SEVERE ANAEMIA IN SUB-SAHARAN AFRICAN CHILDREN:

THE ROLES OF NUTRITION, METABOLISM, AND THE GUT MICROBIOTA IN RECOVERY

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Declaration of Originality

I certify that this report and the research to which it refers are the product of my own work, and any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard reference practices of the discipline.

Disclosure Statement

All TRACT study clinical samples and dietary data were collected by the study research teams based in Mbale Regional Referral Hospital and Soroti Regional Referral Hospital, coordinated by Rita Muhindo and Dr Sophie Uyoga. The 24hr dietary recall tool used in the TRACT study was developed and validated in conjunction with Dr Helen Nightingale. Dietary data were initially uploaded to a secure OpenClinica database by Mudoola Macreen and then Ruth Adong, and secondary input and coding in Dietplan v6 software were completed by the author. Urine samples were prepared and analysed by the author, while plasma samples were prepared with the aid of an automated Gilson Robot operated by Rose Tolson and Nikita Harvey in the Division of Computational and Systems Medicine at Imperial College London, and analysed by the author. Faecal DNA extraction, quantification and dilution were completed by the author, while the 16S rRNA library formation and sequencing were completed by Dr Julie MacDonald, Imperial College London. All statistical analyses were completed by the author. Professor Elaine Holmes and Dr Isabel Garcia-Perez provided assistance with ^{1}H NMR metabolite identification.

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ABSTRACT

Severe anaemia (Hb<5.0g/dl) affects almost 1 in 20 children in sub-Saharan Africa, and up to 1 in 4 child hospital admissions in this region (Muhe et al., 2000; Kiggundu et al., 2013). Micronutrient deficiency, malaria, infections and genetic factors contribute to its development. Severe childhood anaemia is associated with a high risk of mortality (19% within 6 months) (Phiri et al., 2008) and survivors experience long term health effects. Bacterial co-infection from gut-derived pathogenic bacteria is a frequent cause of death (Church and Maitland, 2014). Diet, altered metabolism, and the gut microbiota are thus implicated in severe anaemia, however interactions between these factors are poorly understood.

The Transfusion and treatment of severe anaemia in African children study (TRACT) which assessed optimum transfusion dose, micronutrient supplementation, and prophylactic antibiotic treatment provided an opportunity to address these factors. The aim of this study was to characterise the diet of children in Eastern Uganda, and assess the impact on recovery from anaemia. It also aimed to assess whether interactions between diet and host metabolism or the gut microbiota were significantly altered in severely anaemic children, and whether changes in these were associated with recovery.

A pilot study (n=19) was conducted to validate a 24-hour dietary recall method, which showed that the method had low inter-assessor variability, and high validity when compared to weighed food records. A subset (n=339) of children from the TRACT study in eastern Uganda were recruited to undergo dietary assessment and provide urine, plasma and faecal samples at admission, day 28 and day 180. A majority of children had diets which lacked diversity, reliant on a single carbohydrate-rich but micronutrient-poor food source. Food intake on admission was restricted in energy and micronutrients and was inadequate for a majority of children, but quantity and diversity increased as children recovered.

Urine and plasma metabolic profiles reflected acute inadequate energy intake on admission (ketosis) and metabolic stress associated with critical illness (glycosuria and lipoprotein dysregulation), in addition to condition-specific effects including malaria-associated hyperphenylalaninaemia. These metabolic derangements resolved with treatment as children recovered. The gut microbiota of children in this study on admission lacked diversity and was dominated by potentially pathogenic *Enterobacteriaceae* and *Enterococcus faecium*. High levels of antibiotic use, in addition to low dietary diversity and dietary fibre intake contributed to reduced diversity and richness in species observed.

Alterations in diet, metabolism and the gut microbiota were identified in this study in severely anaemic children, reflecting the causes and severity of this condition. Potential routes to reduce the impacts of these derangements were identified for future research.

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ABBREVIATIONS

1D – One Dimensional

^{1}H – Proton

24hrDR – 24 hour dietary recall

2D – Two Dimensional

BCAA – Branched Chain Amino Acid

BMI – Body Mass Index

BMIFAZ – Body Mass Index-for-Age Z-score

CI – Confidence Interval

CPMG – Carr-Purcell-Meiboom-Gill

DMT1 – Divalent Metal Transporter 1

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

Fe - Iron

G6PD – Glucose-6-phosphate dehydrogenase

HAZ – Height-for-Age Z-score

Hb - haemoglobin

HIV – Human Immunodeficiency Virus

IF – Intrinsic Factor

INF-α – Interferon alpha

JRES – J-resolved

NADH – Nicotinamide adenine dinucleotide (reduced)

NMR – Nuclear Magnetic Resonance

OPLS – Orthogonal Partial Least Squares

OPLS-DA – Orthogonal Partial Least Squares Discriminant Analysis

OTU – Operational Taxonomic Unit

PABA – Para-aminobenzoic acid

PC – Principal Component

PCA – Principal Components Analysis

PCFT – Proton Coupled Folate Transporter

Phe – Phenylalanine

QC – Quality Control

RFC – Reduced Folate Carrier

RNA – Ribonucleic acid

rRNA – Ribosomal ribonucleic acid

RNI – Reference nutrient intake

SAM – severe acute malnutrition

SACN – Scientific Advisory Committee on Nutrition

SD – Standard Deviation

SLC46A1 – Solute Carrier Family 46 Member 1

STOCSY – Statistical Total Correlation Spectroscopy

STORM – Subset Optimization by Reference Matching

TRACT – Transfusion and Treatment of severe Anaemia in African Children: randomised Controlled Trial

Trp – Tryptophan

TSP – Trimethylsilyl Propionic Acid

Tyr – Tyrosine

WAZ – Weight-for-Age Z-score

WHZ – Weight-for-Height Z-score

WHO – World Health Organisation

1. CHAPTER 1 - INTRODUCTION

1.1 GENERAL INTRODUCTION

The burden of childhood diseases, particularly those caused by inadequate nutrition and infection, is predominantly borne by developing regions such as sub-Saharan Africa, and South Asia (Kassebaum, 2016). Severe anaemia is one of the most common and fatal childhood conditions in these regions, and while treatments for mild or moderate anaemia have been successful, children who present with severe anaemia often fail to recover. An estimated 19% of children treated for severe anaemia die during admission or within 6-months of discharge (Brabin et al., 2001; Phiri et al., 2008). In addition to high fatality rates, the cognitive and physical development of children who do survive are permanently affected, negatively impacting potential (Martins et al., 2011; Jauregui-Lobera, 2014). Attempts to understand poor treatment responses have so far failed to deliver improve outcomes, but the application of '-omics' technologies presents renewed opportunities.

Adequate nutrition is a necessary prerequisite for recovery in severe anaemia. Nutrition also has profound effects on metabolism and immunity, and the gut microbiota, yet many studies fail to account for variations in diet. Although our understanding of the role of the gut microbiota in heath and disease and its interaction with human metabolism is still in its infancy, there is reason to believe that gut microbes play important roles in morbidity and treatment response in severe anaemia. Invasive bacterial infections caused by translocation of gut-derived non-typhoidal *Salmonella* are an important cause of death in severely anaemic children, especially those with malaria (Church and Maitland, 2014). Severe malnutrition is common in this region and undernourished children have significantly altered balances of gut microbial species, namely expansion of *Proteobacteria* and contraction of *Bacteroides* and *Firmicutes*, which may affect local gut immunity and response to nutritional therapies (Smith et al., 2013). A combined approach which addresses variations in diet, the gut microbiota, and host metabolism could help to explain poor treatment response in childhood.

Nutrition, metabolism and the gut microbiota are intrinsically linked, yet the ability to effectively assess each and the interplay between them has only recently become possible with the development of '-omics' technologies including metabonomics and metataxonomics. The aim of this thesis is to generate increased understanding of the roles of these three factors in the recovery from severe anaemia in children in sub-Saharan Africa.

1.2 Severe childhood anaemia

Anaemia describes a reduced capacity for blood oxygen transport, and is the product of one, or a combination of the following processes: impaired erythrocyte production, haemolysis, or loss of blood. Diagnosis and severity are classified by circulating haemoglobin concentration, summarised in Table 1.1, defined at sea-level altitude. Although the WHO provides a definition of severe anaemia of Hb<7 g/dl in children under 5 years old and Hb<8.0g/dl in children 5-14 years old (World Health Organisation, 2011b), this is not universally agreed, with many groups instead quoting <5.0g/dl as diagnostic for severe anaemia (Calis et al., 2008; van Hensbroek et al., 2010). Unless otherwise specified, the latter definition for severe anaemia is used throughout this thesis.

Table 1.1 Haemoglobin levels (g/dl) for diagnosis of anaemia in children (adapted from (World Health Organisation, 2011b))

		Anaemia		
Age	Non-anaemic	Mild	Moderate	Severe
6 months to 5 years	≥11.0	10.0-10.9	7.0-9.9	<7.0 or <5.0
5 to 11 years	≥11.5	11.0-11.5	8.0-10.9	<8.0 or <5.0
12 to 14 years	≥12.0	11.0-11.9	8.0-10.9	<8.0 or <5.0

1.2.1 Epidemiology

Pre-school age children (0-5yrs old) have the highest prevalence of anaemia (Hb<11.0g/dl) of all age groups, with 41.7% affected globally in 2016 (World Health Organisation, 2016). In the same report, pre-school children in sub-Saharan Africa and South Asia had the highest prevalence, at 61% and 55% respectively.

Stevens et al. (2013) provide the most detailed overview of the global and regional prevalence of severe anaemia in pre-school age children, using the WHO definition of <7g/dl. They estimate that in 2011, between 2.5-4.9% of children in sub-Saharan Africa were severely anaemic, with the next highest region being South Asia with 2.1%. In both regions the prevalence has significantly decreased from 1995 to 2011: in sub-Saharan Africa by 4.8-7.7%, and in South Asia by 3.8%. In the annual World Malaria Report, the WHO also reported the average prevalence of severe childhood anaemia in 16 sub-Saharan African countries to be 3%, rising to 8% in those where malaria is endemic (World Health Organisation, 2018b).

Using the stricter definition of <5.0g/dl, studies conducted in sub-Saharan African countries report between 1-4% of children in the community to be severely anaemic (Muhe et al., 2000; Desai et al.,

2002). Despite decreasing prevalence, severe anaemia remains an important cause of hospitalisation (Obonyo et al., 2007; Phiri et al., 2008; van Hensbroek et al., 2010). Between 10-27% of hospitalised children in sub-Saharan African countries are severely anaemic (Weber et al., 1997; Biemba et al., 2000; Muoneke et al., 2012; Kiggundu et al., 2013), and they are known to have increased risk of death from all causes (Scott et al., 2014; Kassebaum, 2016).

1.2.2 Aetiology

Some individual and societal factors are associated with an increased likelihood of a child developing anaemia regardless of geographic region: being aged less than 2 years old (Gebreegziabiher et al., 2014; Semedo et al., 2014; Khan et al., 2016; Ray et al., 2016; Kuziga et al., 2017; Ncogo et al., 2017), having an anaemic mother (Khan et al., 2016; Ray et al., 2016; Harding et al., 2018), low maternal education level (Kuziga et al., 2017; Nguyen et al., 2018), low household income/socioeconomic status (Gebreegziabiher et al., 2014; Ncogo et al., 2017; Nguyen et al., 2018), poor sanitation (Semedo et al., 2014; Nguyen et al., 2018), and being underweight or stunted (Gebreegziabiher et al., 2014; Kuziga et al., 2017; Legason et al., 2017; Harding et al., 2018). There is less published literature on the direct causes of severe anaemia, however Calis et al. (2008) reported that bacteraemia (OR 5.3, 95% CI 2.6-10.9), hookworm infection (OR 4.8, 95% CI 2.0-11.8), vitamin A deficiency (OR 2.8, 95% CI 1.3-5.8), low maternal education level (OR 2.5, 95% CI 1.5-4.1), glucose-6-phosphatase deficiency (OR 2.4, 95% CI 1.3-4.4), malaria (OR 2.3, 95% CI 1.6-3.3), vitamin B12 deficiency (OR 2.2, 95% CI 1.4-3.6) and human immunodeficiency virus infection (OR 2.0, 95% CI 1.0-3.8) were associated with severe anaemia. These factors are likely to vary significantly regionally, but many appear to be clustered in sub-Saharan Africa.

The underlying causes of anaemia can be divided into several broad classes: nutritional, infection-related, genetic disorders and haemoglobinopathies, and chronic disease-related. Frequently, and particularly in severe anaemia, the aetiology is multi-factorial (van Hensbroek et al., 2011).

1.2.2.1 Nutrition

1.2.2.1.1 Energy and protein

All micronutrient requirements must be met within the volume of food that meets energy needs. Energy intake is highly correlated with intake of macronutrients (carbohydrate, protein, and fat), the B group of vitamins, and most minerals (Livingstone and Black, 2003). For other vitamins and minerals, total energy intake is less predictive of adequacy since dietary sources tend to be concentrated in specific foods, e.g. vitamin B12 is found almost exclusively in animal source foods. Due to the correlation of energy, macronutrients and micronutrients, it is difficult to assess the direct roles of energy and protein in anaemia pathogenesis in the absence of micronutrient deficiency. Some attempts to do so have been made in animal studies, which suggest that protein-energy malnutrition results in changes in red blood cell morphology leading to haemolysis, reduced red blood cell survival, and reduced red blood cell production (Macdougall et al., 1982; Warrier et al., 1990). In practice it is unlikely for protein-energy malnutrition to occur in the absence of micronutrient deficiencies.

1.2.2.1.2 Iron

Iron has the capacity to exist in numerous oxidation states: in cells most commonly as ferrous iron (Fe^{2+}) or ferric iron (Fe^{3+})(SACN, 2010). Due to its redox potential, iron is ubiquitous in all living organisms, and is used in various electron donation and acceptance reactions. In humans the principal use of iron is in the oxygen-transport protein, haemoglobin (Worwood, 2017). Approximately 65-85% of the 3500-4500mg of iron in an adult male is incorporated into circulating haemoglobin, and a further 8-11% in myoglobin which functions as a reservoir of oxygen in muscles (Worwood, 2017). Iron is also required for DNA synthesis and cell proliferation, drug metabolism enzymes and cofactors, and endocrine and neurotransmitter synthesis (Thejpal, 2015).

Since there is no excretory pathway for iron, iron homeostasis is controlled by regulation of iron absorption (Ganz, 2013). Current iron status has a profound effect on dietary absorption of both haem and non-haem iron: high saturation of transferrin with ferric iron (i.e. adequate iron stores) is sensed by hepatocytes via transferrin receptors 1 and 2, which promotes hepcidin expression, causing internalisation of the enterocyte transmembrane protein ferroportin (Pantopoulos et al., 2012; Ganz and Nemeth, 2015). This effectively prevents iron that has been absorbed from the lumen from passing into systemic circulation.

Assessment of iron status is based on the interpretation of multiple parameters, broadly divided into measurements of functional iron (Hb concentration, mean corpuscular volume (MCV), mean

corpuscular haemoglobin), tissue iron supply (transferrin saturation [serum iron/transferrin concentration x 100], zinc protoporphyrin, serum transferrin receptor), and iron in tissues (serum ferritin, transferrin receptor/ferritin ratio, hepcidin) (Zimmermann et al., 2005; SACN, 2010). Markers of functional iron status are not specific to iron, for example microcytosis (MCV <80fL) may be due to iron deficiency, or other causes such as chronic disease, thalassaemia or sideroblastic anaemia (Massey, 1992). Most markers of tissue iron supply and iron in tissues are also affected by the presence of inflammation and infection (Cassat and Skaar, 2013). Concomitant measurement of biomarkers of inflammation such as C-reactive protein and orosomucoid (alpha-1-acid glycoprotein) may be used to "correct" ferritin levels (Neuberger et al., 2016), but frequently studies concerned with iron status exclude children with evidence of inflammation (Zimmermann et al., 2005). As such, the true prevalence of iron deficiency in children in sub-Saharan Africa may be grossly underestimated (van Hensbroek et al., 2011; Neuberger et al., 2016).

Recommended nutrient intakes (RNIs) for iron, summarised in Table 1.2, are dependent on the bioavailability of iron in the diet. These values will provide sufficient iron for 95% of the population, while estimated average requirements (EARs) used for other nutrients are sufficient for 50% of the population. Doses for treatment of anaemia are discussed in section 1.3 below. Dietary factors that promote iron absorption include consumption of haem-iron from meat and fish, and co-consumption of ascorbic acid (vitamin C). Other dietary factors inhibit iron absorption: co-consumption of calcium or of iron binding phenols such as phytates and tannins (Zijp et al., 2000; Gibson et al., 2010). Studies assessing the bioavailability of diets in sub-Saharan African countries report that common meals incorporating local staple foods (cereals: maize/rice/millet, legumes: beans/peas, roots and tubers: cassava) contained low amounts of non-haem iron and vitamin C, and high levels of phytate, resulting in low overall iron availability (Guiro et al., 1991; Beiseigel et al., 2007; Gibson et al., 2010).

Table 1.2 Recommended nutrient intakes for iron (mg/day) at different bioavailabilities, adapted from (World Health Organisation and Food and Agricultural Organizaion of the United Nations, 2004)

	5% bioavailability	10% bioavailability	15% bioavailability
Infants (0.5-1 years)	18.6	9.3	6.2
Children (1-3 years)	11.6	5.8	3.9
Children (4-6 years)	12.6	6.3	4.2
Children (7-10 years)	17.8	8.9	5.9

Low dietary iron intakes are reported in the few studies assessing diet in sub-Saharan African children in sufficient depth. Harika et al. (2017) reviewed dietary studies which reported iron intake in sub-Saharan African children, the results of which are summarised below in Table 1.3. Large proportions of the children studied failed to meet the recommendations, ranging from 13% in Ethiopia to 100% in

South Africa. The majority of dietary iron these studies came from plant sources that are simultaneously low in iron and high in inhibitory factors such as phytate including cassava, and grains such as millet or maize.

Table 1.3 Dietary iron intake and adequacy of non-breastfed children in sub-Saharan Africa, adapted from (Harika et al., 2017)

Country	Reference	n	Age Group	Iron Intake (mg/day)	Inadequate intake (%)
Ethiopia	(Baye et al., 2013)	14	12-23 months	28 (19)	13
	(Ethiopia Public Health Institute, 2013)	8079	6-35 months	11.6 (12)	34
Kenya	(Gegios et al., 2010)	449	2-5 years	5.9 (3.7)	34
Nigeria	(Gegios et al., 2010)	793	25 years	11.5 (9)	22
	(Ayogu et al., 2015)	80	6-15 years	16.8 (7)	51
	(Hassan et al., 2012)	394	7-11 years	10.9 (3.3)	97
South Africa	(Balogun et al., 2015)	128	12-36 months	5.3 (3.5)	62
	(van Stuijvenberg et al., 2015)	149	2-5 years	8.1 (4.6)	44
	(Grobbelaar et al., 2013)	143	4-18 years	9.2 (1.9)	100

Iron deficiency is the most common micronutrient deficiency in the world and accounts for approximately 50% of childhood anaemia (World Health Organisation, 2008b), however it is nearly always only one of multiple factors contributing to severe anaemia. Calis et al. (2008) reported that iron deficiency was not associated with Hb <5.0g/dl; instead it was inversely associated with severe anaemia (OR 0.4, 95% CI 0.2-0.6), which the authors hypothesise is due to the inverse relationship between bacteraemia and functional iron deficiency in response to infection. Others have reported a putative protective effect of functional iron deficiency against malaria infection (Kumar and Choudhry, 2010), but iron deficiency is also known to increase susceptibility to other infections (Calder, 2013).

1.2.2.1.3 Folate

The term dietary folate includes an array of similar but not identical B9-group vitamins, largely reduced methyl and formyl folylpolyglutamates (SACN, 2017). Structurally, all folates have in common a single or multiple γ-glutamate residues and 4-aminobenzoic acid (PABA) conjugated to fused aromatic pyrimidine and pyrazine rings (pteridine)(Tibbetts and Appling, 2010), as shown in Figure 1. Folic acid (pteroylmonoglutamic acid) is an oxidised synthetic analogue of folate, not normally found in foods, but used in supplementation and food fortification programmes.

Figure 1.1. Structures of folate and folic acid, modified from (Tibbetts and Appling, 2010)

Folic acid

Folate

Pteridine | *p*-Aminobenzoic acid | Poly-γ-glutamate tail

Folate is a critical one-carbon carrier in numerous reactions, including DNA biosynthesis and methylation, amino acid metabolism and vitamin metabolism (Tibbetts and Appling, 2010). It is directly involved in the generation of purine and thymidine for DNA synthesis, and in many methylation reactions via the methylation of homocysteine to re-form methionine (Stover, 2004; Crider et al., 2012). Inadequate supply of intracellular folate predominantly affects rapidly dividing cells of the bone marrow and intestinal epithelium, but through its effects on homocysteine metabolism and methylation reactions, has also been associated with increased risk of cardiovascular disease and certain cancers (Bailey and Gregory, 1999; Khan and Jialal, 2018). Folate deficiency produces a megaloblastic anaemia (MCV >100fl), characterised by large erythrocyte precursor cells caused by failure of haematopoietic stem cell division (Bailey and Gregory, 1999). Excess folate also has negative health impacts, including worsening anaemia in the elderly, masking or aggravating vitamin B12 deficiency (SACN, 2017), and promoting colorectal cancer (Crider et al., 2012). Maternal and intrauterine folate deficiency is also a known risk factor for the development of neural tube defects (Imbard et al., 2013).

Naturally occurring folate is found in a wide variety of foods of both plant and animal origin; offal, mainly liver, and yeast extract are the richest sources of folate but other lower density sources include brassicas, legumes, and wholegrains (Public Health England, 2015). Mandatory fortification of processed grains/flour with folic acid is now practiced in 81 countries worldwide, including many in sub-Saharan Africa, with the primary aim of reducing neural tube defects (Table 1.4).

Table 1.4 Sub-Saharan African countries practicing mandatory folic acid fortification of flour/processed grains, adapted from (Wald et al., 2018)

Benin	Guinea	Senegal
Burkina Faso	Kenya	Sierra Leone
Burundi	Liberia	South Africa
Cameroon	Mali	Tanzania
Cote d'Ivoire	Mauritania	Togo
Djibouti	Mozambique	Uganda
DR Congo	Niger	Zimbabwe
Ghana	Nigeria	

Folate must be deconjugated to a single glutamate residue prior to absorption, a step not required for folic acid (SACN, 2017). Folic acid must be reduced to tetrahydrofolate in the liver, after which it is metabolised identically to dietary folate. Both are methylated to form 5-methyl tetrahydrofolic acid (5-MTHF) which can be taken up by cells for use and conversion into other polyglutamate forms as needed (SACN, 2017). Despite routine fortification, the majority of folate eaten in developing countries is naturally-occurring, the bioavailability of which can be up to 50% lower than folic acid (World Health Organisation and Food and Agricultural Organizaion of the United Nations, 2004). The demethylation of 5-MTHF (the same reaction in which homocysteine is converted to methionine) within cells is catalysed by vitamin B12, and it is this interaction which can result in the masking of B12 deficiency by excess folate (Stover, 2004).

Folate status is assessed directly by serum 5-MTHF concentration, however this is more reflective of recent dietary intakes (SACN, 2006). Measurement of erythrocyte folate is indicative of body stores of folate, and in conjunction with downstream metabolic products dependent on folate, such as homocysteine and methyl malonic acid, can provide a more complete picture of recent functional folate sufficiency (Khan and Jialal, 2018). Vitamin B12 levels must be assessed concurrently due to the close metabolic interaction.

RNIs for folate are estimated on the assumption that all intake is of naturally-occurring sources, with an average bioavailability of 50%, summarised in Table 1.5. Suggested levels of supplementation for treatment are discussed in section 1.2.3 below. Common staple foods eaten in a number of African countries including white rice, millet, cassava and maize are poor sources of folate, and cooking results in considerable folate losses in all dietary sources (SACN, 2006; Verhoef et al., 2017). Studies reporting the dietary intake of folate in sub-Saharan Africa are sparse, but those published suggest that low intake of folate is common. Caswell et al. (2018) report that 57% of 200 children aged 4-9 years in rural central Zambia, had inadequate intake of folate. Barugahara et al. (2013) report that 73% of school age girls in Western Uganda fail to meet recommended intakes, with prevalence in urban

settings greater than in rural. Dapi et al. (2011) also report high levels of inadequate intakes for adolescent boys (80%), and girls (87%) in urban Cameroon. Younger children also appear to have insufficient intakes: 71% of boys and 60% of girls aged 12-36 months in Western Cape province, South Africa had intakes below the estimated average requirement (EAR) (Balogun et al., 2015). Despite low intakes and limited bioavailability, it appears that physiological folate insufficiency is not a frequent finding in cases of severe anaemia (van Hensbroek et al., 2011), however this conclusion is contradicted elsewhere (Mamabolo and Alberts, 2014) and folate deficiency may add to a poor general nutritional state. Folate deficiency also impairs general immunity, contributing to increased risk of infection, providing another route by which folate may increase risk of anaemia (see section 1.2.2.2) (Verhoef et al., 2017).

