appropriate. Where variables are not normally distributed, they will be transformed by taking logarithms. If this is insufficient, then equivalent non-parametric tests will be used. Metabolomic data will be analysed using multivariate statistical techniques including principal components analysis (PCA), projection to latent structures (PLS) and orthogonal PLS (OPLS).

Time Frame/Duration of the Project

Development of the modified feed will begin in August 2017. The study commenced at Mbale RRH in June 2018. We aimed to recruit over a period of 4-6 months (to December). Owing to slow recruitment (34 of 160 children on 1st October 2018) we are opening recruitment at an additional site at SRRH. Primary data analysis and report writing will take a further 4-6 months.

ETHICAL CONSIDERATIONS

For children and/or their parent/guardians

Potential risks:

There are very few risks attached to this study. Initially cowpea was targeted as a locally available legume which contains adequate amounts of the resistant starch of interest to promote gut microbial health. Two significant barriers to their use in the current study were encountered: i) on testing a final cowpea-enriched feed product was found to have significantly less resistant starch than anticipated, and ii) no supplier could be identified who met the strict food safety standards required with respect to certification for contaminants including pesticides, toxins, microbes and metals. Another locally

available alternative, although eaten less frequently in Uganda, is chickpea, which on testing was found to retain its resistant starch content throughout processing and chickpea (gram) flour is readily available from reputable suppliers. Chickpea production in Uganda in 2016 was estimated to be 5085 tonnes in 2016 by the Food and Agricultural Organization of the United Nations, increasing at approximately 100tonnes (2.2%) each year since 2010. Details of the processing and packaging will be addressed by due diligence of the experienced collaborator (Campden BRI). The chickpea flour based interventions we propose are used within the local diets but have been milled/processed in such a way to maximise their ability to ferment within the bowel to create a favourable microbiome. Intolerance or allergy is rare. The G6PD variant in these populations retains >12% of its activity thus not rendering G6PD deficient patients to susceptibility to oxidant stress (as seen in Mediterrean variants rendering them susceptible to 'Favism').

Blood samples are required as part of this study; this includes routine bloods according national guidelines and an additional 12mls over the whole duration of the study. Required volumes of blood will be minimized wherever possible and be within the locally agreed maximum**.** Urine and stool will be collected in a non-invasive manner thus should not cause any distress to the child or their family.

Potential benefits:

The direct benefits to the child and/or family include:

- Closer observation during admission which consequently may allow the clinical team to make important changes to the child's treatment during in hospital stay.
- All routine non-study medications required by the hospital to treat the child will be made available.
- The parents or guardians for the children will be asked to return for follow up at 28 days after admission. This additional visit will include a clinical review thus an opportunity for medical treatment if required. Education regarding nutrition will also be readdressed at this time. Reimbursement for transport cost for this follow-up visit plus any treatment costs required during the visits will be made.

Informed consent:

Written informed consent will be sought from parents or guardians who will receive an information sheet with details of who to contact if they have any further questions. The study team will be given training in procedures for requesting consent from parents/guardians. Parents and guardians will be

continually be able to ask questions and will be provided with information in an appropriate form accordingly.

For Researchers

Potential Risks:

No risks are anticipated other than potential hazards associated with needle stick injuries and biological hazards. All trial personnel will receive pre-study training regarding safe phlebotomy practice and management of needle stick injuries. Any researcher who is required to do laboratory work will have safety and procedural training specific to the laboratory, as per Imperial College policy.

Potential benefits:

Involvement will contribute to the personal professional development of both study and clinical team members. This specifically includes experience in running the study, for example clinical trials training and research training.

Compensation/reimbursement

All costs for clinical tests and for the routine medication that is not available in the hospital at the time of admission will be covered by the study. Any additional procedure required due to a complication or a potential complication will be covered by the study. The children plus their parents/guardians will be asked to return for follow up at the nutrition unit on day 28 and day 90. Reimbursement for transport cost after discharge and for follow up visits plus any treatment costs required during the visits will be made.

Confidentiality

Information will be stored securely and will only be made available to those caring for the child and those directly involved in the study. Any reports will not mention the names of the child or parent/guardian.

Use of stored samples

Blood, urine and stool samples will only be used for the purpose of this study.

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Indemnity

Imperial College London holds negligent harm and non-negligent harm insurance policies, which apply to this study.

Commercial Gain

None to declare.

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