Table 1.5 Estimated average requirements (EARs) and reference nutrient intakes (RNIs) for folate in children and adolescents (World Health Organisation and Food and Agricultural Organizaion of the United Nations, 2004)

Age group	EAR (µg/day)	RNI (µg/day)
7-12 months	65	80
1-3 years	120	150
4-6 years	160	200
7-9 years	250	300
10-18 years	330	400

1.2.2.1.4 Vitamin B12

Vitamin B12, or cobalamin, is a cobalt-containing cofactor for methionine synthase and methylmalonyl coenzyme mutase (World Health Organisation and Food and Agricultural Organizaion of the United Nations, 2004). While the latter enzyme is involved in the catabolism of fatty acids and some amino acids, the former is involved in the folate cycle by catalysing the reaction in which 5-MTHF donates its methyl group to homocysteine to re-form methionine (Krautler, 2012). Deficiency of vitamin B12 leads to accumulation of 5-MTHF, inhibiting purine and thymidine synthesis which results in depressed DNA synthesis and impaired erythrocyte production (Agostoni et al., 2015a). Failure of vitamin B12-dependent methyl-transfer reactions also causes irreversible damage to the nervous system, via defective synthesis of phospholipids required for myelination (Miller et al., 2005). In children these effects can extend to developmental delay and lifelong impaired cognition (Venkatramanan et al., 2016).

Ultimately, all dietary vitamin B12 comes from synthesis by microorganisms. Although some human intestinal bacteria can synthesise cobalamin (*Pseudomonas* and *Klebsiella* species), they contribute insignificant quantities (Green et al., 2017). The gut microbiota of herbivorous animals generates vitamin B12, which then enters the human food chain bound to protein in the form of milk, meat and

eggs (World Health Organisation and Food and Agricultural Organizaion of the United Nations, 2004). Protein-bound cobalamin is released in the low pH environment of the stomach, then bound to salivary glycoprotein transcobalamin I, to prevent acidic denaturation. In the lower pH of the small intestine, these glycoproteins are digested, allowing vitamin B12 to bind to gastric intrinsic factor (also a glycoprotein) facilitating absorption in the terminal ileum (Agostoni et al., 2015a). Maximal absorption of vitamin B12 is approximately 20-40% of that ingested, although this is accounted for in published reference intake values (Green et al., 2017). Both poor dietary intake and impaired absorption are common causes of deficiency.

Frank deficiency and marginal vitamin B12 status, defined as serum B12 concentrations of <148pmol/litre and 148-221pmol/litre respectively, are common in children in sub-Saharan African countries. In a large study of school age Kenyan children (n=555), Siekmann et al. (2003) reported that 31% of children were deficient, while a further 38% were marginal. Mamabolo and Alberts (2014) reported prevalence of B12 deficiency of 10% in non-anaemic children in increasing to 14% in children with anaemia in South Africa. Other studies of children in Botswana, Cameroon and The Gambia included in a recent review report similar prevalence of frank deficiency of approximately 9-17% (Green et al., 2017).

Current WHO-recommended dietary vitamin B12 intakes are summarised in Table 1.6. There are limited published studies reporting dietary vitamin B12 intakes in sub-Saharan African children, however one study (Murphy et al., 1995) reported that 87% of Kenyan children aged 7-9 had low intakes. It is also generally understood that plant foods are the predominant food source for children in these countries, and intake of meat is highly associated with socio-economic status (World Health Organisation and Food and Agricultural Organizaion of the United Nations, 2004; McLean et al., 2007).

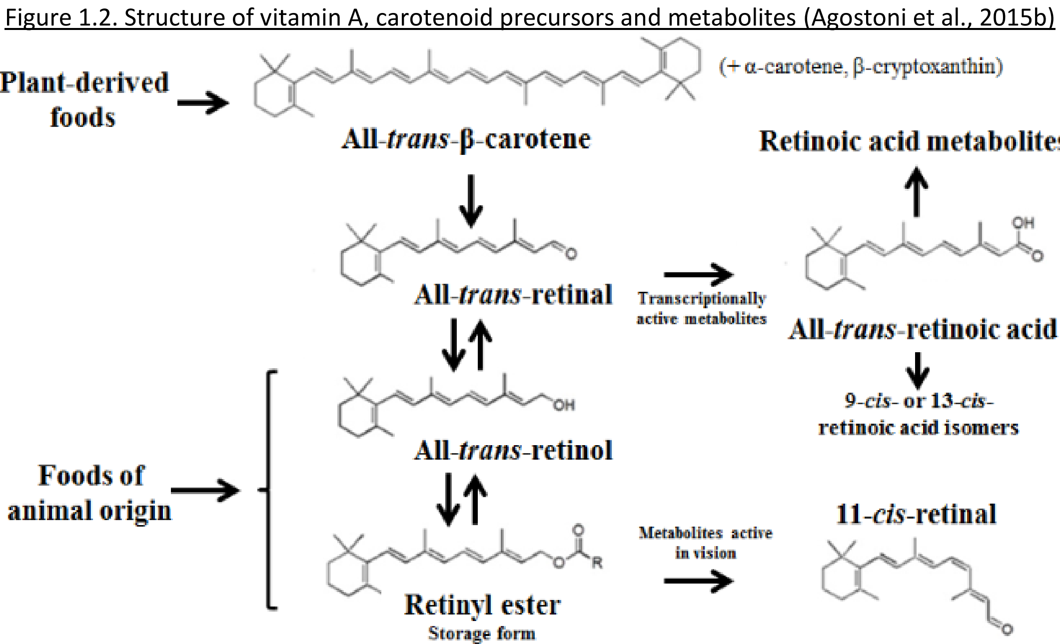
Table 1.6 Estimated average requirements (EARs) and reference nutrient intakes (RNIs) for vitamin B12 in children and adolescents (World Health Organisation and Food and Agricultural Organizaion of the United Nations, 2004)

Age group	EAR (µg/day)	RNI (µg/day)
7-12 months	0.6	0.7
`1-3 years	0.7	0.9
4-6 years	1.0	1.2
7-9 years	1.5	1.8
10-18 years	2.0	2.4

1.2.2.1.5 VITAMIN A

The term Vitamin A encapsulates both pre-formed vitamin A (retinol) from animal source foods, provitamin A carotenoids from plant source foods, and the range of metabolically active intermediate forms. The structure of vitamin A and its subclasses is shown in Figure 2.

Figure 1.2. Structure of vitamin A, carotenoid precursors and metabolites (Agostoni et al., 2015b)



Aside from its well-established role in vision, vitamin A is important for the immune system, cell growth and differentiation (World Health Organisation and Food and Agricultural Organizaion of the United Nations, 2004). There are several mechanisms through which vitamin A and its metabolites may contribute to anaemia: iron homeostasis, immune modulation, erythropoietin expression, and apoptosis of erythrocyte precursors (Semba and Bloem, 2002). Of these possible mechanisms only impaired immunity with the associated increased risk of infection, and iron homeostasis are thought to be of significant clinical importance. Vitamin A affects the rate of proliferation and activation of T- and B-cells and the production of TNF-α within monocytes, and promotes T-helper-2 cell differentiation, among other effects (Mora et al., 2008; Sanjoaquin and Molyneux, 2009). In addition to increased risk of general infection, an increased risk of malaria in vitamin A deficiency has been reported (Sanjoaquin and Molyneux, 2009). Iron absorption is upregulated in vitamin A deficiency, but iron absorbed then becomes functionally unavailable and trapped within the liver and spleen (World Health Organisation, 2017b). In 2013, it was estimated that 48% of children under age 5 in sub-Saharan Africa were vitamin A deficient, with South Asia having the second highest prevalence of 44% (Stevens et al., 2015). A more recent systematic review of vitamin A deficiency in children in Ethiopia, Kenya, Nigeria and South Africa found that prevalence of frank deficiency ranged from 14-42% in the four countries (Harika et al., 2017).

Between 70-90% of retinol is absorbed, whereas provitamin A compounds are less well absorbed (5-65%) with significant variation depending on genetic characteristics, dietary and lifestyle factors (Agostoni et al., 2015b). Due to the different absorption rates, retinol requirements are expressed as retinol equivalents (REs) where 14 units of β-carotene equals 1 unit of retinol and 28 units of other provitamin A carotenoids equal 1 unit retinol, though these conversion rates have been questioned (World Health Organisation and Food and Agricultural Organizaion of the United Nations, 2004). Since excess vitamin A can be highly toxic, only estimated average requirements (EARs) which cover 50% of the population are presented, beside recommended safe intake levels, both summarised in Table 1.7.

Table 1.7 Estimated average requirements (EARs) and safe intake levels for vitamin A in children and adolescents (World Health Organisation and Food and Agricultural Organizaion of the United Nations, 2004)

Age group	EAR (µg RE/day)	Safe Intake Level (µg RE/day)
7-12 months	190	400
1-3 years	200	400
4-6 years	200	450
7-9 years	250	500
10-18 years	330-400	600

Vitamin A has been the focus of global supplementary and fortification programs, however coverage is not 100% and adequate dietary intake remains important. In Africa over 80% of total vitamin A intake comes from plant sources including greens (spinach/amaranth/cow pea leaves), sweet potato, and mango, while the remainder comes from largely from milk and eggs (Codija, 2001). Inadequate intake has been reported in between 45-100% of children aged under 5 years in Ethiopia, Kenya and Nigeria, with mean intakes ranging from 6.5-17% of the EAR (Harika et al., 2017).

1.2.2.2 Infection

Chronic and acute infections are a common factor in the development of severe anaemia (van Hensbroek et al., 2011). Many pathogens have specific effects that contribute to anaemia, e.g. blood loss or haemolysis, however all result in immune activation and inflammation. Prolonged or repeated immune activation, as is the case in seasonal malaria infection, HIV infection, or environmental enteric dysfunction, can contribute to the development of anaemia in a number of ways. Bacterial lipopolysaccharides and cytokines, particularly interleukin-6 (IL-6), promote the release of hepcidin, causing iron to remain bound in ferroportin in enterocytes and cells of the mononuclear phagocyte

system (Madu and Ughasoro, 2017). Interferon-gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), and IL-6 are also known to down-regulate erythropoiesis and inhibit the effect of erythropoietin in the bone marrow (van Hensbroek et al., 2010; Madu and Ughasoro, 2017). Specific pathogens known to contribute to the development of severe childhood anaemia, and their relevance to the sub-Saharan African setting are outlined in the following sections.

1.2.2.2.1 BACTERIA

Tuberculosis (TB) is the leading cause of death worldwide from a single pathogen, and Africa has the highest regional incidence at 237/100,000 (World Health Organisation, 2018a). Due to the difficulty and high cost of testing for TB in children, the true prevalence and incidence in sub-Saharan African countries is unclear. Global estimates of childhood TB prevalence range from 500,000 to 1,100,000, with an estimated mortality rate of 17.6% (Jenkins, 2016; World Health Organisation, 2017a). Anaemia is common in children with tuberculosis, estimated to be present in 32-86% of cases, largely caused by the general effects of chronic immune activation as outlined above (Minchella et al., 2015), although haemolysis has also been reported in rare cases (Rathish and Siribaddana, 2018).

Gastrointestinal pathogens which cause severe diarrhoea, including *Shigella*, *Campylobacter*, *Yersinia*, and *Salmonella* species, are also associated with the development of severe anaemia (Jonker et al., 2017). Diarrhoeal disease is also a leading cause of death in children, accounting for 8% of all deaths in children under 5 years old in 2017, most of which occurred in South Asia and sub-Saharan Africa (Walker et al., 2013; United Nations Children's Fund, 2018). The principal mechanisms through which diarrhoeal disease cause anaemia are via general effects of infection, but also through gastrointestinal blood loss when diarrhoea is severe, and reduced absorption of nutrients required for red cell production. Some bacteria have extra-intestinal effects contributing to anaemia, for example Shigellosis may rarely cause haemolytic anaemia (Chao et al., 2006).

1.2.2.2.2 PARASITES (HELMINTHS, SCHISTOSOMES, PROTOZOA)

Of the three major intestinal nematode pathogens (hookworm, roundworm, and whipworm), hookworm is most associated with the development of severe anaemia (van Hensbroek et al., 2011) Hookworm infection is common in sub-Saharan African children with an estimated 9 million annual infections in children under 5, and 18 million in children between 5 and 10 years old (de Silva et al., 2003). In hookworm infection, larvae from contaminated soil penetrate the skin, initiating infection. Adult hookworms then migrate to and adhere to the gut lining, where they attach and feed on blood,

which is the principal causative mechanism of hookworm-associated anaemia (Whitfield, 1993). Of the two species of hookworm, *Necator americanus* and *Ancylostoma duodenale*, the latter leads to more severe anaemia. In severe *A. duodenale* infections the cumulative blood loss of 0.2ml per worm per day may total up to 100ml of blood lost daily (Awasthi and Bundy, 2007).

Risk of infection from water-borne schistosomes is greatest in Africa, where over 91% of those at risk live (World Health Organisation, 2012). Young children, especially those who are poor or live in rural areas, are also the group most vulnerable to severe schistosomiasis infection, where 60-80% of school age children may be actively infected (Colley et al., 2014; Tchuem Tchuente et al., 2017). Schistosome infection contributes to the development of severe anaemia by inflammatory processes, the digestion of red blood cells by adult worms, and by urinary blood loss in cases where eggs become lodged in the tissue lining the bladder (Colley et al., 2014).

Infection by enteric protozoan species such as *Giardia*, *Cryptosporidium* and *Entamoeba*, is common in children in Africa (Harhay et al., 2010). They may contribute to anaemia by the development of diarrhoea and malabsorption, in addition to inflammation, however they have not been reported to be major determinants of progression to severe anaemia.

1.2.2.2.3 MALARIA

Malaria is caused by infection by *Plasmodium* parasite species, transmitted via *Anopheles* mosquitoes. Humans are the natural intermediate host for four *Plasmodium* species: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* (Peterson and Calamandrei, 2012). *P. falciparum* is the dominant and more virulent species globally; within Africa it accounts for 99.7% of cases (World Health Organisation, 2018b). Indeed the vast majority of global recorded malaria cases (92%) and deaths (93%) in 2017 were in Africa (World Health Organisation, 2018b). Children aged under 5 are particularly susceptible, and they account for 61% of the 435,000 deaths due to malaria (World Health Organisation, 2018b), again with the vast majority occurring in Africa. The parasite initially infects hepatocytes where it matures, then proceeds to infect erythrocytes and multiply causing lysis, leading to the clinical manifestation of malaria (Centres for Disease Control and Prevention, 2017). Malaria contributes to the development of severe anaemia in a variety of disease-specific processes in addition to the general effects of immune activation including: haemolysis, erythrocyte sequestration by the spleen, and impaired erythropoiesis (Perkins et al., 2011). There is a poorly understood individual component which determines whether an individual with malaria will develop associated severe anaemia. Calis et al. (2008) reported that in the rural setting where malaria is holoendemic, only patients with high parasite loads had increased risk of severe anaemia.

1.2.2.2.4 VIRUSES

A number of viruses have been implicated in childhood anaemia. Human immunodeficiency virus (HIV) has a strong association with severe anaemia, with an odds ratio of 2.0 (95% CI 1.0-3.8) (Calis et al., 2008; Redig and Berliner, 2013). Pregnant HIV-positive women have a 15-45% chance to transmit the infection to their children, either in utero or more often during childbirth (Barral et al., 2014). Paediatric HIV is concentrated in sub-Saharan Africa: 90% of children under the age of 15 living with HIV are located in this region equating to approximately 3.1 million children (World Health Organisation, 2011a). It is generally accepted that HIV principally causes anaemia through the general effects of inflammation (Morinet et al., 2011), however there is evidence of a direct effect of both the virus itself and anti-retroviral therapy on the rate of red cell production, though the magnitude of these effects is not known (Redig and Berliner, 2013).

Other common viruses, such as human parvovirus B19, are now known to affect the bone marrow directly. A recent study by Ashaka et al. (2018), reported that 15.7% of non-anaemic Nigerian pre-school children were positive for parvovirus B19 infection, but almost double (29.8%) were positive in anaemic children. Parvovirus has been shown to directly damage DNA and cause apoptosis in erythrocyte progenitor cells, and to reduce erythropoietin signalling (Morinet et al., 2011). It is likely that the true extent of the effect of viruses on the development of severe anaemia in children is currently underestimated.

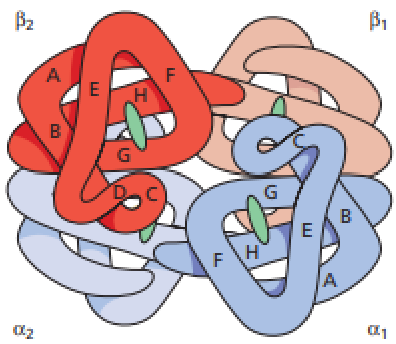
1.2.2.3 GENETIC DISORDERS AND HAEMOGLOBINOPATHIES

Glucose-6-phosphate dehydrogenase (G6PD) is a ubiquitous enzyme, which facilitates the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH), an important antioxidant (Cappellini and Fiorelli, 2008). This is the only method of NADPH production available to erythrocytes, thus reduced G6PD activity results in a restricted ability to prevent oxidative damage, such as occurs in prophylactic treatment for malaria. Defects in the genes encoding G6PD are common, affecting 400 million people globally, mostly in Africa, Asia and the Mediterranean (Cappellini and Fiorelli, 2008). The variants of G6PD found across Africa frequently retain some G6PD activity, while that found in the Middle East and Asia is more severe (Howes et al., 2012). Therefore despite being common, G6PD deficiency contributes less to the development of severe childhood anaemia in Africa than in other regions.

Thalassaemias are a group of genetic disorders which affect the production of globins, substituents of haemoglobin. The most common form of haemoglobin in children over 6 months of age and adults is

haemoglobin A, comprised of two α-globins and two β-globins carrying haem, as shown in Figure 1.3. Genetic deletions or mutations in the genes encoding the globins, result in a deficit of one globin and relative excess of other globins, which in turn may cause reduced erythrocyte production, or haemolysis in mature cells (Bain, 2006). The major thalassaemias are named for the globin which is under expressed, i.e. α-thalassaemia describes low α-globin, while β-thalassaemia refers to reduced β-globin. The milder forms of α-thalassaemia in which there remains some α-globin production are common throughout sub-Saharan Africa (prevalence 10-25%), however the severe forms are found predominantly in southeast Asia and some Mediterranean islands (Williams and Weatherall, 2012). β-thalassaemia has a narrower distribution centred around the Mediterranean, as well as some areas of southeast Asia. The most severe form of thalassaemia, "thalassaemia major", results in erythrocyte production failure, and without regular transfusion will lead to death (Borgna-Pignatti et al., 2005; Taher et al., 2018).

Figure 1.3. Illustration of healthy haemoglobin A, including α- and β-globins and haem (green) (Bain, 2006)



Sickle cell disease (SCD) is an inherited disorder of β-globin synthesis causing conformational changes in the haemoglobin molecule upon deoxygenation. A variety of mutations can lead to the clinical manifestation of SCD, but the most common is homozygous HbSS, followed by HbS/β-thalassaemia (Ware et al., 2017). Erythrocytes carrying the defective deoxyhaemoglobin become elongated and curved, occluding blood vessels and causing local ischaemia and inflammation; ultimately the defective red blood cells lyse or are removed from circulation (Ware et al., 2017). Sickle cell trait is concentrated in historically malaria endemic areas, especially sub-Saharan Africa where up to 30% of the population may be heterozygous (HbAS) carriers (Piel et al., 2013b) and approximately 237,253 homozygous HbSS children are born each year (Piel et al., 2013a). Heterozygosity reduces the risk of death from malaria, however those who are homozygous are at greater risk of death, and malaria is one of the primary causes of hospital admission and fatality in this group (Luzzatto, 2012).

1.2.3 Treatment and outcomes

Severe anaemia is associated with increased risk of mortality, such that every 1.0g/dl Hb increase is associated with a 24% decreased risk of mortality (Scott et al., 2014). The WHO recommends transfusion of 20ml/kg whole blood or equivalent blood product for children with Hb <4g/dl, or <6g/dl with complications such as dehydration, shock, heart failure, difficulty breathing, impaired consciousness or high malaria parasite load (World Health Organisation, 2000). Additional treatment with anti-malarials, iron and folate supplements, or antibiotics are recommended as needed. Even following treatment, an estimated 6.4% of children with severe anaemia die in hospital, while a further 12.6% die within 6 months (Phiri et al., 2008), suggesting that current treatments are only partially effective. A common immediate cause of death in severe childhood anaemia is febrile bacteraemia caused by invasive non-typhoidal *Salmonella* species, an especially common finding in children who have malaria and anaemia (Park et al., 2016; Nyirenda et al., 2018). The two main species responsible, *S. typhimurium* and *S. enteritidis*, are spread via inadequate sanitation, contaminated food and water and contact with animals (Haselbeck et al., 2017). These pathogens normally cause self-limiting gastroenteritis and diarrhoea, however in children with severe anaemia, malaria and impaired immunity, they successfully translocate from the gut to the circulation. Co-infection by *Plasmodium* and invasive bacteria is associated with 24% increased risk of death, compared to malaria alone (Church and Maitland, 2014).

As iron is a crucial virulence-limiting factor for most pathogens, treatment of iron deficiency in children at risk of malaria and subsequent invasive bacterial infection with supplementary oral iron has been the subject of some controversy. Iron deficiency is treated in conjunction with folate deficiency with recommended daily doses of 25mg of iron in children under 2 years of age, and 60mg of iron in children over 2 years, with folate supplementation of 100-400mg daily. Sazawal et al. (2006) reported that routine prophylactic iron supplementation at these doses resulted in increased risk of severe malaria and death. In this study increased mortality was seen only in children who were iron-replete, however, and iron-deficient children did not have increased risks. A recent meta-analysis (Neuberger et al., 2016) of 35 trials and 31,955 children disputed this and reported that in areas with malaria prevention/management services, iron supplementation did not lead to an increase in severe malaria (RR 0.90, 95% CI 0.81-0.98) or any difference in deaths. In areas without these services, iron supplementation may increase the incidence of malaria transmission (RR 1.16, 95% CI 1.02-1.31).

Children who survive severe anaemia are subject to long term negative impacts on physical and mental capacity. Impaired neurological development is commonly reported, especially in iron and B12 deficient children, leading to reduced work capacity and impaired cognition in adulthood (Kassebaum, 2016).

1.3 METABOLIC PROFILING

The development of several analytical chemistry techniques throughout the 20th century has led to their adoption as valuable methods to explore human metabolism in health and disease. Approaches which allow the simultaneous measurement of many different chemical classes formed the basis of a new approach to biomolecular research: metabonomics. Metabonomics was characterised by Nicholson et al. (1999) as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification", while the closely related term metabolomics was defined by Fiehn (2002) as "a comprehensive and quantitative analysis of all metabolites". Although the approaches differed slightly the terms are now used interchangeably and can be encapsulated in the broader term metabolic profiling. In effect, the small molecule environment of biological samples is characterised, and differences between individuals, conditions etc. are assessed using multivariate statistical approaches.

Two platforms form the core of metabolic profiling technologies: nuclear magnetic resonance spectroscopy and mass spectrometry. The principles of NMR will be further outlined in Chapter 2, but briefly atomic nuclei which have intrinsic magnetic properties can be non-destructively analysed by placing them in a magnetic field, applying a finely tuned radio-frequency pulse, and measuring the energy released by the nuclei as they relax (Lindon et al., 2011). Of the atomic nuclei which display this property, hydrogen is the most commonly exploited in metabolomics research. Although biofluids can be directly infused into mass spectrometers, it is frequently conducted after the biofluid has been separated by gas or liquid chromatography or electrophoresis to improve the resolution of the technique (Lindon et al., 2011). Methods may be untargeted, where as many chemical classes as possible are analysed simultaneously, or targeted to a specific class of interest (e.g. lipids – lipidomics).

1.3.1 METABOLIC DERANGEMENTS IN SEVERE ANAEMIA

Since the aetiology of severe anaemia is frequently multifactorial as outlined above, the metabolic signatures of the condition are often aetiology-specific. Several studies have explored the effect of iron deficiency and iron deficiency anaemia on the serum/plasma and urine metabolomes. McClorry et al. (2018) found that mild anaemia (<11.0g Hb/dl) in childhood was associated with decreased serum concentrations of succinate and fumarate, intermediates in the tricarboxylic acid cycle. A reduction in available oxygen for respiration may partially account for this finding, however since the diet of children was not assessed there are numerous other potential reasons. Anaemic male infants in this study also had reduced serum concentrations of the branched chain amino acids (isoleucine,

leucine and valine) as well as other amino acids including histidine and tyrosine, however no reason for this sex-specific finding was given. Although not directly applicable to humans, an animal study by Alexeev et al. (2017) showed that rat pups given oral iron supplementation showed elevations of plasma 3-hydroxybutyrate, alanine, histidine, taurine and dimethylamine, but these metabolites were also reported to be sensitive to diet.

Infectious causes of anaemia have been shown to have distinct metabolic effects which relate to the pathophysiology of the infectious organism. The metabolic sequelae of malaria have been well characterised, and are defined principally by hyperphenylalaninaemia (Enwonwu et al., 1999; Lopansri et al., 2006; Rubach et al., 2015), and arginine deficiency (Olszewski et al., 2009; Chau et al., 2013; Leopold et al., 2019). Leopold et al. (2019) provide a detailed examination of other metabolic derangements in malaria including elevated plasma tryptophan metabolites associated with oxidative stress, elevated plasma lactate and alanine related to acidosis, and multiple other dysregulated amino acids. Other infectious causes of anaemia have been shown to have metabolic effects. Tuberculosis infection is associated with lower plasma concentrations of amino acids (histidine, cysteine, glutamine, tryptophan, citrulline and creatine), medium-chain fatty acids and lysophosphatidylcholines (Weiner et al., 2012; Collins et al., 2018; Weiner et al., 2018). Other amino acids are observed to be elevated in TB such as phenylalanine and kynurenine, a downstream product of tryptophan metabolism associated with metabolic stress (Weiner et al., 2012).

Metabolic alterations associated with sickle cell disease have also been described, however much of this research to date has been conducted in mouse models, therefore its applicability to human metabolism is questionable (Adebiyi et al., 2019). Studies of human erythrocytes have shown upregulation of anaerobic energy metabolism, specifically of glycolysis by means of the pentose phosphate pathway (Darghouth et al., 2011; Adebiyi et al., 2019). Darghouth et al. (2011) also report significant decreases of compounds, cofactors and precursors related to antioxidant metabolic pathways including glutathione and nicotinamide. One study examining the plasma metabolome in sickle cell disease reported reductions in plasma arginine and ornithine concentrations (Papassotiriou et al., 2013).

Some studies have reported other metabolic alterations in anaemia of unspecified aetiology, for example one study reported higher faecal concentrations of acetate and propionate in anaemic children but higher faecal butyrate in non-anaemic children (Paganini et al., 2016).

Most studies outlined above reported metabolic alterations associated with mild anaemia (<11.0g Hb/dl), and have compared anaemic children with non-anaemic controls. No previous studies were identified which reported specifically on the metabolic effects of severe childhood anaemia (<5.0g

Hb/dl), or were concerned with the metabolic changes which take place in association with recovery from this condition.

1.3.2 Diet and metabolic profiling

Metabolomics technologies have been applied to nutritional research in a variety of ways which can broadly be divided into 1) metabolic fingerprinting of nutrition-related conditions, 2) biomarker identification and dietary compliance, 3) host-gut microbe co-metabolism, and 4) food composition.

The International study of Macro- and Micronutrients and blood Pressure (INTERMAP) study demonstrated that dietary patterns such as high vegetable intake or high animal protein intakes resulted in clear urinary metabolic signals (Holmes et al., 2008), which could be associated with raised blood pressure. Those with high vegetable intakes had increased quantities of dimethylamine in 24hr urine samples, while those with higher animal protein intakes had acetylcarnitine, creatine and *N,N,N*-trimethyllysine. Some gut microbial associated compounds such as 4-cresol (microbial metabolite of tyrosine) and trimethylamine (microbial metabolite of choline) have also been demonstrated to be associated with cardiovascular disease in adults (Brial et al., 2018).

Excretion of specific food-related metabolites is utilised as a method to independently assess dietary intake. Heinzmann et al. (2010) described proline betaine as a specific and quantitative marker of citrus fruit intake, that was later validated on a large scale by re-analysing urinary metabolomic data from the INTERMAP study and referencing this to 24hr dietary recalls. Garcia-Perez et al. (2017) has demonstrated that tartaric acid is a specific quantitative marker of grape intake, but also that urine metabolic profiles can predict adherence to a specified dietary pattern. In this instance they compared the urine metabolic profiles of subjects eating a "healthy" diet as per WHO guidelines in a controlled environment, to the profiles of those same subjects eating "unhealthy" diets. The relative adherence to this dietary pattern was shown to result in clear urinary metabolic signatures.

Metabolic profiling has also been used to explore metabolic dysregulation in states of impaired nutritional status. In the context of children in a low and middle income setting, a recent review provides an excellent overview of the metabolic signatures of childhood undernutrition (Mayneris-Perxachs and Swann, 2019). Stunting, defined by height-for-age z-score <-2, is associated with decreased serum concentrations of amino acids and sphingomyelins, while underweight (weight-for-age z-score <-2) is characterised by reduced urinary betaine and dimethylglycine, but elevated gut microbial metabolites such as phenylacetylglutamine and 3-indoxyl sulfate. More acute undernutrition, for example wasting (weight-for-height z-score <-2) is associated with elevated plasma

ketones and non-esterified fatty acids. Studies have shown that acute undernutrition is associated with lower levels of plasma amino acids. In Ugandan children with severe acute malnutrition, Bartz et al. (2014) report decreased levels of some amino acids in malnourished children, but after treatment branched chain amino acids (BCAAs: isoleucine, leucine and valine) and other amino acids such as alanine, tryptophan and cysteine are elevated. McMillan et al. (2017) report decreased blood concentrations of BCAAs and other amino acids including arginine, tyrosine, and kynurenine in children with severe acute malnutrition compared to well-nourished controls.

Metabolic profiling can therefore shed light on both pathophysiological mechanisms in severe anaemia, and dietary intake and general nutrition status making it a valuable and previously little utilised tool to characterise a global metabolic response to severe anaemia and environmental factors.

1.4 The gut microbiome

The human gut microbiota are dominated by bacteria, although other microorganisms including fungi, viruses/phages, and amoebae form a significant part of this community. Colonisation is thought to begin during birth, however there is mounting evidence to suggest that prenatal *in utero* colonisation occurs (Tanaka and Nakayama, 2017; Walker et al., 2017). The microbiota in early infancy are characterised by *Bacteroides*, *Bifidobacteria*, and *Clostridia*, with changing proportions of dominant species evident up to the third year of life (Nicholson et al., 2012; Tanaka and Nakayama, 2017). Exposure to the mother's microbiota, in addition to environmental exposures to other species and the effects of nutrition, caesarean vs. vaginal delivery, illness and medications all contribute to the development of the eventual stable gut microbiome (Nicholson et al., 2012).

1.4.1 Gut microbiota in severe anaemia

Although the characterisation of gut microbial populations in the context of severe anaemia is a relatively new area of research, some studies which describe the gut microbiota in the context of mild anaemia (Hb <11.0g/dl), iron deficiency and iron supplementation provide an initial insight. Kenyan children with anaemia of undefined aetiology aged 2-5yrs were shown by Paganini et al. (2016) to have higher proportions of *Prevotella* than non-anaemic children (4.5% vs. 2.0%). Non-anaemic children in this study had higher proportions of *Streptococcus* compared to those with anaemia (6.3% vs. 3.9%). Higher haemoglobin concentrations and better iron status were associated with lower relative frequency of *Escherichia coli* (Paganini et al., 2016).

Muleviciene et al. (2018) compared the gut microbiota of children aged 6-34 months with iron deficiency anaemia (IDA, n=10) to those with normal haemoglobin and iron status (n=10). IDA was associated with elevated *Enterobacteriaceae* (4.4% vs. 3.0% in controls), *Veillonellaceae* (13.7% vs. 3.6%) and decreased proportions of *Coriobacteriaceae* (3.5% vs 8.8%). Lee et al. (2017) studied the effect of oral versus intravenous iron treatment in adults with irritable bowel disease, and found that oral iron was associated with decreases in *Collinsella aerofaciens*, *Faecalibacterium prausnitzii*, *Ruminococcus bromii*, and *Dorea* species. Conversely the proportion of *Bifidobacterium* in oral-iron treated individuals was higher than in those treated with intravenous iron. Treatment with iron in anaemic children has been associated with expansion of *Bacteroides fragilis* which is strongly dependent on haem availability (Yilmaz and Li, 2018). *Enterobacteriaceae*, *Streptomycetaceae* and *Bacillaceae* secrete siderophores to scavenge iron when availability is limited (Yilmaz and Li, 2018). A recent in vitro study exploring the effect of iron availability on the growth and proliferation of pathogenic gut bacteria demonstrated that *E. coli* and *Salmonella typhimurium* are both strongly inhibited by a lack of iron, while other beneficial species such as *Lactobacillus rhamnosus* are not affected (Parmanand et al., 2019).

Other important causes of severe anaemia in children have been independently associated with the gut microbiome. One study by Yooseph et al. (2015) examined the gut microbiota of children in Mali and Malawi and risk of contracting *Plasmodium falciparum* in the subsequent 6 months. They found that children at the lowest risk of contracting malaria had relatively higher proportions of *Enterobacteriaceae Escherichia/Shigella*. Hookworm infection has been associated with concomitant elevations in *Verrucomicrobiaceae* and *Enterobacteriaceae*, but decreased proportions of *Bacteroidiaceae* when compared to non-infected individuals (Jenkins et al., 2017). Interestingly the same study found no differences in the diversity or richness of the gut microbiota between those with active hookworm infection compared to those without.

Sickle cell disease has been associated with an increased risk of infection with intestinal non-typhoidal *Salmonellae*, which recent research has linked to dysregulation of the gut barrier (Dutta et al., 2019). Only two studies report changes in the gut microbiome in subjects with sickle cell disease. Lim et al. (2018) reported that the gut microbiota of individuals with sickle cell disease (n=25) compared to those without (n=15) was relatively enriched with *Alistipes*, but showed no difference in species diversity. In a brief report Brim et al. (2017) describe relatively greater proportions of *Bifidobacteria*, *Campylobacter*, *Veillonella*, *Actinomyces*, *Scardovia* and *Atopobium* species in subjects with sickle cell disease (n=14) when compared to controls (n=14).

1.4.2 Diet and the gut microbiota

Host diet has a profound effect on the gut microbiota, since the vast majority of substrates required for microbial metabolism and proliferation are dietary components. Different dietary patterns and components have been associated with characteristic gut microbial populations, however few studies of gut microbial populations in anaemia report concurrent dietary intake. In infancy exclusive breast feeding is associated with enrichment of *Bifidobacterium* and *Lactobacillus* species (Matamoros et al., 2013), apparently due to their preferential metabolism of oligosaccharides in breast milk (Musilova et al., 2014). Infants fed with formula milk tend to have greater diversity in gut bacterial species, with higher proportions of *Enterobacteriaceae*, *Clostridia* and *Bacteroides* than those exclusively fed with human breast milk (Harmsen et al., 2000; Penders et al., 2006). Weaning onto solid foods is associated with rapid increases in the diversity of species, and the dominant species of gut microbiota shift from *Bifidobacterium* to species within the phylum *Firmicutes* (Stewart et al., 2018).

One study conducted by De Filippo et al. (2010) compared the development of the gut microbiota in children from a rural village in Burkina Faso to children in European countries. Children from Burkina Faso had a notable enrichment in *Actinobacteria* (10.1% vs. 6.7%), *Bacteroidetes* (57.7% vs. 22.4%) and depletion in *Firmicutes* (27.3% vs. 63.7%) and *Proteobacteria* (0.8% vs. 6.7%) when compared to children from Europe. Two genera associated with dietary fibre metabolism were uniquely abundant in the Burkina Faso children: *Prevotella* and *Xylanibacter*. *Enterobacteriaceae* (*Shigella*/*Escherichia*) were underrepresented in the children from Burkina Faso compared to European children, which the authors suggest provides protection from inflammation and gastrointestinal disease (De Filippo et al., 2010).

Dietary fibre content is well established to have an effect on the gut microbiota of adults (Walker et al., 2011; Yatsunenko et al., 2012; Scott et al., 2013), although it has been mostly studied in the context of obesity. Rapid changes in the gut microbiota in response to different quantities of dietary fibre have been described in under 4 days (Yatsunenko et al., 2012). Depending on the type of dietary fibre, different species have been observed to become enriched in studies. *Ruminococcus*, *Bifidobacterium* and *Eubacterium rectale* appear to respond predominantly to resistant starch (Abell et al., 2008; Martinez et al., 2010; Walker et al., 2011). Fructans and galacto-oligosaccharides selectively stimulate *Bifidobacteria*, *Faecalibacteria*, and *Collinsella* (Tannock et al., 2004; Ramirez-Farias et al., 2009).

Dietary fat and protein also selectively enrich bacterial species with the capability of metabolising these substrates. For example tyrosine and phenylalanine in the gut lumen are fermented by the gut bacteria to products phenylpropionate and phenylacetate, and higher dietary intakes result in increased proportions of bacteria capable of metabolising these amino acids such as *Clostridia* and

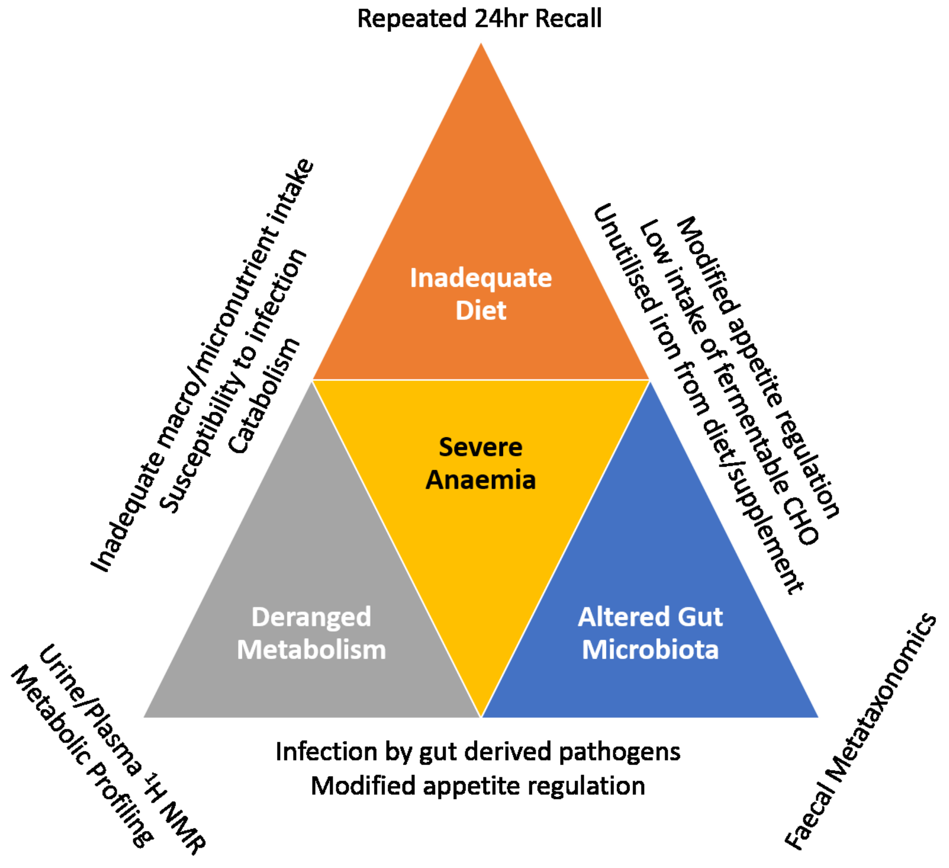
Enterobacteria (Yatsunenko et al., 2012; Zhao et al., 2019). Increased gut microbial metabolism of protein has been associated with proliferations of potential pathogens (Ma et al., 2017). Higher fat diets have been associated with lower diversity of bacterial species in the gut, decreases in the proportion of *Faecalibacterium*, and increases in *Alistipes* and *Bacteroides* (Wan et al., 2019).

Some dietary compounds such as polyphenols such as tannins, flavones and proanthocyanidins are also used by bacterial species as substrates for energy and proliferation, since up to 90-95% of these compounds are not absorbed by the host (Monagas et al., 2010). In some cases these compounds may be transformed by the gut microbiota and further modified by human metabolism, for example flavan-3-ols converted by *Clostridia* and *Eubacteria* into benzoic acids, further metabolised to hippurate in the liver (Monagas et al., 2010; Lees et al., 2013). Diets with higher proportions of these compounds have been associated with increases in species capable of utilizing the specific dietary phenols such as *E. coli*, *Enterococcus*, *Bifidobacterium*, *Lactobacillus*, *Bacteroides* and *Eubacterium* (Selma et al., 2009; Cardona et al., 2013).

1.5 Scope of thesis

Diet, metabolic dysregulation and alterations in the gut microbiota may play important roles in the development of and recovery from severe anaemia, however these have previously only been partially examined, or explored in mild anaemia. Therefore the overarching goal of this project was to characterise the diet and associated metabolic profiles and gut microbiota of children recovering severe anaemia, in addition to exploring interactions between these factors and their impact on recovery, as illustrated in Figure 1.4. The first stage was to develop and validate a dietary assessment method in children in the eastern region of Uganda (Nightingale et al., 2016). This validated tool was then used in a large interventional trial in this region (Transfusion and treatment of severe anaemia in African children study, TRACT described fully in Section 2.2.1 below), to characterise the diet of children recovering from severe anaemia and to explore its role in recovery. The next section aimed to examine metabolic derangements in severe anaemia, and to establish if these were associated with diet, treatment or outcome. Finally the gut microbiota of TRACT study children were characterised, and interactions between diet, treatment and outcome were explored.

Figure 1.4 Schematic representation of the areas of investigation, interaction and methods of this study



2. Chapter 2 – Methods

2.1 Introduction

General materials and methods used are described in the following sections including study design, dietary assessment, validation of dietary assessment methods, nuclear magnetic resonance spectroscopy for metabolic profiling, multivariate statistical approaches and metabolite identification strategies for NMR data, and gut microbial profiling by 16S rRNA sequencing.

2.2 Study design

2.2.1 Transfusion and treatment of severe anaemia in African children study (TRACT)

The TRACT study design has been fully described elsewhere (Mpoya et al., 2015), and is attached as Appendix I; briefly it was a randomised controlled trial conducted in 4 hospital sites in Uganda and Malawi. Ethical approval was granted by the Imperial College Research Ethics Committee (ICREC_13_1_11), the Ugandan Makerere University School of Medicine Research and Ethics Committee (#REC ref 2013-050), and the Malawian College of Medicine Research and Ethics Committee (P.03/13/1365). TRACT was primarily designed to test the effectiveness of the current WHO-recommended transfusion strategies for severely anaemic children (Hb <4g/dl) or less severely anaemic (Hb 4-6g/dl) with complications: liberal versus conservative transfusion (30ml/kg versus 20ml/kg). Children who had uncomplicated severe anaemia (Hb 4-6g/dl) were also randomised to receive no immediate transfusion vs. triggered transfusion in the event the child developed more severe anaemia or complications.

Secondary post-discharge randomisations in TRACT for all children were: iron and folate supplementation versus multi-vitamin multi-mineral (MVMM) supplementation (also containing iron and folate in lower concentrations), and post-discharge cotrimoxazole prophylaxis versus none. These interventions were initiated on discharge from hospital and continued for three months. In the iron and folate arm, the dosages were age-dependent: age <2 years were provided 25mg iron/day and 100ug of folic acid/day, and age 2-12 years received 60mg iron/day and 400µg folic acid/day. Iron was provided as either syrup or tablet format. Children who were randomised to receive MVMM were given a micronutrient powder taken daily which provided: 10mg iron, 90µg folic acid, 4.1mg zinc, 400µg vitamin A, 0.5mg thiamine, 0.5mg riboflavin, 6.0mg niacin, 0.5mg pyridoxine, 0.9µg vitamin B12, and 30mg vitamin C. The MVMM also contained 0.5µg vitamin D, 5mg vitamin E, 0.6mg copper, 90.0µg iodine, and 17.0µg selenium. In the antimicrobial prophylaxis arm, for those randomised to

receive cotrimoxazole, dosage was age-dependent: those aged 6-60 months received 240mg cotrimoxazole/day, and those aged 5-12 years received 480mg/day. Cotrimoxazole was given as dispersible tablets containing 240mg of cotrimoxazole, comprised of 40mg trimethoprim and 200mg sulphamethoxazole. All children received one 500mg dose of the anti-helminthic mebendazole.

Comprehensive, structured reviews alongside biological sample were undertaken on admission, day 28, day 90 and day 180 post admission. Data collected during the review visits included anthropometric measures, clinical observations, laboratory indices, and post-discharge intervention adherence. TRACT successfully recruited 3983 children between September 2014 and May 2017, with results currently in submission for publication. The recruitment of children to the sub-study took place from January 2015-November 2015, therefore any effects of seasonality on children's diets was evenly distributed throughout the data.

Prior to the commencement of the trial it was identified that sub-studies could be undertaken to provide added insight and value to the main trial. The current study was one of those approved and was conducted in a subset (n=339) of children recruited to the main trial. Eligibility for the sub-study closely matched that of the main trial, summarised in table 2.1, with the additional exclusion criterion of any partially breastfed children, to ensure that dietary assessment could be undertaken accurately. In addition to the assessments at admission, day 28, and day 180 (day 90 was not included in the sub-study) were added: 24hr dietary recall, additional plasma sample collection, urine sample collection and faecal sample collection. Plasma samples were drawn per local guidelines, aliquoted to a labelled sample storage tube, and stored at -80°C. A spot urine sample was collected at the earliest available time during each study visit using a single use sterile sample container, then aliquoted to a labelled sample storage tube and stored at -80°C no more than 30 minutes after collection. Faecal samples were collected at the earliest available time using a single use sterile sample container, held on ice on the ward for <30 minutes before transfer to a labelled sample storage tube and storage at -80°C.

Table 2.1 TRACT study eligibility criteria, adapted from (Mpoya et al., 2015)

Inclusion criteria	Exclusion criteria
Aged 2 months to 12 years	Malignancy of other terminal illness
Severe anaemia (Hb <6g/dl) on the day of admission	Acute trauma or burns
Care-giver willing and able to provide consent	Surgery as main reason for admission
	Chronic renal or liver failure
	Signs of bi-ventricular heart failure
	Known congenital or valvular heart disease
	Exclusively breastfed children
	Partially breastfed children (sub-study only)

A flow chart of the children enrolled onto this sub-study is displayed in Table 2.2 below. At the time this thesis was being prepared, the results of the TRACT trial had not been published. Some outcomes were made available to the author by the TRACT study to enable more detailed analysis including randomisation information, haemoglobin concentration and recovery, and survival and the trial results are summarised in Chapters 3 and 4.

Table 2.2 Flow of children in the TRACT sub-study with samples and dietary data provided per time point (n=339)

All		Admission (n=339)	Died (n=13)	Day 28 (n=326)	Died (n=13)	Day 180 (n=313)
Mbale	Total	182	10	172	5	167
	24hrDR	162		152		142
	Plasma	133		123		119
	Urine	137		112		88
	Faeces	96		107		76
Soroti	Total	157	3	154	8	146
	24hrDR	152		92		80
	Plasma	79		76		72
	Urine	136		73		77
	Faeces	118		75		70

2.2.2 TRACT Study Setting

The TRACT study was conducted in four centres across two countries, Uganda and Malawi. Of the four centres only two Ugandan sites, Mbale Regional Referral Hospital and Soroti Regional Referral Hospital, were selected to take part in the dietary sub-study. Mbale and Soroti districts, named for their largest municipalities, are located in Eastern Uganda with Soroti municipality being located approximately 100km north of the larger Mbale. The population of Soroti district recorded in the most recent census in 2014 (Uganda Bureau of Statistics, 2014) was 296,833, however it was projected to reach 351,400 by 2019. Soroti district is predominantly rural with only 16.7% of the population residing within the urban Soroti municipality. In contrast Mbale has a larger population estimated at 488,960 in the 2014 census, projected to rise to 568,800 by 2019. Compared to Soroti, Mbale's population is more likely to reside within urban areas where 27.7% of the district's population live. Both districts are largely rural with agriculture being the main economic activity, and subsistence farming being common. Other than anecdotal, there is a dearth of published information regarding the dietary patterns and intakes of children or adults in these regions. Some generalised insight can be gleaned from the Ugandan Food Tables (Hotz et al., 2012), outlined in more depth below in section 2.3.2, and the most recent Ugandan Census of Agriculture (Uganda Bureau of Statistics, 2009) . Briefly the chief staples consist of matooke (plantain), maize, millet, rice, potato, sweet potato, and cassava. Most staples are boiled and/or fried, while maize and millet can be prepared with water to a stiff consistency to form posho (aka ugali) and millet bread. Beans, peas, nuts, fish, beef, goat and chicken constitute

the major dietary sources of protein, while dairy and eggs are less prevalent. The main vegetables and fruits eaten include greens of numerous variety (e.g. amaranth, cow pea), tomato, onion, spinach, sweet banana, mango, and passion fruit juice.

2.3 Dietary assessment

The measurement of nutritional intake is a balance between respondent burden and accuracy, and the method chosen should be validated for use in the target population group. Three main methods of dietary assessment are widely used in nutrition research: weighed food diaries, single or repeated 24-hour dietary recalls (24hrDR), and qualitative or semi-quantitative food frequency questionnaires (FFQs). Each method is also subject to specific limitations that must be considered when choosing the best approach. FFQs are retrospective and can be used to qualitatively or semi-quantitatively assess dietary patterns over long periods of time, but are subject to inaccurate reporting both from the respondent and from poor question design and knowledge of local diets (Thompson and Subar, 2013). Weighed food records are prospective and considered relatively accurate, but they are also affected by inaccurate reporting by respondents, caused by observer effect and require high literacy and motivation (Thompson and Subar, 2013). 24hr DR provides dietary information from the preceding 24 hour period. This method is dependent on the respondent's recall ability (Thompson and Subar, 2013), and ability to estimate quantities, however additional tools may be used to maximise accuracy such as food models or atlases (Frobisher and Maxwell, 2003). As a major aim of this thesis is to assess interactions between diet, metabolism and the gut microbiota, 24hr DR was chosen as the most suitable method for use in the TRACT sub-study, since urinary metabolic profiles and gut microbial activity are affected by recent dietary intake (Walsh et al., 2006; Conlon and Bird, 2015; Xu and Knight, 2015).

2.3.1 24-hour dietary recall

The full standard operating procedure (SOP), source document (SD), case report form (CRF) and volume/portion size to weight conversion table for the dietary recall sub-study of TRACT are attached in Appendices II-V, with relevant details described below. This 24hr DR method is based on that developed by Gibson and Ferguson (2008), with modifications to reflect local dietary practices, and inclusion of additional portion size estimation techniques. A study to establish method validity was conducted between June and August 2014 (Nightingale et al., 2016), details of which are provided in Chapter 3. Structured training in the validated method was provided to local nursing and support staff by the author over a period of 2 weeks prior to sub-study recruitment commencing.

24hr DRs were conducted in a triple pass approach, which has been shown to maximise recall fidelity (Ma et al., 2009). In the first pass, the guardian/parent and child are requested to freely report all food

and drink taken by the child in the previous 24hr period. In the second pass the interviewer requests further detail regarding the time, type and quantity of food or drink taken including detailed portion size estimation. In the final pass the interviewer reviews the information provided to allow for correction or addition of any omitted items.

Portion size estimation was aided by: 1) sample household volume measures for liquids or dry pouring foods such as rice (cups, bowls, tablespoons and teaspoons), 2) volume of playdough for semi-solid foods such as boiled maize flour (posho or ugali) or millet flour (atap or millet “bread”), 3) reference to local standard sized foods with associated price e.g. 1 medium-sized bread roll (ban) costs 200UGX.

Once the final pass was complete, volumetric data was converted to weight using a volume-weight conversion database created for this purpose, e.g. 100ml of atap weighs 136g (Appendix IV). Standard food sizes were treated in a similar fashion, i.e. 1 medium-sized ban weighs 50g, based on the average of 3 samples measured (Appendix IV). All dietary data with weight in grams was then transcribed onto the CRF.

2.3.2 Data entry and dietary analysis

Dietary data from CRFs was double-entered onto a purpose-built, secure online database hosted on OpenClinica (OpenClinica LLC, Massachusetts USA), developed by the TRACT data management team. Data from both Mbale and Soroti were independently entered by two data management team members on-site in Mbale Regional Referral Hospital to minimise any data entry errors. This data was then reviewed by the author to check that volume and food-item to weight conversions were calculated correctly, and resolve any discrepancies between the double-entered data. Discrepancies were resolved by reference to the source documents in the first instance, or by discussion with the staff member if this was not possible.

Common issues encountered were recorded in a coding protocol to ensure consistency in approach. Where the parent/guardian respondent was not present with the child for the 24hr prior, the dietary recall was rejected, which occurred more frequently with school age children. Any 24hr DR missing >25% of portion size estimates of all foods, or missing more than one portion size estimate for a staple food were not included in the final study. Standard portion sizes and cooking methods were developed for use to replace missing details in otherwise complete recalls, by reference to data generated in the validation study. A summary of the issues encountered and resolutions is shown in Appendix VI.

Data generated from the 24hr DR assessments were entered into Dietplan v6.0 (Forestfield Software Ltd, Horsham UK). Food composition data were taken from “A Food Composition Table for Central and

Eastern Uganda" (Hotz et al., 2012), in the first instance. The Ugandan food composition tables compiled data for local dishes and composite meals common in Uganda. Composition analysis for local foods not available in the United States Department for Agriculture (USDA) databases were also added (United States Department of Agriculture, 2018). Although many foods and ingredients retained the USDA composition values, the Ugandan food tables are the most comprehensive repository for Ugandan food data. When dishes or food items not included in the Ugandan food tables were encountered, a local standardised recipe was created from averaged information sourced from a minimum of three local staff members (Appendices VII and VIII).

Nutrient density of each 24hr DR per 1000kcal was calculated for macro- and micronutrients as a marker of dietary quality in the presence of differing age-related requirements and variable intakes. While dietary energy is presented as MJ in this thesis, density per 1000kcal was chosen to express density to enable comparison to other data. This approach is often taken to compare the dietary value of individual foods, but can also be applied holistically to diets to assess the likelihood of micronutrient deficiency in the presence of energy sufficiency, or vice versa (Drewnowski and Fulgoni, 2014).

2.3.3 VALIDATION OF THE 24HR DIETARY RECALL

The validity of the 24hr DR method described above was assessed by conducting a pilot study. In this study, children aged 6 months to 12 years who had been admitted to Mbale and Soroti Regional Referral Hospitals but who had been treated and were considered healthy, were recruited. With parental consent, an independent trained researcher completed a full day weighed food record in the child's home. An independent researcher visited the child's home on an agreed day from early morning prior to the child waking (07:00) to evening after the child went to sleep for the night or no later than 21:00. The researcher observed the child during all daytime hours, and noted the time and every type of food or drink given. The food/drink container was weighed using digital scales prior to the child eating/drinking, and then again after the child had finished. The final recorded food/drink weight was calculated by subtracting any remaining food/drink from the original amount.

Validity of the 24hr DR was assessed in five ways, to simultaneously address several different facets of validity as recommended by Lombard et al. (2015). Inter-interviewer agreement was assessed by Bland-Altman analysis (Bland and Altman, 1986) of mean difference in estimates for macro- and micronutrients. This approach assesses the presence, direction and extent of bias at the group level. Briefly, the mean difference between the estimates is calculated by subtraction, and then the limits of agreement which are equivalent to the 95% confidence interval are determined. These results are plotted together to allow visualisation of the agreement between quantitative methods. Bland-

Altman analysis was also used to assess the quantitative agreement between the 24hr DR and weighed food records estimates.

The strength and direction of association between the 24hr DR method and weighed food records were analysed by Pearson product moment correlation (*r*). A related correlation method, two-way random model intraclass correlation (ICC), was also applied. ICC reflects both the degree of correlation and the strength of agreement between two methods (Koo and Li, 2016). Results of ICC are classified as followed: ICC < 0.5 indicates poor reliability, ICC 0.5-0.75 indicate moderate reliability, ICC 0.75-0.9 indicate good reliability, and ICC > 0.9 indicate excellent reliability (Koo and Li, 2016).

Agreement between 24hr DR and weighed food records was also assessed by quartile cross classification. Quartiles of estimated intakes of macro- and micronutrients were determined, and individual classification into each quartile determined for each method. Classification in the same quartile was described as 'correct', classification into a quartile above or below the correct quartile was described as 'adjacent', while classification into a quartile two or more above or below the correct quartile was described as 'grossly misclassified'. The outcome of this process is presented as the percentages of estimates which were correct, adjacent, or grossly misclassified.

Classification agreement assessment by Cohen's weighted Kappa (κ) was also applied to measure the agreement between 24hr DR and weighed records. The criteria to interpret this statistic was as described by Landis and Koch (1977) and summarised in Table 2.4.

Table 2.4 Interpretation of Cohen's weighted Kappa (κ) statistic (Landis and Koch, 1977)

κ	Interpretation
<0	Poor agreement
0.0-0.20	Slight agreement
0.21-0.40	Fair agreement
0.41-0.60	Moderate agreement
0.61-0.80	Substantial agreement
0.81-1.0	Almost perfect agreement

2.3.4 Dietary diversity score

Dietary diversity score (DDS) was calculated for each 24hr DR according to WHO guidelines (World Health Organisation, 2008a). DDSs have been shown to be good simple indicators of the micronutrient adequacy of diets in children (Steyn et al., 2006). The food groups included in this assessment are: 1) grains, roots and tubers, 2) legumes and nuts, 3) dairy products, 4) flesh foods, 5) eggs, 6) fruit and vegetables rich in vitamin A and precursors, and 7) other fruit and vegetables. A score was given for the consumption of food from one of seven food groups in any quantity, with a minimum score of 0 and maximum score of 7. Consumption of food from four or more groups in 24 hours was classified as

diverse. Examples of the most common foods reported in each group are displayed in Table 2.3. Fruit and vegetables providing 120 Retinol Equivalents (REs) per 100g of food were classified as rich in vitamin A (World Health Organisation, 2008a).

In addition to these seven food groups used to calculate diversity score, two others were created to classify the remaining foods that could not be included in any WHO recommended groups: sugar and highly sweetened foods, and other. These food groups were used for descriptive purposes only, and are not included within diversity score statistics. Examples of foods in the high-sugar foods category include doughnuts, biscuits, cake, soda, and fats or sugar added to other foods such as spreads or sugar added to porridge. Foods categorised as “other” were largely soups and liquid from stews, and non-nutritive drinks including “dry” or black tea, coffee and water. Where milk and sugar were used in preparation of porridge, tea, or coffee, these were categorised in the appropriate groups. Special foods for management of malnutrition such as F-75/F-100 therapeutic milks and PlumpyNut were also categorised as “other”, however their use was reported in only 3 dietary recalls, and as such they did not contribute significantly to aggregated results.

Table 2.3. World Health Organisation food groups for assessing dietary diversity in children, with examples from the TRACT study (World Health Organisation, 2008a)

WHO Food Group	Examples from Eastern Uganda
1. Grains, roots and tubers (inc. starchy bananas)	Atap/millet bread (boiled cassava and millet flours) Cassava (boiled/fried) Matooke, starchy banana (boiled/steamed) Porridge (maize or millet) Posho (boiled maize flour) Sweetcorn (boiled/roasted) Potato (boiled/fried) White bread (baked) White rice (boiled/fried)
2. Legumes and nuts	Bean stew (blackeye bean) Groundnuts/peanuts (roasted/plain) Groundnut/peanut sauce/paste, peas (boiled)
3. Dairy products	Bongo (fermented milk) Whole milk (raw/boiled)
4. Flesh foods	Beef (stew/stir fried) Calf's liver (fried) Chicken (fried/boiled) Dried omena/silverfish (fried, boiled) Goat meat (roasted) Fresh fish/tilapia (boiled, pasted*)
5. Eggs	Eggs (boiled/fried)
6. Vitamin A-rich fruit and vegetables	Cabbage (boiled/fried) Greens inc. amaranth/cow pea/spinach (boiled/fried/pasted*) Mangoes (raw/juice) Passion fruit (juice) Paw-paw (raw) Sweet potato (boiled/steamed)
7. Other fruit and vegetables	Aubergine (fried) Avocado (raw) Bananas (raw) Cabbage (boiled/fried) Guava (raw) Jackfruit (raw) Oranges (raw/juice) Pineapple (raw)

*pasted refers to foods cooked with groundnut/peanut paste

2.3.5 DIETARY PATTERN ANALYSIS

Dietary pattern analysis is a complementary descriptive approach to traditional nutrient-based dietary analysis methods. Dietary patterns can provide additional qualitative information on the effect of whole diets, rather than the effect of single foods or nutrients. Analysis based on single foods or nutrients fail to account for the interaction of nutrients within various foods e.g. inhibition of iron absorption by tannin, and for the collinearity of many nutrients (Hu, 2002). Dietary patterns may be described by adherence to predefined patterns e.g. the Mediterranean diet score or by identifying patterns from dietary data (Cespedes and Hu, 2015). The latter approach was taken in this thesis, as no ideal dietary patterns have been described in acute illness in children, or in the recovery from

severe anaemia. Principal components analysis (PCA) was used to conduct dietary pattern analysis; the theory behind PCA is discussed in detail in section 2.4, but briefly it examines the correlation matrix to identify a number of latent variables (factors) which can successfully describe variation within the data (Reedy et al., 2010). These latent variables may be comprised of several correlated foods or food groups that summarise major dietary traits. Dietary pattern analysis was conducted in IBM SPSS Statistics v.24 (IBM, New York USA).

2.3.6 CALCULATION OF DIETARY REQUIREMENTS

Requirements for energy, protein and micronutrients were estimated using age- and weight-specific equations published by the WHO, United Nations University, and the Food and Agricultural Organisation of the United Nations (World Health Organisation et al., 2002; World Health Organisation and Food and Agricultural Organizaion of the United Nations, 2004; World Health Organisation et al., 2004). Energy requirements were based on a low physical activity level (PAL) during hospital admission, and a moderate PAL if an outpatient at later study visits, and include energy required for growth.

2.3.7 DETECTION OF OVER- AND UNDERREPORTING

As dietary data is subject to inaccuracy caused by both under- and over-reporting, approaches have been developed to reduce this impact on data interpretation. Objective biomarkers exist for several nutrients and foods, however these are frequently burdensome and expensive e.g. 24-hour urine nitrogen for protein intake (Hedrick et al., 2012). These methods were not suitable for the current study due to cost and time considerations in respect of emergency medical treatment in a resource-limited environment. Goldberg cut off values are a statistical approach to identify over- or under-reporters, based on the assumption that in a healthy weight stable adult, energy intake will equal requirements (Black, 2000a; Black, 2000b). As the current study was conducted in acutely unwell malnourished children with poor appetites and frequently a history of weight loss, or later experiencing catch up growth at variable rates, this approach would likely dramatically overestimate the number children classified as under-reporting at admission, and over-reporting on review. It was therefore decided not to apply these cut-offs in the current study, so this must be borne in mind when interpreting the results of the dietary data analysis.

2.4 METABOLIC PROFILING

Increasingly humans are viewed as a metaorganism, with the health and metabolic activity of both human and microbial physiologies being highly inter-dependent (Turnbaugh et al., 2007; Bosch and McFall-Ngai, 2011). Disturbances in either the host or associated microbiome have been shown to have profound effects on the other, with the health of the metaorganism depending on mutual cooperation (Holmes et al., 2012; Nicholson et al., 2012). Omics approaches attempt to collectively assess all constituents of a biological system in tandem, to holistically assess biological response to stimuli. The metabolome is therefore an ideal target for holistically assessing metaorganism physiology in health and disease. Metabolism can be considered the interface between external environmental exposures, referred to as the exposome, and internal physiological factors, and its basic constituents are the product of metabolic processes: metabolites. Measurement of the metabolic response to stimuli, referred to interchangeably as metabonomics or metabolomics, has been defined as the "quantitative measurement of the dynamic multiparametric response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al., 1999) or more simply as "a comprehensive and quantitative analysis of all metabolites" (Fiehn, 2002).

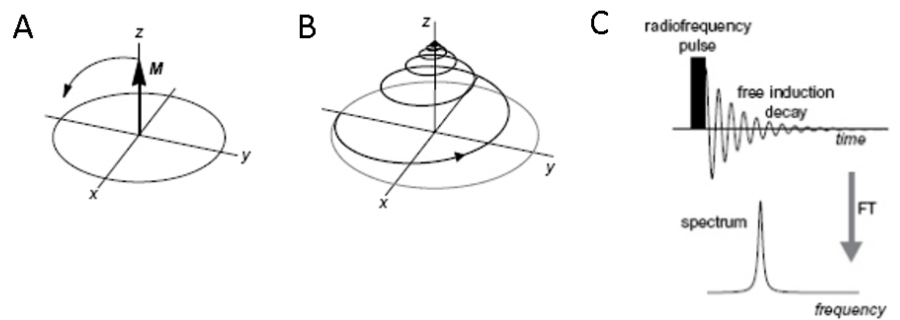
Profiles of the metabolites within a biofluid are frequently obtained using either nuclear magnetic resonance (NMR) spectroscopy, or chromatographic or electrophoretic separation coupled to mass spectrometry (MS). Proton (^{1}H) NMR spectroscopy is highly reproducible and capable of broadly assessing many low molecular weight metabolites (<1500 Daltons) of different chemical classes both from human and microbial origin. ^{1}H NMR is also rapid and requires minimal sample handling and preparation, making it an effective untargeted approach for biofluid metabolic profiling in large studies.

2.4.1 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Atomic nuclei hold an intrinsic angular momentum known as spin (I); non-zero spin results in the generation of a nuclear magnetic moment ($M = \gamma_X$), where γ_X is the gyromagnetic ratio of atomic nucleus X. Hydrogen is found in high abundance in biological compounds, and its nucleus contains a single proton with spin quantum number of ½ (I = ½). Hydrogen nuclei under the influence of a static magnetic field (B_0) therefore orientate either parallel or antiparallel to the direction of the field (z). Both antiparallel (β) and parallel (α) nuclei circulate around the direction of the field at a specific rate referred to as the Larmor frequency of the field, dependent on the strength of the magnetic field. The two possible orientations (α and β) have different energy states, and the proportion (N) of nuclei in

each energy state follows a Boltzmann distribution ($N_\beta/N_\alpha = \exp(-\Delta E/kT)$, where ΔE = energy difference between orientations, k = Boltzmann constant [1.38 x 10^{-23} m^2 kg s^{-2} K^{-1}] and T = temperature in Kelvin). The energy difference between the parallel and anti-parallel states (ΔE) can be calculated from the nuclear gyromagnetic ratio (γ): $\Delta E = \gamma h B_0/2\pi$, where h = Planck's constant [6.626 x 10^{-34} m^2kg/s]. At thermal equilibrium there are always slightly more nuclei (approximately 0.0048% more) in the lower energy parallel (α) orientation than the higher energy (β) orientation. Low energy nuclei can enter the high energy orientation by the application of a pulse of electromagnetic radiation with a frequency of $\Delta E/h$, referred to as the nuclear resonance frequency. In standard one-dimensional experiments the electromagnetic pulse is calibrated to re-orient nuclei at 90° to the magnetic field, summarised in Figure 2.1 below. Removal of this pulse allows nuclei to return to their original orientations, releasing the energy absorbed in a process referred to as free induction decay (FID) which can be detected and quantified (Ross et al., 2007). This information is Fourier transformed and digitised to form an NMR spectrum.

Figure 2.1 Basic one-dimensional nuclear magnetic resonance spectroscopy (Hore, 2015)



A: effect of 90° pulse on nucleus orientation within magnetic field (M), B: nucleus realigns to magnetic field orientation, C: illustration of free induction decay during B and conversion to nuclear magnetic resonance spectrum by Fourier Transform (FT)

Although theoretically ^{1}H nuclei have identical resonance frequencies, in molecules this is not the case, owing to variation in local electromagnetic forces caused by other atomic nuclei and shielding caused by electrons. The relative resonance frequency of a nucleus compared to a standard nucleus is referred to as the chemical shift, which permits the identification of nuclei from different chemical environments or functional groups. A reference compound is added to each sample to enable chemical shift calculation, in this case TSP (3-(Trimethylsilyl)propionic-2,2,3,3-d4). Neighbouring nuclei within molecules can also have different orientations, and therefore have different energy states. If these nuclei are less than four bonds apart, they can interact with one another, causing changes in the

multiplicity of peaks in the NMR spectrum, thus providing additional structural information (Ross et al., 2007; Hore, 2015).

Biofluid samples contain a high proportion of water, hence a large number of ^1H nuclei, which can dominate NMR spectra. In standard one-dimensional NMR experiments (e.g. Nuclear Overhauser Effect Spectroscopy), an additional low power continuous pulse is applied which effectively reduces the signal from water (Dona et al., 2014). Some samples, e.g. plasma, may also contain significant amounts of larger molecules such as proteins or lipids, which would mask signals from lower concentration metabolites. A modified pulse sequence (Carr-Purcell-Meiboom-Gill sequence) can also be used in these samples to exploit the rapid signal loss of nuclei in macromolecules, effectively removing macromolecule signals (Dona et al., 2014).

Two-dimensional experiments can assist with compound identification by elucidation of multiplicity and relationships between ^1H nuclei. J-resolved (JRES) ^1H NMR experiments are simple 2D experiments that separate chemical shifts into two time domains, enabling elucidation of peak structure, overlapping and multiplicity (Hore, 2015).

2.4.2 Sample preparation

Sample preparation for NMR spectroscopy in all studies was as described by Dona et al. (2014), briefly described below. Sample preparation order and experimental run order were randomised using Excel functions (Microsoft, Redmond USA) to limit any systematic effects. In order to check equipment stability, quality control samples for each sample type were prepared as follows. An aliquot of each sample being prepared was transferred to a falcon tube, which was then vortex mixed and centrifuged at 12000g for 5 minutes at 4°C. Quality control samples were drawn from this and prepared as described below, and run every 12-24 samples.

2.4.2.1 Urine preparation

Urine buffer (1.5M KH_2PO_4) was prepared as follows and stored at 4°C: 20.4g of KH_2PO_4 was dissolved in 80ml of D_2O. 100mg of reference compound (TSP) and 13mg of NaN_3 were dissolved in 10ml of D_2O, then both solutions were mixed well. pH was adjusted to 7.4 by the addition of potassium hydroxide pellets, the solution transferred to a 100ml flask and volume adjusted to 100ml with D_2O.

Samples were thawed and vortex mixed, then centrifuged at 12000*g* for 5 minutes at 4°C. 540µl of supernatant was transferred to a SampleJet 5mm NMR tube (Bruker, Karlsruhe Germany), to which was added 60µl of urine buffer, and then mixed well. NMR tube caps were sealed with POM balls.

2.4.2.2 *Plasma preparation*

Plasma buffer (0.075M NaH_2PO_4) was prepared as follows and stored at 4°C: 5.32g of NaH_2PO_4 was dissolved in 380ml of water. 0.4g of reference compound (TSP) was added to this solution and dissolved. 5ml of 4% NaN_3 solution was added and mixed, then 100ml of D_2O added. pH was adjusted to 7.4 using 1M HCl solution. The solution was transferred to a 500ml flask and volume adjusted to 500ml with water.

Samples were thawed and vortex mixed, then centrifuged at 12000*g* for 5 minutes at 4°C. 350µl of sample and 350µl of plasma buffer were added to a microcentrifuge tube that was then vortex mixed and centrifuged for 12000*g* at 4°C for 5 minutes. 600µl of supernatant was transferred into a SampleJet 5mm NMR tube (Bruker, Karlsruhe Germany), then sealed with POM balls.

2.4.3 ^{1}NMR spectroscopy data pre-processing

^{1}H NMR spectroscopy was conducted using a Bruker 600Mhz Avance Spectrometer (Bruker, Karlsruhe Germany). Processing of spectral data was conducted using Topspin v3.1 (Bruker, Karlsruhe Germany). Fourier transformed spectra were automatically phased and baseline corrected, then visually inspected and manually re-phased where necessary. Throughout the experiment, the shim-quality of the spectra was assessed by measuring the peak width at half height of TSP (for urine) or lactate (plasma). Where this was found to be in excess of 0.8Hz, the experiment was repeated, and the better of the two spectra kept.

2.4.4 Multivariate statistical analysis

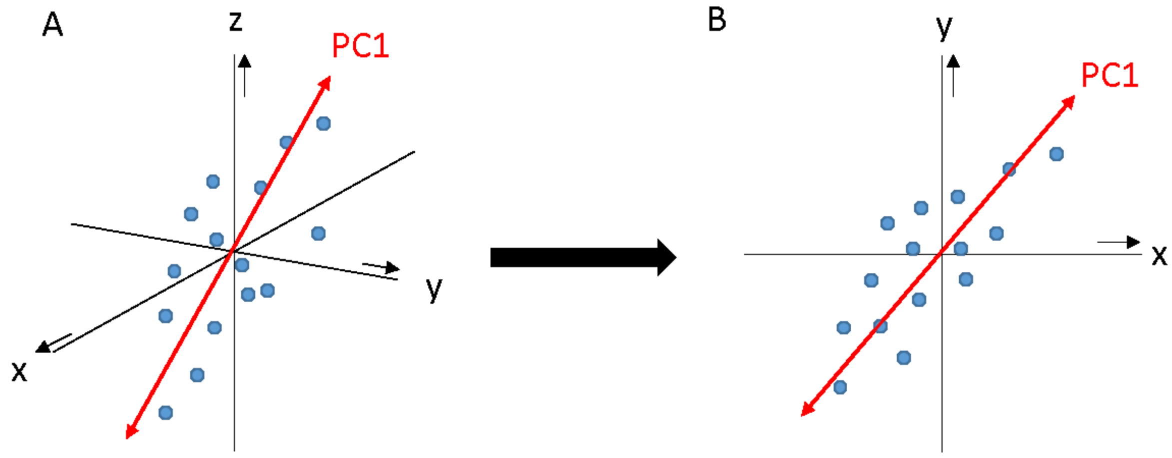
Data generated from ^{1}H NMR spectroscopy experiments are complex owing to the large number of NMR signals generated, and the highly collinear nature of many metabolites. In order to sensibly extract meaningful, interpretable information, multivariate statistical approaches have been developed. A high resolution NMR spectrum may contain upwards of 20-30K data points per sample, meaning that the number of variables per sample is highly likely to be greater than the number of samples in each dataset, referred to as high dimensionality data. Broadly speaking, the aim of multivariate methods are to reduce the dimensionality of these datasets, which they achieve by effectively summarising the variation within the data through identification of latent variables. Both SIMCA (Umetrics, Umea Sweden) and Matlab (The MathWorks Inc., Natick USA) were used to generate

multivariate models and associated scores and loadings plots in this thesis. Two approaches are generally taken when interrogating high dimensional datasets: unsupervised and supervised.

2.4.4.1 UNSUPERVISED MULTIVARIATE ANALYSIS

Unsupervised multivariate analysis does not rely on any a priori class information to determine the latent variables which express the greatest amount of variation within the dataset. Principal component analysis (PCA) is a common approach for this purpose, where each latent variable is termed a principal component (PC)(Wold et al., 1987). Data are ordered in a 2D sample and variable data matrix, the X matrix. Covariant variables which display high levels of combined variation are considered collectively, and the first PC will described the greatest variation within the data matrix, denoted R^2 (Jolliffe, 2010). Subsequent PCs will explain progressively less of the variation, and therefore progressively have smaller R^2 values. Samples may be better or worse explained by each component, which is quantified by a score; scores can be used to visualise how each sample relates to others with respect to the selected principal components, as shown in Figure 2.2. An optimal number of PCs can be achieved which explains a high amount of variation, in a small number of variables, effectively reducing the data dimensionality.

Figure 2.2 Illustration of dimension reduction by principal components analysis (PCA)



Data dimension reduction by PCA is shown. Principal component 1 (PC1) explains the greatest variation within the dataset (A). The data can be successfully summarised in two dimensions (B), retaining a large amount of information from PC1

2.4.4.2 SUPERVISED MULTIVARIATE ANALYSIS

Supervised methods supply class information a priori, and attempt to maximise the covariance in the X matrix and class membership, i.e. find the latent variables which explain the greatest differences, if present, between two classes supplied in a Y-matrix. Supervised methods can be used to detect

metabolic differences between discrete classes (anaemic vs. non-anaemic) or for regression against a continuous variable (haemoglobin concentration). Partial least squares (PLS) is a supervised multivariate statistical method used for regression (PLS-R) and for class separation or discriminant analysis (PLS-DA). Orthogonal PLS (OPLS) is an extension of the PLS method, which excludes sources of variation unrelated to the specified Y-matrix, by excluding the effect of variables orthogonal to the calculated predictive variable (Cloarec et al., 2005b).

2.4.4.3 Multivariate model quality

Both unsupervised and supervised methods generate measures of the quality both of the model overall and of individual components. It is important to refer to these parameters to establish if the model explains sufficient variation to be of use (R^2). Supervised models must also be tested for overfitting and how well the model is able to accurately predict class membership or continuous variable values (Q^2). Q^2 is calculated by cross-validation of the model, in which successive 1/7ths of the X-matrix are excluded to check how well the remaining data match the original model, until all data has been excluded once (Worley and Powers, 2013). Both parameters are expressed in percentage terms such that an R^2 value of 0.35 means the model or latent variable explains 35% of the variation in the dataset, while a Q^2 value of 1.0 means the model has perfect predictive ability. If more components are added than are necessary, the R^2 will continue to improve, but the Q^2 value will begin to decrease, indicating overfitting. Generally speaking a Q^2 value of 0.5 or more is considered indicative of a robust model, however many studies in free-living human populations report permutation-validated models with Q^2 values of 0.3 or more. Permutation is a further method to assess whether a given model may have arisen by chance. In this procedure, Y-matrix values or class membership are randomised, and the model rebuilt multiple times (999 in this thesis), to assign a p-value (cross-validated analysis-of-variance, CV-ANOVA) describing the probability that the non-permuted model arose by chance (Worley and Powers, 2013).

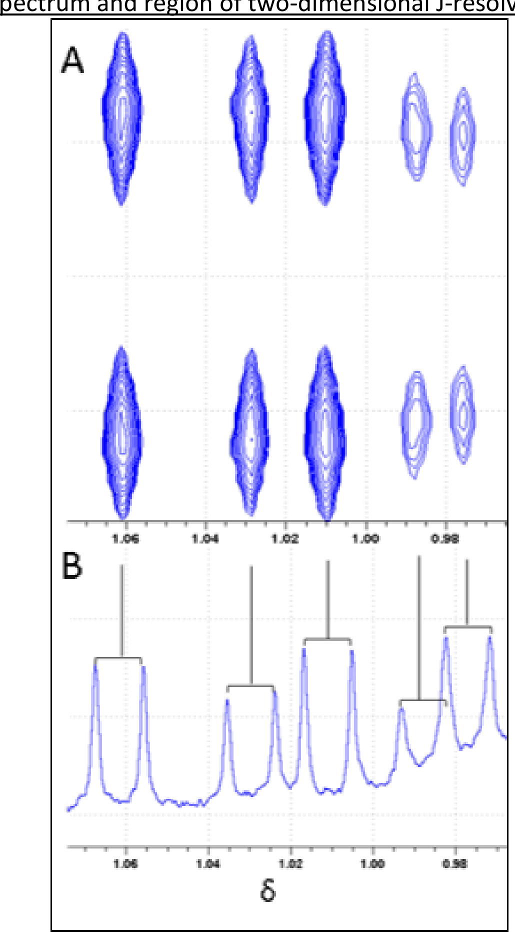
2.4.5 Metabolite identification

Due to the convolved nature of spectral peaks in NMR spectra of complex mixtures, and multiple signals arising from different ^{1}H nuclei in metabolites, it is necessary to adopt a methodical approach to metabolite identification. Chemical shifts identified by multivariate models provide a limited amount of information about the underlying metabolites. Identifying other NMR-active nuclei within the same metabolite can be achieved by the application of statistical techniques. Statistical correlation

spectroscopy (STOCSY) (Cloarec et al., 2005a), takes a chemical shift value as input, and by analysis of all available spectra, locates other chemical shifts that are correlated with the original peak. An extension of this approach (subset optimisation by reference matching (STORM) (Posma et al., 2012)), has been developed which limits the available spectra to those which contain higher concentrations of the metabolite of interest, thus maximising the correlation with associated peaks. Both methods were used throughout this thesis to retrieve chemical shift patterns.

As described in section 2.4.1 above, the signal retrieved from ^{1}H nuclei may be subject to interaction with other nuclei, causing peak splitting in characteristic patterns, which can be used to assist in metabolite identification (Dona et al., 2016). In some instances visual inspection of the feature can indicate the multiplicity, however frequently in biofluid metabolic profiles, there is significant peak overlap. In these instances, 2D J-resolved spectra can be used to deconvolve overlapping peaks and identify the peak multiplicity. An example of an overlapped region of a plasma ^{1}H NMR spectrum with the associated JRES spectrum is displayed below in Figure 2.3.

Figure 2.3 Example of peak convolution in one-dimensional CPMG ^{1}H NMR plasma spectrum and region of two-dimensional J-resolved spectrum



J-resolved (A) and CPMG (B) spectra of human plasma showing:
alpha-hydroxyisovalerate (doublet δ0.98), valine (doublets δ0.99 and δ1.03),
isoleucine (doublet δ1.01) and alpha-ketoisovalerate (doublet δ1.06)

The chemical shift and peak multiplicity patterns of each NMR active ^1H nucleus in metabolites is highly characteristic, and by comparison to reference databases such as the Human Metabolome Database (Wishart et al., 2018), or an in-house database, can be used to identify metabolites.

2.5 METATAXONOMIC PROFILING

Sequencing the 16S ribosomal ribonucleic acid (rRNA) gene is a commonly used method for culture-independent profiling of microbial species. The 16S rRNA gene encodes the 16S rRNA portion of the 30S subunit of prokaryotic ribosomes, responsible for protein synthesis within cells. Due to its essential function, the 16S rRNA gene is highly conserved across prokaryotic species, however it contains highly distinctive variable regions (V1-9). Identification and classification of microbial species within a sample can be achieved by targeted sequencing of the variable regions and comparison to phylogenetic databases.

2.5.1 SAMPLING AND DNA EXTRACTION

Faecal samples were collected from children in the TRACT study as outlined previously, for subsequent profiling. Samples were immediately placed on ice, then transferred to the on-site research laboratory for aliquoting into microcentrifuge storage tubes, and freezing at -80°C within 1 hr of collection, before temperature controlled transport to Imperial College London.

QIAamp PowerFecal DNA Kits (Qiagen, Hilden Germany) were used by the author to extract faecal DNA as described in the protocol. Briefly, inside a biosafety cabinet, 250mg of defrosted faecal sample was transferred into a safe-lock microcentrifuge tube. To this glass beads were added and then 750µl of bead solution and 60µl of solution C1. Tubes were then heated at 65°C for 10minutes, and placed into the Bullet Blender Storm instrument (Next Advance Inc., New York USA), and bead beaten for 3 minutes at speed 8. Tubes were removed and centrifuged for 1 minutes at 13,000g, after which supernatant was transferred to a clean microcentrifuge tube. 250µl of solution C2 was added to the supernatant, then vortex mixed, and incubated at 4°C for 5 minutes. The tubes were then re-centrifuged at 13,000g for 1 minute, and up to 750µl of supernatant transferred to a new tube, while avoiding the pellet. 1200µl of solution C4 was added to the supernatant and then vortex mixed. The mixture was loaded onto a spin filter and centrifuged at 13,000g for 1 minute in 3 x 650µl batches discarding the flow through until all had been filtered. 500µl of solution C5 was then loaded onto the filter, and centrifuged at 13,000g for 1 minute. The extracted DNA was then eluted from the filter by

adding 100µl of solution C6, and centrifuging for 1 minute at 13,000g. Aliquots of 25µl volume of the eluted DNA was transferred to new tubes and stored at -80°C.

Prior to sequencing, the concentration of DNA was determined using Qubit dsDNA BR assay kits (Thermo Fisher Scientific, Massachusetts USA) as per the protocol, and where necessary DNA was diluted to below 5µg/ml. Assessment of DNA concentration was completed by the author.

2.5.2 Illumina MiSeq 16S rRNA sequencing and data processing

MiSeq sequencing of faecal DNA in this thesis was completed by the Marchesi Laboratory at Imperial College London. In MiSeq amplicon sequencing of the 16S rRNA gene, forward and reverse primers are selected which target the region of interest, in this case the V1-V2 variable regions. The region is then amplified, followed by removal of primer sequences and artefacts which may have formed. Fluorescent nucleotides are sequentially added to the amplified complementary DNA strands, emitting a distinctive signal for each base (Clarridge, 2004). Sample libraries were prepared according to Illumina's 16S protocol (Illumina, 2017), and details of the primers used are presented in Chapter 4 results.

These complementary base sequences form the basis of 16S rRNA-based microbial profiling, however several data processing steps are necessary before analysis. There are several methods to achieve this, for example QIIME (Caporaso et al., 2010) or Mothur (Kozich et al., 2013): in this thesis the Mothur data processing pipeline was applied based on the online SOP accessed 14th July 2017. Paired read sequences are combined to maximise the information available for phylogenetic classification, and those with ambiguous bases or of the wrong length excluded. Similar paired sequences are clustered together as operational taxonomic units (OTUs), and classified by comparison to reference databases including SILVA (Quast et al., 2013), Greengenes (DeSantis et al., 2006) and the Ribosomal Database Project (Cole et al., 2014). OTUs with low count numbers (<10 reads per OTU across all samples), or singletons (OTUs with only 1 read) were combined into "rare" and "singleton" variables. Read depth and coverage were used as a measure of sequencing success, and any sample with total reads <16,509 was discarded. Specific details of the sequencing quality are given in the corresponding results chapter.

2.5.3 Statistical analysis of microbial profiles

OTU data generated from the Mothur pipeline with corresponding taxonomy information was analysed in the following ways. Univariate comparisons based on associated metadata were

undertaken in STAMP software (statistical analysis of taxonomic and functional profiles) (Parks et al., 2014). Two group comparisons were conducted using two-sided White's non-parametric t-test, with Benjamini-Hochberg false discovery rate (FDR) (Benjamini and Hochberg, 1995) applied to correct for multiple testing. Multiple group comparisons were conducted by the Kruskal-Wallis H-test, also using Benjamini-Hochberg FDR correction.

Mothur scripts were used to calculate alpha and beta diversity statistics, which were then compared using general statistical methods described in section 2.6 below. In-house R scripts run in RStudio v1.0.143 (RStudio Inc., Boston USA) with R version 3.5.0 were also used to conduct multivariate analysis of 16S data based on the UniFrac weighted distance matrix generated from Mothur, chiefly non-metric dimensional scaling (NMDS) with associated permutational analysis of variance (PERMANOVA) statistical tests. Where multiple PERMANOVA were performed within a multi-group analysis, p-values were adjusted using FDR. Summary dietary data were integrated with metataxonomic data as follows: for nutrients and food groups where sufficient numbers had intakes to enable separation into tertiles, the highest and lowest tertiles were compared. Where intake of a food group was reported by less than 75% of children, those who had any intake (>0g) were compared to those with no intake. For the remaining food groups a pragmatic cut off was used of the 10th percentile of intake, and those with intakes above and below this level were compared. These values are reported in the relevant results sections.

2.6 General statistical methods

Calculation of anthropometric indices to classify wasting (WHZ), underweight (WAZ) and stunting (HAZ) were completed in Excel, Microsoft Office 15 (Microsoft, Corporation, Washington USA). These were calculated by comparison to gender- and age-specific reference data, using the LMS method to calculate the z-score based on distance from the median population value (World Health Organisation, 2007). Statistical analyses of dietary data, 24hr DR method validation, subject characteristics and outcomes were conducted in IBM SPSS Statistics v24 (IBM, New York USA). Normality of data distribution was assessed using Q-Q plots and the Kalmogorov-Smirnov statistic. Non-normally distributed data were analysed using the relevant non-parametric equivalent tests, noted in the results chapters. Outcome prediction by individual and combined dietary factors was interrogated using unadjusted binomial regression for discrete variables and unadjusted linear regression for continuous variables.

3. Chapter 3 – Nutrition in Recovery from Severe Anaemia

3.1 Introduction

Chapter 3 details the validation of the 24 hour dietary recall method and the results of its subsequent application in the TRACT clinical trial. As previously discussed, dietary assessment methods are subject to limitations, and a tool developed for use within one population may be unsuitable for use in other groups. The validity of several methods of dietary assessment has been assessed in Africa, but rarely in children, such as food frequency questionnaires (Lin et al., 2007; Amare et al., 2012), 24 hour dietary recall (Kigutha, 1997; Alemayehu et al., 2011; Jariseta et al., 2012), weighed food records (Kigutha, 1997) and household consumption surveys (Jariseta et al., 2012). FFQs have been found to be poor measures of daily intake, while prospective weighed records have been found to be highly accurate in providing quantitative dietary information. Prospective weighed records are burdensome and unsuitable if the dietary exposure of interest has already occurred, but can be used as a "gold standard" comparison method for the validation of other quantitative methods, which was the approach taken in the current study. The method validation study included a period of 24hrDR tool development and validation within children and parents from Mbale and Soroti municipalities in Eastern Uganda, and was undertaken between January-August 2014 (Nightingale et al., 2016), Appendix IX.

The second portion of this chapter deals with the application of the validated 24hr dietary recall tool to the TRACT study described in Chapter 2 sections 2.2.1-2.2.3. The thematic background and relevant literature can be found in Chapter 1 sections 1.2.2 and 1.2.3.

Aims:

- To develop and validate a method to accurately assess dietary intake in children in sub-Saharan Africa
- To describe the dietary traits of children in a specific region of sub-Saharan Africa: Eastern Uganda, and assess how this may interact with haematological status
- To assess the impact of acute dietary intake on recovery from severe childhood anaemia
- To generate dietary data which can be used to explain variation in metabolic and gut microbial profiles

Hypothesis:

- Recent dietary intake can affect the outcome of children being treated for severe anaemia

3.2 Results

3.2.1 24 hour dietary recall method validation

3.2.1.1 General results

In total 24 children (14 from Mbale and 10 from Soroti) were recruited to the study, however three were later excluded due to illness and a further two were excluded as they failed to complete the 24hrDR interviews. Of the remaining 19 children who were included in the study, 63% were female (n=12), the mean age was 3.4 years (± 2.6), and the mean weight was 14.0kg (± 5.6). Three children were classified as underweight, with WAZ scores <-2.0, while the mean WAZ score was -0.23 (±1.57). Full details of subject characteristics are displayed in Table 3.1 below. Power was determined in a post-hoc analysis based on mean energy consumption of 6563kJ (SD 1706kJ) showing the study had 80% power to detect a difference of 1097kJ (16.7%) at a significance level of 0.05.

Table 3.1 Subject characteristics of children (n=19) who took part in the 24 hour dietary recall validation study

	Overall (n=19)	Mbale (n=10)	Soroti (n=9)
	Mean (SD)	Mean (SD)	Mean (SD)
Age (years)	3.4 (2.6)	3.7 (2.8)	3.0 (2.3)
Gender (% female)	63.2	70.0	55.6
Weight (kg)	14.0 (5.6)	14.9 (7.0)	13.1 (3.6)
Weight-for-age z-score	0.23 (1.57)	0.54 (1.45)	-0.12 (1.71)

3.2.1.2 Estimated requirements and dietary intake

Requirements and estimated intake are presented for macronutrients and major micronutrients for information. The median estimated energy requirement in the 19 children was 4046kJ [IQR 3582-5277kJ] per day, while median energy intakes exceeded this in both dietary recalls (6339kJ, IQR 4010-7211kJ and 5104kJ, IQR 4030-6747kJ) and the weighed food record (6021kJ, IQR 4231-7109). Based on the most accurate estimate from the weighed food record, dietary energy intake was 149% of estimated requirements. Further comparisons of intakes to requirements made below are also based on weighed food record estimates, which are presented alongside requirements and dietary recall estimates in Table 3.2 below. Protein intakes (29.9g, IQR 23.0-48.3g) exceeded requirements (12.4g/day IQR 10.9-14.2g/day), by a factor of 2.4. Among the nutrients traditionally thought of as affecting haematological status (iron, vitamin B12, and folate), median intakes of vitamin B12 (1.0µg, IQR 0.2-1.7µg) and folate (119µg, IQR 62-210µg) were in excess of requirements (0.7µg and 80µg respectively), while iron intakes (5.3mg, IQR 3.1-8.7) were only 76% of estimated requirements (7.0mg, IQR 7.0-10.0mg). For brevity, formal assessments of agreement are shown for energy, protein and iron only.

Table 3.2 Nutrient intake and requirements of children (n=19) recruited to the 24 hour diet recall validation study

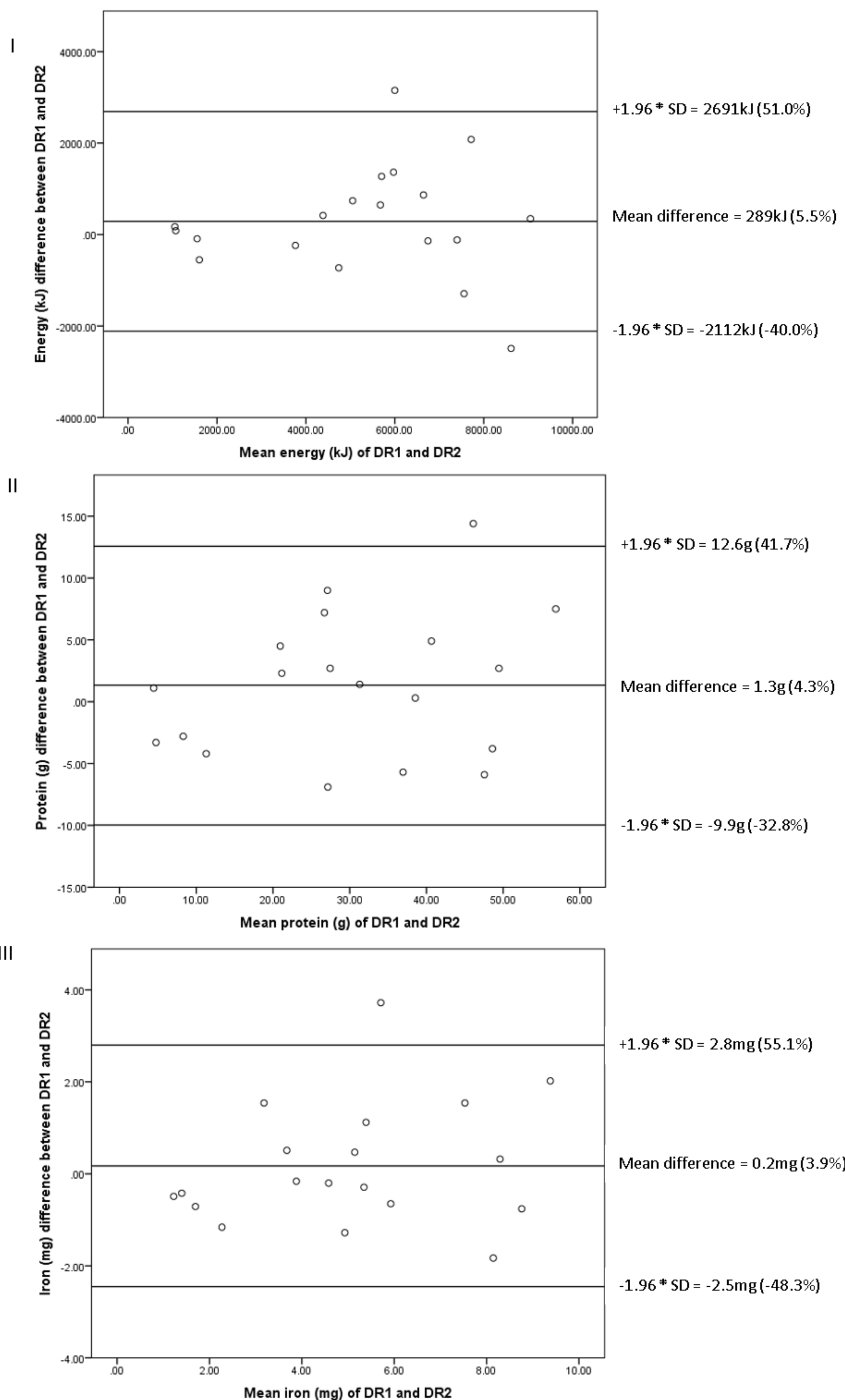
Nutrient	Requirements Median [IQR]	First dietary recall Median [IQR]	Second dietary recall Median [IQR]	Weighed food record Median [IQR]
Quantity (g)	-	1317 [719-1509]	1203 [776-1512]	1269 [804-1485]
Water (g)	-	792 [477-1118]	968 [523-1097]	954 [559-1105]
Energy (kJ)	4602 [3836-5208]	6339 [4010-7211]	5104 [4030-6747]	6021 [4231-7109]
Protein (g)	12.4 [10.9-14.2]	31.6 [22.8-43.9]	30.6 [19.4-39.4]	29.9 [23.0-48.3]
Nitrogen (g)	-	2.6 [1.7-3.8]	3.5 [2.1-3.9]	2.9 [1.9-3.9]
Fat (g)	-	30.8 [15.9-59.3]	34.2 [17.5-48.9]	36.5 [17.2-53.3]
Carbohydrate (g)	-	155.5 [95.4-198.4]	131.0 [75.6-187.2]	145.7 [101.1-185.4]
Starch (g)	-	47.2 [21-92.5]	34.8 [18.9-82.8]	41.4 [19.6-88.5]
Sucrose (g)	-	39.5 [18.6-49.0]	41.1 [24.1-53.0]	32.7 [18.2-64.0]
Fibre (AOAC) (g)	-	3.4 [1.2-9]	3.6 [2.0-8.2]	5.4 [1.1-9.8]
Sodium (mg)	-	110 [63-435]	315 [98-679]	97 [50-272]
Potassium (mg)	-	777 [422-1678]	634 [524-1601]	1004 [349-1643]
Calcium (mg)	450 [450-500]	257 [109-516]	230 [88-374]	262 [123-521]
Magnesium (mg)	54 [54-60]	70 [25-105]	53 [27-115]	76 [19-120]
Phosphorus (mg)	-	311 [146-357]	271 [186-379]	318 [140-386]
Iron (mg)	7.0 [7.0-10.0]	5.2 [3.9-7.4]	4.8 [3.1-6.5]	5.3 [3.1-8.7]
Copper (mg)	-	0.3 [0.2-0.6]	0.3 [0.1-0.6]	0.4 [0.1-0.6]
Zinc (mg)	4.1 [4.1-4.1]	4.1 [2.6-6.2]	4.1 [2.2-6.0]	3.7 [2.7-5.8]
Chloride (mg)	-	178 [94-652]	447 [121-999]	145 [67-381]
Manganese (mg)	-	0.9 [0.3-1.8]	1.0 [0.5-2.0]	1.3 [0.3-1.8]
Selenium (µg)	10 [10-17]	7.4 [2.9-15.4]	9.1 [5-13.8]	9.9 [2.4-14.3]
Iodine (µg)	140 [140-140]	15.8 [6.8-22.6]	18.8 [5.3-26.9]	12.3 [5.1-21.0]
Retinol (µg)	190 [190-200]	57 [13-203]	96 [9-176]	59 [2-225]
Carotene (µg)	-	438 [46-1552]	589 [326-1138]	318 [98-1034]
Vitamin D (µg)	5 [5-5]	0.2 [0.1-0.6]	0.3 [0.2-0.7]	0.1 [0.1-0.7]
Vitamin E (mg)	-	0.7 [0.2-2.8]	1.6 [0.6-2.4]	1.2 [0.3-5.3]
Thiamine (mg)	0.3 [0.3-0.5]	0.7 [0.4-1.0]	0.7 [0.4-0.8]	0.7 [0.4-1.0]
Riboflavin (mg)	0.4 [0.4-0.5]	0.5 [0.2-0.9]	0.4 [0.2-0.9]	0.5 [0.2-1.0]
Niacin (mg)	4 [4-6]	5.5 [2.9-7.2]	6.0 [3.9-7.3]	5.7 [3.7-8.2]
Vitamin B6 (mg)	0.3 [0.3-0.5]	0.8 [0.5-1.2]	0.8 [0.5-1.1]	0.9 [0.6-1.2]
Vitamin B12 (µg)	0.7 [0.7-0.9]	1.0 [0.3-1.3]	0.9 [0.4-1.3]	1.0 [0.2-1.7]
Folate (µg)	80 [80-150]	116 [65-166]	84 [79-153]	119 [62-210]
Pantothenate (mg)	1.8 [1.8-2.0]	0.8 [0.4-1.6]	1.0 [0.4-1.6]	0.7 [0.5-1.7]
Biotin (µg)	6 [6-8]	4.3 [1.9-5.5]	4.7 [2.6-7.1]	3.4 [1.2-6.7]
Vitamin C (mg)	30 [30-30]	52 [25-116]	48 [23-137]	37 [26-137]

Abbreviations: AOAC, Association of official Analytical Chemists ; IQR, interquartile range

3.2.1.3 INTER-INTERVIEWER AGREEMENT

Bland-Altman plots showing the agreement between each interviewer are shown in Figure 3.1 for energy, protein and iron intake estimates. Mean energy intake estimate differed by 289kJ, 95% CI -2112-2691kJ, equivalent to 5.5% (-40.0% to 51.0%). For protein, mean estimates differed by 1.3g (4.3%) on average, 95% CI -9.9-12.6g (-32.8% to 41.7%). Lastly, iron intake estimates differed by 0.2mg (3.9%) on average, 95% CI -2.5-2.8mg (-48.3% to 55.1%). ICC shows good agreement between interviewers for energy (ICC = 0.802, 95% CI 0.429-0.933) and iron (ICC = 0.868, 95% CI 0.618-0.955). Agreement in estimating protein intake was excellent with ICC of 0.925 (95% CI 0.779-0.975).

Figure 3.1. Bland-Altman plots displaying inter-interviewer agreement for: (I) energy, (II) protein, and (III) iron (n=19)



3.2.1.4 Correlation between 24hrDR and weighed food records

The weighed food records and 24hrDRs showed a high degree of correlation for energy, protein and iron, summarised in Table 3.3. Estimates of energy intake had excellent intraclass correlation (ICC) of 0.98 (95% CI 0.90-0.98), and Pearson correlation coefficient (*r*) of 0.96 ($p<0.001$). Protein intake was similarly excellently correlated with an ICC of 0.97, 95% CI 0.90-0.99 and r of 0.985 ($p<0.001$). Reported intakes of iron also showed excellent correlation with ICC of 0.94 (95% CI 0.84-0.98) and r of 0.91, $p<0.001$.

Table 3.3 Correlation analyses of weighed food records and 24 hour dietary recalls (n=19)

Nutrient	Weighed Food Record		Combined Dietary Recalls			
	Mean	SD	Mean	SD	ICC† (95% CI)	*r*
Energy (kJ)	5429	2720	5279	2503	0.98 (0.90-0.98)	0.960*
Protein (g)	33.2	17.9	30.3	15.9	0.97 (0.90-0.99)	0.985*
Iron (mg)	5.5	3.2	5.1	2.5	0.94 (0.84-0.98)	0.910*

SD, standard deviation; ICC, intraclass correlation coefficient; CI, confidence interval; *r*, Pearson correlation coefficient

*p<0.001

†ICCs compared absolute agreement of average measures, using a two-way random model

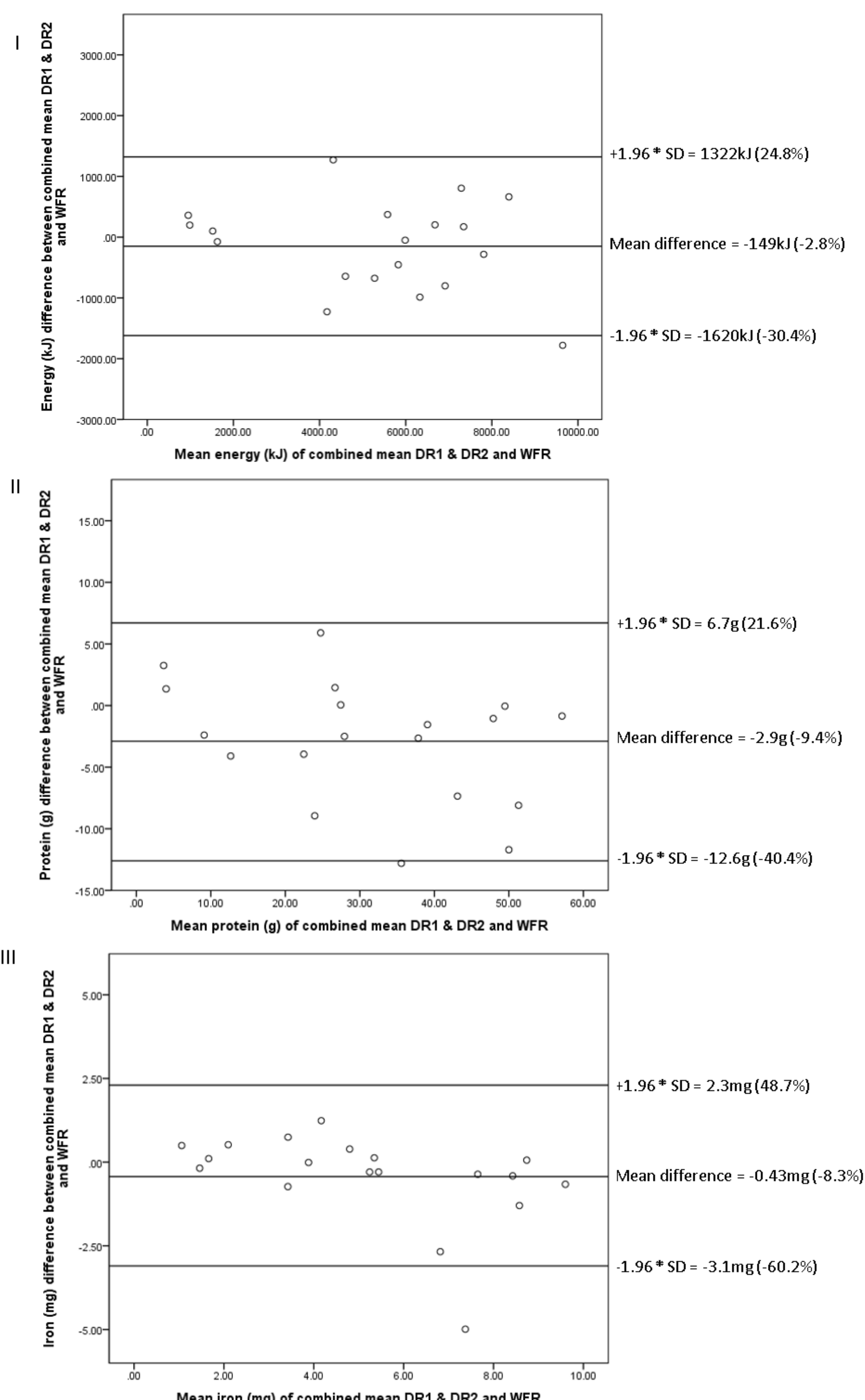
3.2.1.5 Quartile cross-classification

Classification into quartiles of intake for selected nutrients is summarised in Table 3.4, and interpretation of Cohen's κ is as per Table 2.4. Intake estimates from the 24hr DR correctly classified energy intake in 79% of cases (n=15), with the remainder being classified adjacently (21%), with substantial agreement indicated by Cohen's κ of 0.719. Protein and iron intakes were better classified by the 24hr DR method, with 89% (n=17) being correctly classified, while the remainder (11%) were classified into an adjacent quartile. Both protein and iron intake quartile cross-classification showed almost perfect agreement with the weighed food record (Cohen's κ = 0.859 in both cases).

Table 3.4 Quartile cross-classification of estimated dietary intake (n=19)

Nutrient	Classified correctly (%)	Classified adjacently (%)	Grossly misclassified (by ≥2 quartiles) (%)	Cohen's Kappa κ (p-value)
Energy	15 (79)	4 (21)	0	0.719 (<0.001)
Protein	17 (89)	2 (11)	0	0.859 (<0.001)
Iron	17 (89)	2 (11)	0	0.859 (<0.001)

Figure 3.2. Bland-Altman plots displaying agreement between combined 24hrDR and weighed food records for: (I) energy, (II) protein, and (III) iron (n=19)



3.2.1.6 VALIDITY OF 24 HOUR DIETARY RECALL RELATIVE TO WEIGHED FOOD RECORDS

The 24hr DR method agreed closely with weighed food records in estimating energy intake, underestimating energy by only -149kJ (-2.8%) on average, with limits of agreement of -1620kJ (24.8%) to 1322kJ (-30.4%). Protein intake was subject to greater underestimation by 24hr DR compared to weighed records, with a mean difference of -2.9g (-9.4%), and a greater tendency to underestimation suggested by limits of agreement ranging from -12.6g (-40.4%) to 6.7g (21.6%). Estimates of iron intake by 24hr DR were similar to those from weighed food records, but still subject to underestimation of 0.43mg (-8.3%) on average, limits of agreement: -3.1mg (-60.2%) to 2.3mg (48.7%). Figure 3.2 displays the results of the Bland-Altman analysis comparing the dietary recall method to weighed food records.

3.2.2 TRACT DIETARY SUB-STUDY RESULTS

3.2.2.1 SUB-STUDY SUBJECT CHARACTERISTICS, ANTHROPOMETRY, AND RANDOMISED TREATMENT

Of the n=339 children recruited to the TRACT sub-study, valid dietary data were collected for 782/1017 study visits. Valid recalls were available for n=314/339 children on admission, n=244/339 children at day 28, and n=224/339 children at day 180. Of the 230 missing diet recalls, 170 were not collected as the adult was not with the child for the previous 24hrs, 34 were excluded due to missing portion size information, and 26 were not present due to the death of the child. There was significant attrition in the dietary recall completion rate in Soroti, with 60/152 children having missing or incomplete dietary recalls at day 28, and 72/152 at day 180. In the 782 24hr dietary recalls conducted, information was provided by the child’s mother in 450 (58%) cases, by the child’s father in 234 (30%) cases, by the child’s grandmother in 63 (8%) cases, by the child’s aunt in 19 (2%) cases. In the remaining 16 (2%) cases information was provided by other family members. Demographic and anthropometric data for the children included in the dietary analysis are presented in Table 3.5. Randomisations for transfusion strategy, nutritional intervention and antibiotic prophylaxis were balanced. During the period of sub-study recruitment, there were low numbers of children with less severe or uncomplicated severe anaemia who would be entered into the transfusion vs. no transfusion study arm, resulting in greater numbers of children with severe and complicated anaemia in this sub-study.

Children presenting to Mbale (n=162) were younger than those in Soroti (n=152), with a median age of 39 months (IQR 16) compared to 52 months (IQR 19) ($p<0.001$), and significantly more children were aged under two years in Mbale (19.8% vs. 8.6%, $p=0.001$). 44.1% of children presenting to Soroti were school age, compared to only 25.9% in Mbale, $p=0.001$. A similar proportion of children in both sites were male (56.8% in Mbale and 58.9% in Soroti, $p=0.700$).

Approximately one fifth (19.8%) of children presenting to Mbale were considered underweight on admission (WAZ <-2) with a median WAZ of -1.3 (IQR 1.3). The percentage of underweight children being admitted to Soroti hospital was slightly higher (22.4%), but not significantly so (p = 0.904), and the median WAZ was also similar (WAZ -1.4, IQR 1.7) p=0.195. There was no change in median WAZ between admission, day 28 and day 180 in children from Mbale (0 vs. 28 p = 0.106; 0 vs. 180 p=0.686; 28 vs. 180 p=0.469), but children in Soroti showed significant improvement in WAZ from -1.4 on admission to -0.7 (IQR 2.0) by day 28, p=0.001. In Soroti, WAZ did not significantly change from day 28 to day 180 (WAZ -0.8, IQR 1.0), p=0.783, but by day 180 it remained lower than admission (p<0.001).

On admission, stunting (HAZ <-2) was more prevalent in children from Mbale region compared to Soroti region (29.4% vs. 15.9% respectively), p=0.004, and median admission HAZ score was significantly lower in Mbale (-1.4, IQR 1.8 vs. -0.6, IQR 1.6), p<0.001. Stunting continues to be more prevalent in Mbale at by day 28 (32.2% vs. 15.3%, p<0.001) and day 180 (25.8% vs. 12.6%, p=0.004). On the contrary wasting (WHZ <-2) was more prevalent in Soroti than in Mbale at admission (31.8% vs. 13.7%, p=0.001), day 28 (39.5% vs. 16.9%, p<0.001) and day 180 (58.4% vs. 30.5%, p<0.001). Median WHZ was also significantly lower in Soroti compared to Mbale at all time points.

Mid-upper arm circumference did not indicate acute malnutrition in children from either study site, with median MUAC greater than the diagnostic cut off of 11.5cm in all children at all time points, except one child in Mbale at day 28. At all study visits, median MUAC was greater in children from Soroti compared to Mbale: admission 15.4cm (IQR 1.7) vs. 14.5cm (IQR 1.8) p<0.001, day 28 15.4cm (IQR 1.6) vs. 14.8cm (IQR 1.7) p<0.001, and day 180 15.8cm (IQR 1.7) vs. 15.2cm (IQR 1.8) p=0.010.

On enrolment to the TRACT sub-study, 74.1% of children from Mbale and 61.2% from Soroti had Hb<5.0g/dl, meeting the widely used definition of severe anaemia. Distribution of anaemia severity was not different between the two hospitals at any study time point: admission p=0.069, day 28 p=0.763, day 180 p=0.087. Despite this, median haemoglobin concentration was lower in Mbale compared to Soroti at day 0 (3.9g/dl vs. 4.5g/dl, p=0.015), and at day 28 (8.9g/dl vs. 10.0g/dl, p<0.001), although this had disappeared by day 180 (9.9g/dl vs. 10.4g/dl, p=0.168).

Malaria was diagnosed in 71% of children in both centres (p=0.953) on admission, reflective of the holoendemic nature of malaria in this region. Malaria was also found in 8.7-14.8% of children at study follow up visits, which did not vary significantly by site. Sickle cell disease was confirmed by genotyping in 32.1% and 25% of children in Mbale and Soroti respectively (p=0.107). A total of 26/339 (7.7%) of children recruited to the TRACT sub-study died.

Table 3.5. Subject characteristics for children enrolled in TRACT dietary sub-study, arranged by study site and time point (n = 782)[1]

Characteristic	Day 0 (n=314)			Day 28 (n=244)			Day 180 (n=222)		
	Mbale (n=162)	Soroti (n=152)	p-value	Mbale (n=152)	Soroti (n=92)	p-value	Mbale (n=142)	Soroti (n= 80)	p-value
Age (months)	39 [16]	52 [19]	**<0.001**	41 [22]	54 [21]	**<0.001**	46 [18]	58 [19]	**0.003**
% Under 24	19.8	8.6		14.5	8.7		9.1	1.2	
% 24-60	54.3	47.4	**0.001**	59.2	43.5	**0.003**	59.4	54.3	**0.021**
% Over 60	25.9	44.1		26.3	47.8		31.5	44.4	
Gender (% male)	56.8	58.9	0.700	55.3	54.3	0.889	58.0	59.3	0.859
WAZ	-1.3 [1.3]	-1.4 [1.7]	0.195	-1.0 [1.6]	-0.7 [2.0]	0.441	-0.9 [1.6]	-0.8 [1.0]	0.679
% Underweight (<-2SD)	19.8	22.4	0.904	17.8	21.7	0.454	16.1	17.3	0.755
HAZ	-1.4 [1.8]	-0.6 [1.6]	**<0.001**	-1.6 [1.7]	-0.6 [1.7]	**<0.001**	-1.4 [1.8]	-0.6 [1.6]	**<0.001**
% Stunted (<-2SD)	29.4	15.9	**0.004**	32.2	15.3	**<0.001**	25.8	12.6	**0.004**
WHZ	-0.7 [1.4]	-1.3 [1.9]	**<0.001**	-0.6 [1.6]	-1.5 [2.0]	**<0.001**	-1.5 [1.4]	-2.3 [2.3]	**0.001**
% Wasted (<2SD)	13.7	31.8	**0.001**	16.9	39.5	**<0.001**	30.5	58.4	**<0.001**
MUAC (cm)	14.5 [1.8]	15.4 [1.7]	**<0.001**	14.8 [1.7]	15.4 [1.6]	**<0.001**	15.2 [1.8]	15.8 [1.7]	**0.010**
% <11.5cm	0.0	0.0	-	0.7	0.0	0.326*	0.0	0.0	-
Haemoglobin (g/dl)	3.9 [1.5]	4.5 [2.4]	**0.015**	8.9 [4.0]	10.0 [4.1]	**<0.001**	9.9 [4.6]	10.4 [4.1]	0.168
WHO: Severe (%)	98.1	95.9		29.4	22.5		29.5	24.0	
of which <5g/dl (%)	74.1	61.2		3.9	4.3		3.5	0	
WHO: Moderate (%)	1.3	2.7	0.069*	32.2	30.3	0.763	23.0	25.4	0.087*
WHO: Mild (%)	0.6	0		13.8	15.7		10.8	5.3	
WHO: Non-Anaemic (%)	0	1.4		24.7	31.5		36.7	45.4	
Malaria (% Present)	71.0	71.1	0.953	9.2	8.7	0.875	9.1	14.8	0.192
Sickle Cell Disease (% Present)	32.1	25.0	0.107	33.6	29.3	0.362	34.3	27.2	0.205
TRACT A (20ml/kg:30ml/kg)	63:61	61:57	-	60:57	37:38	-	60:55	34:27	-
TRACT B (Transfusion:None)	21:17	14:20	-	19:16	11:6	-	12:15	8:11	-
Nutritional strategy (IF:MVMM)	88:74	75:77	-	83:69	46:46	-	79:63	41:39	-
Cotrimoxazole (No:Yes)	70:92	77:75	-	66:86	48:44	-	64:78	37:43	-

[1]Continuous variables are presented as median [IQR], p-values from Independent Samples Mann-Whitney U Test; categorical variables are presented as percentage of n, p-values from Pearson Chi-Square test, or Likelihood Ratio where assumptions are violated, indicated by *

Abbreviations: WAZ, weight for age z-score; HAZ, height for age z-score; WHZ, weight for height z-score; MUAC, mid-upper arm circumference, IF, iron and folate supplementation; MVMM, multivitamin multi-mineral supplement.

3.2.2.2 TRACT DIETARY SUB-STUDY RESULTS – MBALE

Due to significant differences in dietary recall completion rates and baseline characteristics known to confound dietary intake (notably age) between the two study sites, it was decided to conduct an interim analysis of dietary data. This revealed significant differences in dietary patterns and foods, therefore in order to accurately represent the dietary data it was necessary to present data from each study site separately. TRACT dietary recall results are summarised in Tables 3.6 (Mbale) and 3.7 (Soroti). Adequacy in terms of the proportion of children meeting requirements for specific nutrients is presented in Table 3.8. It should be noted that micronutrient data presented for **day 28 is inclusive of supplementation** to accurately represent intake.

In Mbale, the median energy intake of children was at, or in excess of estimated requirements at all study time points. On admission, median energy requirement was 4.2MJ (IQR 1.2), similar to median requirements (4.1MJ, IQR 3.2), p=1.000. By day 28 median energy intake was in excess of requirements at 5.5MJ (IQR 3.4), p<0.001. Energy intake subsequently fell to 5.1MJ (IQR 2.7) at day 180, which was not significantly different to requirements (4.7MJ, IQR 1.4) at this stage, p=1.000. Carbohydrate contributed the greatest amount of energy of all macronutrients at all time points, ranging from 68.7% to 73.0%, which did not change significantly over time (p=1.000). Intake of sugar as a proportion of total carbohydrate intake did vary significantly, and contributed 13.4% of energy on admission, significantly more than at day 28 and day 180 (9.3% and 8.4% respectively), p<0.001. The percentage of energy derived from fat decreased insignificantly over the course of the study visits: 22.3% (IQR 18.4) at admission, 19.5% (IQR 17.4) at day 28, and 16.4% (IQR 17.1) at day 180, p=0.272. Protein's contribution to energy intake peaked at day 28 at 11.3%, but this did not vary between study time points, p=0.075. Reported protein intake was substantially greater than requirements at all time points: on admission 21.9g of protein were consumed (180% requirements, p<0.001), on day 28 protein intake was 32.0g (248% requirements, p<0.001), and on day 180 28.7g (214% requirements, p<0.001). Protein intake at both review appointments was significantly greater than at admission, p<0.001.

In light of adequate median energy intake, it is interesting to note that median intake of many micronutrients was inadequate. Calcium intake was insufficient at all time points, ranging from 32.4 to 36.6% of requirements, p<0.01 in all cases. Dietary intake of iron was low on admission (3.6mg IQR 3.4, requirement 5.8mg IQR 0.5, p<0.001), and at day 180 (4.8mg IQR 3.2, requirement 5.8mg IQR 0.5, p<0.001), but median intake at day 28 (28.6mg IQR 49.5) was in excess of requirement (5.8mg IQR 0.5), p<0.001, due to supplementation at this time point. Intake of zinc was inadequate on admission (3.5mg, IQR 3.3), but was similar to requirements at later time points. Among the B-complex vitamins,

median reported intakes of thiamine, riboflavin and vitamin B6 were sufficient at all time points, while median intakes of niacin, pantothenate, biotin, folate and vitamin B12 were significantly below recommended intakes at most time points. On day 28, supplementation with iron and folate or multivitamin multimineral was ongoing, therefore intakes of many micronutrients at this time point are artificially elevated. An exception to the broad pattern of insufficient intakes at non-supplemented time points is biotin at day 180 (intake 5.6µg, IQR 8.2 vs. requirements of 8.0µg/day, $p=0.525$).

Table 3.6 Nutrient intake and estimated requirements for children reviewed at Mbale Regional Referral Hospital, presented by study time point

	Day 0 (n=162)			Day 28 (n=152)			Day 180 (n=142)			
Nutrient	Requirement median [IQR]	Intake median [IQR]	Corrected p-value*	Requirement median [IQR]	Intake median [IQR]	Corrected p-value*	Requirement median [IQR]	Intake median [IQR]	Corrected p-value*	Between Time Point Corrected p-value**
Energy (MJ/day)	4.2 [1.2]	4.1 [3.2][b,c]	1.000	4.4 [1.2]	5.5 [3.4][a]	**<0.001**	4.7 [1.4]	5.1 [2.7][a]	1.000	**<0.001**
% from carbohydrate		68.7 [17.1]			69.2 [19.7]			73.0 [21.0]		1.000
% from sucrose		13.4 [13.8][b,c]			9.3 [8.5][a]			8.4 [9.9][a]		**<0.001**
% from fat		22.3 [18.4]			19.5 [17.4]			16.4 [17.1]		0.272
% from protein		9.0 [5.1]			11.3 [5.3]			10.6 [4.3]		0.075
Protein (g/day)	12.2 [4.4]	21.9 [23.4][b,c]	**<0.001**	12.9 [4.0]	32.0 [26.8][a]	**<0.001**	13.4 [4.9]	28.7 [24.1][a]	**<0.001**	**<0.001**
Calcium (mg/day)	500.0 [50.0]	162.0 [206.0]	**<0.001**	500.0 [50.0]	183.0 [192.0]	**0.002**	500.0 [50.0]	163.5 [213.0]	**0.001**	1.000
Iron (mg/day)	5.8 [0.5]	3.6 [3.4][b,c]	**<0.001**	5.8 [0.5]	28.6 [49.5][a,c]	**<0.001**	5.8 [0.5]	4.8 [3.2][a,b]	**4.45e-9**	**<0.001**
Zinc (mg/day)	4.1 [0.7]	3.5 [3.3][b,c]	**<0.001**	4.1 [0.7]	6.3 [5.5][a]	**<0.001**	4.1 [0.7]	3.5 [3.5][a]	0.075	**<0.001**
Vitamin A (µg RE/day)	200.0 [0]	76.5 [249.0][b,c]	**0.015**	200.0 [0.0]	429.0 [525.0][a,c]	**<0.001**	200.0 [0.0]	130.0 [423.0][a,b]	1.000	**<0.001**
Thiamine (mg/day)	0.5 [0.1]	0.5 [0.5][b,c]	0.090	0.5 [0.1]	1.1 [0.9][a,c]	**<0.001**	0.5 [0.1]	0.7 [0.7][a,b]	0.81	**<0.001**
Riboflavin (mg/day)	0.5 [0.1]	0.5 [0.6][b]	0.810	0.5 [0.1]	0.8 [0.7][a,c]	**<0.001**	0.5 [0.1]	0.4 [0.5][b]	1.000	**<0.001**
Niacin (mg NE/day)	6.0 [2.0]	1.9 [3.0][b,c]	**<0.001**	6.0 [1.9]	10.1 [6.2][a]	**<0.001**	6.0 [2.0]	2.1 [2.0][a]	**<0.001**	**<0.001**
Vitamin B6 (mg/day)	0.5 [0.1]	0.6 [0.8][b,c]	0.210	0.5 [0.1]	1.5 [1.2][a]	**<0.001**	0.5 [0.1]	1.1 [1.0][a]	**<0.001**	**<0.001**
Vitamin B12 (µg/day)	0.9 [0.3]	0.4 [0.8][b]	**<0.001**	0.9 [0.3]	0.9 [1.4][a,c]	0.308	0.9 [0.3]	0.5 [1.0][b]	**0.001**	**<0.001**
Folate (µg/day)	150.0 [50.0]	116.0 [119.0][b,c]	**<0.001**	150.0 [50.0]	329.3 [346.3][a,c]	**<0.001**	150.0 [50.0]	131.5 [105.0][b,c]	**<0.001**	**<0.001**
Pantothenate (mg/day)	2.0 [1.0]	0.7 [0.9][b,c]	**<0.001**	2.0 [1.0]	0.9 [1.1][a]	**<0.001**	2.0 [1.0]	0.9 [1.0][a]	**<0.001**	**0.034**
Biotin (µg/day)	8.0 [4.0]	5.9 [7.1]	**<0.001**	8.0 [4.0]	5.1 [7.9]	**<0.001**	8.0 [4.0]	5.6 [8.2]	0.525	1.000
Vitamin C (mg/day)	30.0 [0.0]	40.0 [69.0][b,c]	**0.045**	30.0 [0.0]	99.0 [103.0][a]	**<0.001**	30.0 [0.0]	72.0 [90.0][a]	**<0.001**	**<0.001**

* Related-Samples Friedman's Two-Way Analysis of Variance by Ranks, Bonferroni corrected for multiple tests

** Wilcoxon Signed Rank Test, Bonferroni corrected for multiple tests; within model pairwise significance ($p<0.05$) is denoted [a, b, c], indicating significant difference from Day 0, Day 28, and Day 180 respectively

3.2.2.3 TRACT DIETARY SUB-STUDY RESULTS – SOROTI

The median energy intake of children in Soroti was significantly lower than estimated requirements at all time points, in contrast to the situation in Mbale. On admission, median energy requirement was 2.6MJ (IQR 2.4), below median requirements of 4.8MJ (IQR 1.1), p<0.001. By day 28 energy intake significantly increased to 3.3MJ (IQR 1.9), p=0.047, but remained below estimated requirements of 5.0MJ [IQR 1.4], p<0.001. Median energy intakes subsequently reverted to levels similar to admission (2.5MJ IQR 2.7), also significantly below requirements of 5.1MJ (IQR 1.5), p<0.001.

As in Mbale, the majority of dietary energy was derived from carbohydrate sources at all time points (76.6%, 80.6% and 86.6% at admission, day 28 and day 180), while the proportion of energy coming from sucrose dropped from 14.5% at admission to 10.3% by day 28 (p=0.001), remaining lower (11.1%) by day 180. Energy from fat decreased over time (14.6%, 10.2% and 5.3% respectively), as the proportion from carbohydrate increased. The total amount of energy coming from protein did not differ over time, however absolute protein intake peaked at day 28 (17.2g, IQR 13.3) which was significantly greater than protein intake at admission (12.4g, IQR 17.0) or day 180 (12.9g, IQR 8.3). Protein intakes were similar to requirements at admission and day 28, but at day 180, median intake of 12.9g was significantly below requirements (15.1g, IQR 7.1), p<0.001.

As expected with generally low energy intakes, median intakes of most micronutrients were below requirements. The following nutrients were persistently below requirements for all time points: calcium, pantothenate, and biotin. For iron, zinc, vitamin A, riboflavin, niacin, vitamin B12, and folate, intakes were insufficient on admission and at day 180, in contrast to day 28 when supplementation was ongoing. Of note are those few nutrients whose intakes were sufficient in the context of an energy-poor diet. Thiamine intake was similar to requirements at day 180 (0.7mg [0.5] vs. 0.6mg [IQR 0.4], p=1.000). On admission, median intake (0.5mg, IQR 0.5) was lower than requirements (0.6mg, IQR 0.1), p<0.001, but not to a clinically significant degree. Vitamin B6 intake had a similar pattern with slightly lower intake on admission (intake of 0.5mg [IQR 0.7] vs. requirement of 0.6mg/day [0.1], p<0.001), but similar to requirements on day 180 (0.7mg [IQR 0.5] vs. 0.6mg/day [IQR 0.5], p=1.000). Vitamin C intake was consistently greater than estimated requirements of 30mg/day, at 48.5mg [IQR 76.0] on admission (p<0.001), 99.0mg [IQR 103.0] on day 28 (p-6.59e-13), and 71.5mg [IQR 55.0] on day 180 (p<0.001).

Table 3.7 Nutrient intake and estimated requirements for children reviewed at Soroti Regional Referral Hospital presented by study time point

	Day 0 (n=152)			Day 28 (n=92)			Day 180 (n=80)			
Nutrient	Requirement median [IQR]	Intake median [IQR]	Corrected p-value*	Requirement median [IQR]	Intake median [IQR]	Corrected p-value*	Requirement median [IQR]	Intake median [IQR]	Corrected p-value*	Between Time Point Corrected p-value**
Energy (MJ/day)	4.8 [1.1]	2.6 [2.4][b]	**<0.001**	5.0 [1.4]	3.3 [1.9][a, c]	**<0.001**	5.1 [1.5]	2.5 [1.7][b]	**<0.001**	**0.047**
% from carbohydrate		76.6 [16.9][c]			80.6 [12.6][c]			86.6 [6.7][a, b]		**<0.001**
% from sucrose		14.9 [15.7][b, c]			10.3 [10.0][a]			11.1 [12.1][a]		**0.001**
% from fat		14.6 [17.8][c]			10.2 [13.7][c]			5.3 [4.8][a, b]		**<0.001**
% from protein		8.8 [4.3]			9.2 [4.6]			8.1 [2.5]		1.000
Protein (g/day)	14.6 [5.6]	12.4 [17.0][b]	1.000	15.3 [5.8]	17.2 [13.3][a, c]	0.510	15.1 [7.1]	12.9 [8.3][b]	**<0.001**	**0.014**
Calcium (mg/day)	550 [50]	85.5 [108.0]	**<0.001**	550.0 [200.0]	99.0 [127.0]	**<0.001**	550.0 [200.0]	65.5 [50.0]	**<0.001**	0.227
Iron (mg/day)	6.3 [0.5]	2.4 [3.0][b]	**<0.001**	6.3 [3.1]	22.8 [50.2][a]	**<0.001**	6.3 [3.1]	2.7 [1.6]	**<0.001**	**<0.001**
Zinc (mg/day)	4.8 [0.7]	1.8 [3.0]	**<0.001**	4.8 [1.5]	4.7 [4.6]	**0.039**	4.8 [1.5]	1.5 [1.0]	**<0.001**	**<0.001**
Vitamin A (µg RE/day)	200.0 [0.0]	28.0 [130.0]	**<0.001**	200.0 [50.0]	402.0 [433.0]	**<0.001**	200.0 [50.0]	7.0 [13.0]	**<0.001**	**<0.001**
Thiamine (mg/day)	0.6 [0.1]	0.5 [0.5][b, c]	**<0.001**	0.6 [0.4]	1.0 [0.6][a]	**<0.001**	0.6 [0.4]	0.7 [0.5][a]	1.000	**<0.001**
Riboflavin (mg/day)	0.6 [0.1]	0.3 [0.4]	**<0.001**	0.6 [0.4]	0.6 [0.6]	0.393	0.6 [0.4]	0.2 [0.2]	**<0.001**	**<0.001**
Niacin (mg NE/day)	8.0 [2.0]	1.9 [3.1]	**<0.001**	8.0 [6.0]	8.1 [6.4]	0.995	8.0 [6.0]	1.9 [1.7]	**<0.001**	**<0.001**
Vitamin B6 (mg/day)	0.6 [0.1]	0.5 [0.7][b, c]	**<0.001**	0.6 [0.5]	1.0 [0.7][a]	**<0.001**	0.6 [0.5]	0.7 [0.5][a]	1.000	**<0.001**
Vitamin B12 (µg/day)	1.2 [0.3]	0.6 [1.5]	**0.015**	1.2 [0.9]	1.3 [2.2]	0.070	1.2 [0.9]	0.7 [1.6]	0.165	5.30e-4
Folate (µg/day)	200.0 [50.0]	57.5 [62][b]	**<0.001**	200.0 [150.0]	341.5 [319.0][a]	**<0.001**	200.0 [150.0]	64.5 [51.0]	**<0.001**	**<0.001**
Pantothenate (mg/day)	3.0 [1.0]	0.4 [0.6]	**<0.001**	3.0 [2.0]	0.4 [0.8]	**<0.001**	3.0 [2.0]	0.3 [0.4]	**<0.001**	1.000
Biotin (µg/day)	12.0 [4.0]	2.4 [5.3]	**<0.001**	12.0 [12.0]	2.0 [6.9]	**<0.001**	12.0 [12.0]	1.0 [2.5]	**<0.001**	0.675
Vitamin C (mg/day)	30.0 [0.0]	48.5 [76.0][b, c]	**<0.001**	30.0 [5.0]	100.0 [67.0][a]	**<0.001**	30.0 [5.0]	71.5 [55.0][a]	**<0.001**	**<0.001**

* Related-Samples Friedman's Two-Way Analysis of Variance by Ranks, Bonferroni corrected for multiple tests

** Wilcoxon Signed Rank Test, Bonferroni corrected for multiple tests; within model pairwise significance ($p<0.05$) is denoted [a, b, c], indicating significant difference from Day 0, Day 28, and Day 180 respectively

3.2.2.4 NUTRIENT ADEQUACY

The proportions of children in both Mbale and Soroti who met the recommended intakes for each nutrient, and the average intakes of adequate versus inadequate consumers, are listed in Table 3.8.

Despite median values for energy intake being adequate at all time points for children seen in Mbale, a minority (42.6%) are meeting requirements on admission, although this proportion rises to 59.2% by day 28, falling slightly to 53.8% by day 180, χ^2 p=0.002. In Soroti, far fewer children have adequate energy intake at admission (15.8%), day 28 (17.4%) and day 180 (7.4%), varying less over time, χ^2 p=0.144. The discrepancy between median data and numbers meeting requirements is explained by extreme variation of intakes. On average, children in Mbale who did not meet energy requirements, consumed only 66% of their estimated requirements, while those who did consumed 150%. In Soroti the discrepancy was similarly pronounced with children who failed to meet requirements taking 50% of requirements on average, while those who did meet requirements took 125%.

Median intake of protein in both sites is adequate or in excess of requirements, however extremely high intakes mask high numbers of children with poor intake. In Mbale a high proportion of children are meeting protein requirements (73.5%, 80.3% and 83.2% at admission, day 28 and day 180), which significantly improves over time (χ^2 p=0.010). Average intake of those who do not meet requirements are low, only taking 59% of requirements, while those who are meeting requirements take 267% of requirements. Fewer children in Soroti are meeting protein requirements at admission (47.4%), day 28 (56.6%) and day 180 (23.5), χ^2 p<0.001, and those who met requirements were consuming approximately 3 times as much protein as those with inadequate intakes (178% vs. 57% requirements).

Minorities of children met requirements for most micronutrients on admission in either study site, however fewer children in Soroti were meeting requirements for all micronutrients except vitamin C. Broadly speaking, adequate intakes of most micronutrients followed the same pattern as energy intake. Intakes of most micronutrients at day 28 are vastly improved, however this is owing to the micronutrient supplementation randomisation in TRACT, and not entirely related to changes in dietary intake. In Mbale, more children were meeting estimated requirements for all micronutrients by day 180, when compared to admission intakes. For example, only 15.7% of children met estimated requirements for iron on admission, rising to 25.9% by day 180; 21.0% of children met estimated requirements for folate on admission, rising to 32.2% by day 180. In Soroti, the opposite trend appears to be prevalent, with the proportion of children with adequate micronutrient intakes falling over time. Only 7.9% of children in Soroti were meeting dietary iron requirements on admission, falling to 2.5%

of children by day 180. This trend is not universal, the proportions of children meeting requirements for thiamine, vitamin B6 and vitamin C increasing over time.

As expected, those meeting requirements, i.e. taking >95% of estimated requirement, have greater intakes than those who are not, however in many instances the differences are large. In Mbale children who were meeting requirements for iron took on average 138% of their requirements, while those who were not took only 54%. In Soroti the difference in iron intake between those meeting vs. not meeting requirements is starker: 125% vs. 36%. Nutrients concentrated in specific foods display even greater disparity between those with inadequate vs. adequate intake. For example, those with inadequate vitamin A intake took 26% of their requirement in Mbale (14% in Soroti), while those with adequate intakes took 553% of requirement (629% in Soroti). A similar pattern is seen for calcium, riboflavin, vitamin B6, vitamin B12, biotin and vitamin C, where those with adequate intake took many times their estimated requirement, and those with inadequate intake frequently took less than 50% of their requirement.

Table 3.8 Percentage of children from Mbale and Soroti meeting requirements for energy, protein and micronutrients, with average intakes of inadequate (I) versus adequate (A) consumers expressed as percentage of requirement

	Mbale (% meeting requirement)					Soroti (% meeting requirement)				
Nutrient	Day 0	Day 28	Day 180	χ^2 test p-value	% req met I:A†	Day 0	Day 28	Day 180	χ^2 test p-value	% req met I:A†
Energy	42.6	59.2	53.8	**0.002**	66:150	15.8	17.4	7.4	0.144	50:125
Protein	73.5	80.3	83.2	**0.010**	59:267	47.4	56.6	23.5	**<0.001**	57:178
Calcium	16.0	19.1	21.7	0.507	28:250	3.9	8.7	2.5	0.149	18:162
Iron	15.7	100.0	25.9	**<0.001**	54:138	7.9	100.0	2.5	**<0.001**	36:125
Zinc	31.4	68.4	40.6	**<0.001**	51:158	13.8	48.4	2.5	**<0.001**	33:157
Vitamin A	30.1	69.2	42.0	**<0.001**	26:553	13.8	54.9	11.1	**<0.001**	14:629
Thiamine	36.6	82.7	53.8	**<0.001**	57:193	32.9	86.8	53.1	**<0.001**	57:155
Riboflavin	41.2	66.2	35.7	**<0.001**	54:236	17.9	46.2	6.4	**<0.001**	39:155
Niacin	23.5	66.2	38.5	**<0.001**	56:162	11.2	50.5	4.9	**<0.001**	42:136
Vitamin B6	52.3	88.0	73.4	**<0.001**	56:260	32.2	84.6	53.1	**<0.001**	49:177
Vitamin B12	22.2	51.9	25.9	**<0.001**	25:470	34.2	57.1	29.6	**<0.001**	29:292
Folate	21.0	91.7	32.2	**<0.001**	51:158	5.9	68.1	0.0	**<0.001**	31:123
Pantothenate	4.9	7.2	11.2	0.155	33:136	2.0	2.2	2.5	0.962	16:133
Biotin	24.7	24.3	29.4	0.763	39:288	8.6	15.2	1.2	**0.005**	16:227
Vitamin C	51.6	87.2	75.5	**<0.001**	39:366	60.5	96.7	85.2	**<0.001**	39:282

†I:A, percentage of req (requirements) met by those with inadequate intake: adequate intake

3.2.2.5 Nutrient density

Table 3.9 presents the concentration of each nutrient per 1000kcal; as this is a measure of nutrient density within the diet, day 28 data omits supplementary micronutrient intake. The concentration of protein in the diets of children from Mbale was similar across all time points with median densities of 22.0g/1000kcal at admission, 26.6g/1000kcal at day 28, and 24.7g/1000kcal at day 180, p=1.000. In Soroti, protein density was also similar across all study visits: 21.1g/1000kcal, 21.6g/1000kcal and 19.2g/1000kcal, p=1.000. While median total protein intakes in Mbale are higher than intakes in Soroti at all study time point, both diets are of similar quality with respect to protein concentration at admission and day 28 (p=1.000 and p=0.075), but protein density is significantly greater in Mbale at day 180 (p=0.001).

Other nutrients whose density in the diet does not change over time in either site include: fibre, calcium, zinc, vitamin A, riboflavin, vitamin B12, pantothenate and biotin (Related-Samples Friedman's Two-Way Analysis of Variance by Ranks p-values >0.05). Although the concentration of these nutrients remains stable over time, several show geographical variation. Fibre is present in greater concentration in the diet of children in Mbale region compared to Soroti at all time points: 3.5g vs. 1.0g/1000kcal at admission (p<0.001), 5.3g vs. 2.2g/1000kcal at day 28 (p=0.001), and 5.7g vs. 2.2g/1000kcal at day 180 (p<0.001).

Of the haematinic nutrients the concentration of iron in the diet in each region is similar and varies little over time. At 4.4mg/1000kcal, dietary iron concentration on day 28 in Mbale stands out as being significantly greater than on admission or day 180 (p<0.001), and also greater than the corresponding time point in Soroti (3.9mg/1000kcal, p=0.045). The concentration of vitamin A is similar on admission between the two sites (77.6µg/1000kcal in Mbale [IQR 219.8], and 288µg/1000kcal in Soroti [IQR 189.6], p=0.105), and does not vary over time within each site (p=0.255 in Mbale and p=1.000 in Soroti). However, dietary vitamin A concentration is significantly greater in Mbale than Soroti at day 28 (147.1µg vs. 18.7µg/1000kcal, p<0.001) and day 180 (106.4µg vs. 11.4µg/1000kcal, p<0.001). Median vitamin B12 concentrations are generally significantly lower in Mbale diets, compared to Soroti diets except at day 28: 0.3µg vs. 0.8µg/1000kcal at admission (p=0.007), 0.4µg vs. 0.8µg/1000kcal at day 28 (p=0.060), and 0.3µg vs. 1.0µg/1000kcal on day 180 (p=0.001). In Mbale, dietary folate concentration peaks at day 28 (137.1µg/1000kcal, p=0.030), but there is no significant change over time in children's diets in Soroti. On admission and day 28, folate concentration is greater in Mbale vs. Soroti: 95.7µg vs. 75.3µg/1000kcal (p=0.003), 137.1µg vs. 88.8µg/1000kcal (p<0.001), however they become similar by day 180, p=0.465.

Table 3.9 Nutrient density (per 1000kcal) of diets of children from Mbale and Soroti regions

	Mbale				Soroti				Between site comparison**		
Nutrient/1000kcal	Day 0 Median[IQR]	Day 28 Median[IQR]	Day 180 Median[IQR]	corrected p-value*	Day 0 Median[IQR]	Day 28 Median[IQR]	Day 180 Median[IQR]	corrected p-value*	Day 0 p-value	Day 28 p-value	Day 180 p-value
Protein (g)	22.0 [12.7]	26.6 [13.2]	24.7 [10.6]	1.000	21.1 [10.9]	21.6 [11.5]	19.2 [6.0]	1.000	1.000	0.075	**0.001**
Fibre (g)	3.5 [5.4]	5.3 [6.2]	5.7 [7.0]	0.090	1.0 [3.9]	2.2 [5.0]	2.2 [3.5]	0.255	**<0.001**	**0.001**	**<0.001**
Calcium (mg)	141.6 [125.9]	138.1 [187.6]	128.1 [307.6]	1.000	115.9 [57.6]	109.6 [63.6]	103.4 [32.0]	1.000	**0.005**	0.075	**0.015**
Iron (mg)	3.3 [2.0][b]	4.4 [2.0][a,c]	3.9 [1.6][b]	**<0.001**	3.3 [2.0]	3.9 [1.4]	4.3 [1.4]	0.150	1.000	**0.045**	0.810
Zinc (mg)	2.9 [2.1]	3 [1.6]	3 [1.3]	1.000	2.2 [2.5]	2.4 [1.3]	2.3 [1.1]	1.000	0.255	**<0.001**	**<0.001**
Vitamin A (µg)	77.6 [219.8]	147.1 [226.0]	106.4 [319.3]	0.255	28.8 [189.6]	18.7 [99.7]	11.4 [18.4]	1.000	0.105	**<0.001**	**<0.001**
Thiamine (mg)	0.5 [0.3][b]	0.7 [0.5][a,c]	0.5 [0.4][b]	**<0.001**	0.8 [0.4][b,c]	1.0 [0.4][a]	1.1 [0.4][a]	**0.003**	**<0.001**	**<0.001**	**<0.001**
Riboflavin (mg)	0.4 [0.4]	0.4 [0.4]	0.4 [0.3]	1.000	0.4 [0.2]	0.4 [0.2]	0.3 [0.1]	1.000	1.000	**<0.001**	0.120
Niacin (mg)	1.4 [2.9][b]	2.6 [4.0][a,c]	1.3 [2.5][b]	**0.003**	3.0 [3.7]	3.1 [3.2]	3.1 [2.9]	1.000	**<0.001**	0.150	**<0.001**
Vitamin B6 (mg)	0.6 [0.5][b,c]	1 [0.6][a]	0.9 [0.7][a]	**0.001**	0.6 [0.7][b,c]	1.2 [0.5][a]	1.2 [0.5][a]	**0.015**	1.000	0.240	**0.002**
Vitamin B12 (µg)	0.3 [0.7]	0.4 [1.5]	0.3 [0.9]	1.000	0.8 [1.7]	0.8 [2.2]	1.0 [2.1]	1.000	**0.007**	0.060	**0.001**
Folate (µg)	95.7 [89.6][b]	137.1 [83.4][a,c]	107.6 [76.6][b]	**0.030**	75.3 [58.3]	88.8 [52.0]	95.2 [58.1]	0.120	**0.003**	**<0.001**	0.465
Pantothenate (mg)	0.7 [0.7]	0.8 [0.8]	0.7 [0.9]	1.000	0.4 [0.9]	0.6 [0.8]	0.6 [0.8]	1.000	**0.001**	0.18	0.570
Biotin (µg)	6.3 [8.3]	4.3 [7.0]	4.8 [8.1]	0.930	2.1 [6.6]	1.3 [6.5]	1.1 [4.5]	1.000	**<0.001**	**0.003**	**<0.001**
Vitamin C (mg)	32 [63.1][b]	70.2 [78.7][a]	55.5 [72.1]	**0.015**	68.1 [79.0][c]	103.3 [54.8]	111.8 [43.6][a]	**0.045**	**0.002**	**0.001**	**<0.001**

*Related-Samples Friedman's Two-Way Analysis of Variance by Ranks, Bonferroni corrected for multiple tests; within model pairwise significance ($p<0.05$) is denoted [a, b, c], indicating significant difference from Day 0, Day 28, and Day 180 respectively

**Independent samples Kruskal-Wallis Test, Bonferroni corrected for multiple tests

Abbreviations: IQR, interquartile range

3.2.2.6 FOOD GROUPS CONTRIBUTION TO DIETARY INTAKE

Tables 3.10 and 3.11 show the contribution of the seven WHO-food groups (FGs), and additional two groups (FG8 sugar and highly sweetened foods, and FG9 'other'), to nutrient intakes in Mbale and Soroti respectively. Broadly speaking, starchy foods were the principal sources of most nutrients, even those which are found in low levels in these foods. Other than sucrose and vitamin B12, starchy foods were the principal source of all other nutrients. On admission, children from Mbale received 44% ±23 of their total energy from starchy foods, with a further 28% ±21 coming from sugar and highly sweetened food and drinks, which is broadly in line with Soroti (46% ±23 from starchy food, 31% ±21 from sweet foods). The proportion of energy from these starchy foods increased over time, in Mbale rising to 55% at day 28 and remaining at this level by day 180. This was accounted for by a concomitant decrease in energy from sugary foods to 13-14%. In Soroti the increase of energy coming from starchy foods is more pronounced, rising to 63% by day 28 and 73% by day 180. The increase by day 28 is accounted for entirely by a reduction in reliance on sugary foods for energy (14%), but by day 180 when energy from sweetened foods remains stable (13%), the further increase in starchy foods' contribution to energy intake comes from relative reductions in all other food groups.

In Mbale, dairy foods were a significant source of energy (8-9%), protein (11-14%), fat (14-15%), calcium (19-20%), zinc (11-13%), vitamin A (18-19%), riboflavin (16-19%), and vitamin B12 (21-28%) at all time points. Dairy is a less important component in the diets of children from Soroti at all time points, accounting for less than 5% of energy intake. Dairy contributes most to the diet of children from Soroti at day 28, but it becomes less important by day 180.

In Mbale dietary iron mostly comes from starchy staples in FG1 (41-51%), with much of the remainder coming from legumes/nuts (FG2, 14-22%) and vitamin A rich fruit and vegetables (FG6, 7-11%). On admission sweetened foods also contributed significantly (FG8, 14%). In Soroti, iron came from FG1 (37%), FG 6 (10%) and FG8 (19%), but also flesh foods (FG4 16%). Over time, FG1 contributed even greater quantities of iron to the diet of children in Soroti, accounting for 72% at day 180.

Sugar and sweetened foods were a significant source of many nutrients on admission in both Mbale and Soroti, but their importance decreased by day 28. For instance, between both sites FG8 contributed 28-31% of energy, 20-25% of calcium, 14-19% of iron and 14-28% of vitamin B12 on admission.

Flesh foods (FG4) was relatively a more important component of the diet in children from Soroti, compared to those in Mbale, providing 29-31% of protein in Soroti compared to 9-13% in Mbale.

Table 3.10 Contribution of food groups to nutrient intakes in children from Mbale region

Nutrient (%)	1. Grains, roots & tubers (& starchy bananas)	2. Legumes & nuts	3. Dairy products	4. Flesh foods	5. Eggs	6. Vitamin A-rich fruit & vegetables	7. Other fruit & vegetables	8. Sugar & highly sweetened foods	9. Other
Mbale Day 0									
Energy	44 (±23)	6 (±10)	9 (±19)	2 (±6)	1 (±4)	3 (±8)	5 (±11)	28 (±21)	2 (±6)
Protein	40 (±26)	13 (±18)	14 (±28)	9 (±19)	4 (±11)	4 (±9)	4 (±9)	8 (±16)	3 (±8)
Fat	37 (±33)	7 (±20)	15 (±30)	5 (±12)	5 (±14)	1 (±2)	6 (±16)	19 (±30)	5 (±13)
Carbohydrate	48 (±23)	3 (±5)	5 (±12)	0 (±1)	0 (±0)	3 (±10)	5 (±13)	35 (±21)	1 (±6)
Sucrose	2 (±3)	0 (±2)	0 (±0)	0 (±0)	0 (±0)	5 (±17)	9 (±21)	83 (±27)	0 (±0)
Fibre	65 (±40)	11 (±21)	0 (±0)	1 (±9)	0 (±0)	8 (±20)	16 (±28)	0 (±0)	0 (±0)
Calcium	34 (±30)	9 (±15)	20 (±37)	2 (±5)	3 (±9)	5 (±14)	6 (±14)	20 (±25)	1 (±4)
Iron	41 (±27)	15 (±19)	6 (±16)	5 (±12)	4 (±12)	7 (±16)	7 (±14)	14 (±21)	1 (±3)
Zinc	42 (±28)	12 (±17)	13 (±26)	9 (±21)	3 (±10)	7 (±18)	4 (±11)	8 (±17)	1 (±2)
Vitamin A	20 (±35)	10 (±24)	19 (±36)	2 (±10)	12 (±28)	14 (±31)	10 (±25)	13 (±29)	1 (±8)
Thiamine	51 (±32)	13 (±18)	9 (±21)	2 (±6)	2 (±6)	3 (±9)	7 (±15)	12 (±22)	1 (±3)
Riboflavin	26 (±25)	6 (±9)	17 (±33)	4 (±11)	5 (±14)	6 (±15)	7 (±15)	13 (±24)	15 (±18)
Niacin	48 (±27)	13 (±19)	4 (±13)	8 (±17)	4 (±11)	8 (±17)	6 (±12)	8 (±14)	1 (±4)
Vitamin B6	55 (±29)	11 (±17)	7 (±19)	4 (±10)	2 (±6)	3 (±10)	12 (±22)	4 (±8)	1 (±2)
Vitamin B12	0 (±1)	0 (±0)	28 (±36)	26 (±36)	18 (±30)	0 (±0)	0 (±0)	28 (±36)	0 (±0)
Folate	30 (±24)	24 (±28)	8 (±18)	2 (±7)	3 (±9)	3 (±9)	8 (±17)	14 (±21)	9 (±14)
Pantothenate	40 (±31)	15 (±21)	0 (±0)	5 (±15)	9 (±22)	2 (±12)	12 (±23)	0 (±0)	17 (±24)
Biotin	34 (±32)	10 (±25)	0 (±0)	4 (±14)	10 (±24)	0 (±0)	10 (±22)	0 (±0)	32 (±33)
Vitamin C	33 (±36)	8 (±21)	7 (±20)	0 (±0)	0 (±0)	14 (±30)	12 (±25)	24 (±36)	1 (±6)

Table 3.10 Contribution of food groups to nutrient intakes in children from Mbale region continued

Nutrient (%)	1. Grains, roots & tubers (& starchy bananas)	2. Legumes & nuts	3. Dairy products	4. Flesh foods	5. Eggs	6. Vitamin A-rich fruit & vegetables	7. Other fruit & vegetables	8. Sugar & highly sweetened foods	9. Other
					Mbale Day 28				
Energy	55 (±19)	10 (±11)	8 (±16)	4 (±7)	1 (±4)	4 (±9)	3 (±6)	13 (±14)	1 (±3)
Protein	37 (±20)	21 (±20)	11 (±23)	16 (±21)	2 (±10)	6 (±10)	2 (±4)	2 (±6)	3 (±9)
Fat	33 (±29)	13 (±21)	15 (±29)	10 (±16)	2 (±8)	2 (±4)	10 (±20)	7 (±19)	7 (±15)
Carbohydrate	65 (±20)	6 (±7)	4 (±10)	0 (±0)	0 (±0)	5 (±9)	2 (±4)	17 (±15)	0 (±0)
Sucrose	4 (±14)	4 (±15)	0 (±0)	0 (±0)	0 (±0)	13 (±21)	5 (±14)	73 (±31)	0 (±0)
Fibre	45 (±36)	18 (±28)	0 (±0)	1 (±8)	0 (±0)	24 (±32)	12 (±21)	0 (±0)	0 (±0)
Calcium	32 (±25)	16 (±18)	19 (±36)	4 (±9)	2 (±8)	13 (±21)	5 (±11)	7 (±14)	1 (±2)
Iron	46 (±21)	22 (±19)	4 (±10)	7 (±13)	2 (±9)	9 (±16)	4 (±6)	4 (±11)	1 (±2)
Zinc	40 (±23)	19 (±19)	11 (±22)	14 (±24)	2 (±9)	6 (±15)	3 (±5)	3 (±10)	1 (±1)
Vitamin A	11 (±22)	20 (±33)	18 (±33)	1 (±9)	5 (±19)	31 (±40)	11 (±24)	2 (±11)	1 (±5)
Thiamine	56 (±25)	20 (±19)	5 (±12)	3 (±9)	1 (±4)	7 (±12)	4 (±8)	3 (±9)	1 (±3)
Riboflavin	32 (±22)	9 (±11)	16 (±31)	8 (±13)	3 (±11)	11 (±17)	4 (±8)	3 (±10)	14 (±16)
Niacin	41 (±22)	21 (±22)	2 (±5)	14 (±20)	2 (±9)	12 (±18)	4 (±6)	2 (±5)	1 (±3)
Vitamin B6	59 (±25)	13 (±15)	4 (±10)	7 (±12)	1 (±4)	8 (±12)	6 (±12)	1 (±4)	1 (±2)
Vitamin B12	0 (±0)	3 (±13)	21 (±34)	55 (±46)	7 (±20)	0 (±0)	0 (±0)	10 (±24)	4 (±14)
Folate	32 (±20)	37 (±28)	4 (±11)	3 (±8)	2 (±8)	7 (±13)	5 (±10)	3 (±9)	6 (±11)
Pantothenate	28 (±28)	28 (±30)	0 (±0)	7 (±16)	4 (±15)	10 (±18)	10 (±19)	0 (±0)	12 (±18)
Biotin	17 (±25)	24 (±38)	0 (±0)	7 (±20)	6 (±20)	0 (±0)	9 (±19)	0 (±0)	35 (±36)
Vitamin C	55 (±33)	7 (±16)	3 (±9)	1 (±6)	0 (±0)	21 (±27)	9 (±20)	3 (±14)	0 (±0)

Table 3.10 Contribution of food groups to nutrient intakes in children from Mbale region continued

Nutrient (%)	1. Grains, roots & tubers (& starchy bananas)	2. Legumes & nuts	3. Dairy products	4. Flesh foods	5. Eggs	6. Vitamin A-rich fruit & vegetables	7. Other fruit & vegetables	8. Sugar & highly sweetened foods	9. Other
Mbale Day 180									
Energy	55 (±20)	7 (±10)	8 (±15)	3 (±4)	0 (±2)	7 (±11)	5 (±10)	14 (±13)	1 (±2)
Protein	41 (±21)	14 (±16)	12 (±23)	13 (±19)	1 (±5)	10 (±15)	3 (±7)	3 (±7)	2 (±3)
Fat	36 (±31)	13 (±24)	14 (±27)	7 (±13)	2 (±8)	3 (±7)	11 (±22)	11 (±24)	5 (±12)
Carbohydrate	66 (±19)	3 (±4)	4 (±9)	0 (±0)	0 (±0)	5 (±11)	4 (±10)	17 (±13)	0 (±0)
Sucrose	2 (±9)	2 (±10)	0 (±0)	0 (±0)	0 (±0)	6 (±19)	13 (±28)	76 (±34)	0 (±0)
Fibre	41 (±36)	14 (±23)	0 (±0)	2 (±11)	0 (±0)	28 (±34)	15 (±24)	0 (±0)	0 (±0)
Calcium	31 (±28)	10 (±14)	20 (±37)	3 (±6)	1 (±7)	18 (±26)	8 (±17)	8 (±14)	0 (±1)
Iron	51 (±20)	14 (±15)	4 (±9)	6 (±10)	1 (±5)	11 (±17)	6 (±11)	6 (±9)	0 (±1)
Zinc	46 (±23)	13 (±16)	11 (±22)	10 (±18)	1 (±5)	11 (±16)	5 (±10)	3 (±5)	1 (±1)
Vitamin A	12 (±26)	11 (±25)	19 (±35)	3 (±15)	4 (±19)	34 (±43)	15 (±31)	1 (±6)	0 (±3)
Thiamine	54 (±25)	13 (±16)	6 (±13)	3 (±6)	0 (±3)	10 (±14)	7 (±13)	5 (±11)	1 (±1)
Riboflavin	32 (±24)	6 (±7)	17 (±33)	7 (±13)	2 (±8)	10 (±17)	6 (±16)	5 (±11)	14 (±15)
Niacin	43 (±25)	18 (±22)	2 (±5)	12 (±18)	1 (±6)	14 (±19)	6 (±11)	3 (±7)	1 (±2)
Vitamin B6	58 (±27)	11 (±15)	4 (±9)	5 (±8)	1 (±3)	11 (±17)	9 (±18)	1 (±4)	0 (±1)
Vitamin B12	0 (±4)	0 (±1)	25 (±34)	52 (±43)	6 (±18)	0 (±0)	0 (±0)	14 (±24)	3 (±8)
Folate	35 (±22)	24 (±24)	5 (±10)	3 (±5)	1 (±4)	13 (±19)	8 (±15)	4 (±8)	6 (±7)
Pantothenate	28 (±29)	19 (±26)	0 (±0)	7 (±17)	2 (±12)	14 (±28)	13 (±24)	0 (±0)	16 (±23)
Biotin	23 (±29)	20 (±35)	0 (±0)	7 (±22)	3 (±11)	0 (±0)	11 (±24)	0 (±0)	36 (±37)
Vitamin C	49 (±37)	5 (±18)	4 (±11)	0 (±1)	0 (±0)	22 (±31)	15 (±27)	4 (±18)	0 (±0)

Table 3.11 Contribution of food groups to nutrient intakes in children from Soroti region

Nutrient (%)	1. Grains, roots & tubers (& starchy bananas)	2. Legumes & nuts	3. Dairy products	4. Flesh foods	5. Eggs	6. Vitamin A-rich fruit & vegetables	7. Other fruit & vegetables	8. Sugar & highly sweetened foods	9. Other
Soroti Day 0									
Energy	46 (±23)	2 (±6)	4 (±13)	7 (±8)	1 (±4)	4 (±10)	5 (±12)	31 (±21)	0 (±1)
Protein	36 (±23)	4 (±11)	6 (±20)	31 (±27)	3 (±12)	6 (±16)	4 (±10)	9 (±15)	1 (±9)
Fat	27 (±30)	3 (±10)	7 (±22)	21 (±25)	3 (±14)	3 (±12)	6 (±15)	28 (±36)	1 (±8)
Carbohydrate	53 (±25)	1 (±3)	2 (±7)	0 (±1)	0 (±0)	4 (±11)	5 (±12)	34 (±21)	0 (±0)
Sucrose	1 (±8)	0 (±0)	0 (±0)	0 (±0)	0 (±0)	5 (±14)	10 (±20)	84 (±34)	0 (±0)
Fibre	31 (±37)	11 (±23)	0 (±0)	10 (±21)	0 (±0)	14 (±27)	34 (±37)	0 (±0)	0 (±0)
Calcium	38 (±27)	4 (±9)	9 (±26)	6 (±9)	3 (±11)	8 (±18)	7 (±15)	25 (±25)	0 (±1)
Iron	37 (±28)	5 (±12)	4 (±15)	16 (±20)	3 (±11)	10 (±24)	6 (±15)	19 (±26)	0 (±1)
Zinc	31 (±26)	4 (±11)	6 (±21)	27 (±31)	3 (±13)	10 (±25)	6 (±15)	12 (±23)	0 (±1)
Vitamin A	3 (±12)	8 (±23)	10 (±26)	17 (±35)	8 (±22)	23 (±37)	21 (±34)	10 (±26)	0 (±0)
Thiamine	67 (±29)	3 (±8)	3 (±13)	3 (±6)	1 (±4)	4 (±12)	6 (±13)	12 (±19)	0 (±1)
Riboflavin	38 (±28)	2 (±6)	7 (±23)	14 (±18)	4 (±14)	9 (±21)	7 (±15)	15 (±22)	4 (±12)
Niacin	41 (±25)	4 (±11)	2 (±9)	24 (±24)	3 (±11)	10 (±22)	6 (±13)	9 (±16)	0 (±2)
Vitamin B6	49 (±32)	4 (±13)	6 (±20)	15 (±19)	2 (±9)	3 (±8)	15 (±25)	5 (±17)	0 (±4)
Vitamin B12	0 (±0)	0 (±0)	8 (±22)	70 (±46)	6 (±19)	0 (±0)	0 (±0)	14 (±25)	1 (±9)
Folate	31 (±27)	9 (±21)	5 (±18)	11 (±16)	3 (±12)	4 (±12)	13 (±23)	20 (±25)	3 (±11)
Pantothenate	28 (±32)	11 (±23)	0 (±0)	18 (±30)	8 (±20)	1 (±8)	24 (±33)	0 (±0)	10 (±23)
Biotin	24 (±33)	10 (±24)	0 (±0)	19 (±31)	8 (±23)	0 (±0)	22 (±33)	0 (±0)	17 (±28)
Vitamin C	49 (±43)	2 (±13)	6 (±19)	0 (±3)	0 (±0)	16 (±31)	15 (±26)	12 (±27)	0 (±0)

Table 3.11 Contribution of food groups to nutrient intakes in children from Soroti region continued

Nutrient (%)	1. Grains, roots & tubers (& starchy bananas)	2. Legumes & nuts	3. Dairy products	4. Flesh foods	5. Eggs	6. Vitamin A-rich fruit & vegetables	7. Other fruit & vegetables	8. Sugar & highly sweetened foods	9. Other
					Soroti Day 28				
Energy	63 (±20)	5 (±9)	5 (±13)	6 (±5)	1 (±3)	5 (±10)	1 (±4)	14 (±15)	0 (±1)
Protein	40 (±21)	10 (±15)	8 (±19)	30 (±21)	2 (±7)	7 (±11)	1 (±2)	2 (±6)	1 (±6)
Fat	34 (±29)	12 (±24)	10 (±25)	23 (±23)	2 (±10)	1 (±3)	7 (±17)	10 (±23)	0 (±0)
Carbohydrate	75 (±18)	2 (±3)	2 (±7)	0 (±0)	0 (±0)	4 (±11)	0 (±1)	17 (±16)	0 (±0)
Sucrose	6 (±22)	7 (±22)	0 (±0)	0 (±0)	0 (±0)	9 (±26)	1 (±5)	76 (±42)	0 (±0)
Fibre	30 (±37)	20 (±31)	0 (±0)	9 (±22)	0 (±0)	33 (±39)	9 (±20)	0 (±0)	0 (±0)
Calcium	55 (±29)	6 (±11)	12 (±30)	6 (±7)	1 (±7)	10 (±17)	2 (±7)	7 (±14)	0 (±0)
Iron	65 (±20)	8 (±12)	2 (±7)	9 (±9)	2 (±8)	8 (±14)	2 (±4)	5 (±9)	0 (±0)
Zinc	48 (±24)	11 (±17)	8 (±20)	18 (±19)	2 (±7)	8 (±13)	1 (±4)	3 (±6)	0 (±0)
Vitamin A	13 (±27)	19 (±32)	15 (±32)	13 (±31)	6 (±18)	20 (±35)	15 (±30)	0 (±0)	0 (±0)
Thiamine	74 (±19)	5 (±8)	3 (±8)	6 (±10)	1 (±3)	4 (±7)	1 (±4)	3 (±7)	4 (±8)
Riboflavin	55 (±28)	4 (±7)	11 (±26)	16 (±14)	2 (±8)	7 (±12)	1 (±5)	4 (±11)	0 (±0)
Niacin	48 (±22)	11 (±18)	1 (±4)	23 (±20)	2 (±8)	11 (±17)	1 (±5)	2 (±6)	0 (±0)
Vitamin B6	74 (±19)	5 (±9)	2 (±6)	10 (±11)	1 (±4)	6 (±11)	2 (±6)	1 (±6)	0 (±0)
Vitamin B12	0 (±2)	0 (±0)	7 (±20)	82 (±40)	4 (±17)	0 (±0)	0 (±0)	4 (±16)	2 (±5)
Folate	54 (±24)	12 (±20)	4 (±10)	6 (±7)	2 (±8)	9 (±16)	3 (±8)	5 (±12)	6 (±17)
Pantothenate	26 (±37)	21 (±35)	0 (±0)	18 (±34)	4 (±16)	8 (±23)	8 (±22)	0 (±0)	14 (±31)
Biotin	28 (±35)	33 (±39)	0 (±0)	24 (±34)	6 (±17)	0 (±0)	9 (±21)	0 (±0)	0 (±0)
Vitamin C	85 (±23)	1 (±2)	1 (±4)	0 (±0)	0 (±0)	9 (±21)	3 (±8)	1 (±7)	0 (±1)

Table 3.11 Contribution of food groups to nutrient intakes in children from Soroti region continued

Nutrient (%)	1. Grains, roots & tubers (& starchy bananas)	2. Legumes & nuts	3. Dairy products	4. Flesh foods	5. Eggs	6. Vitamin A-rich fruit & vegetables	7. Other fruit & vegetables	8. Sugar & highly sweetened foods	9. Other
Soroti Day 180									
Energy	73 (±15)	2 (±3)	3 (±9)	5 (±5)	0 (±2)	3 (±7)	1 (±4)	13 (±11)	0 (±0)
Protein	49 (±19)	6 (±9)	5 (±16)	29 (±20)	1 (±7)	7 (±9)	1 (±3)	1 (±6)	1 (±1)
Fat	47 (±27)	5 (±12)	6 (±19)	34 (±26)	2 (±9)	1 (±6)	2 (±6)	4 (±16)	0 (±1)
Carbohydrate	80 (±14)	1 (±2)	1 (±4)	0 (±0)	0 (±0)	2 (±7)	1 (±5)	14 (±11)	0 (±0)
Sucrose	16 (±35)	2 (±13)	0 (±0)	0 (±0)	0 (±0)	1 (±9)	3 (±14)	78 (±42)	0 (±0)
Fibre	47 (±40)	10 (±22)	0 (±0)	8 (±23)	0 (±0)	27 (±36)	7 (±20)	0 (±0)	0 (±0)
Calcium	63 (±25)	5 (±8)	7 (±24)	6 (±7)	1 (±6)	7 (±14)	4 (±12)	6 (±12)	0 (±0)
Iron	72 (±17)	6 (±9)	1 (±4)	10 (±12)	1 (±6)	6 (±12)	1 (±4)	3 (±6)	0 (±0)
Zinc	57 (±22)	6 (±9)	5 (±16)	21 (±21)	1 (±6)	7 (±11)	1 (±5)	2 (±4)	0 (±0)
Vitamin A	6 (±20)	37 (±45)	11 (±28)	16 (±34)	4 (±18)	14 (±30)	11 (±28)	0 (±0)	0 (±0)
Thiamine	85 (±16)	4 (±7)	1 (±5)	4 (±9)	0 (±1)	3 (±5)	1 (±5)	2 (±7)	0 (±0)
Riboflavin	56 (±26)	3 (±5)	6 (±22)	16 (±16)	2 (±9)	5 (±10)	2 (±7)	2 (±10)	8 (±15)
Niacin	60 (±20)	6 (±10)	1 (±3)	22 (±19)	1 (±6)	8 (±13)	1 (±5)	1 (±5)	0 (±0)
Vitamin B6	82 (±15)	2 (±4)	1 (±5)	8 (±9)	0 (±1)	4 (±9)	2 (±8)	0 (±1)	0 (±0)
Vitamin B12	0 (±0)	0 (±0)	7 (±20)	86 (±43)	3 (±14)	0 (±0)	0 (±0)	3 (±14)	0 (±0)
Folate	63 (±19)	12 (±17)	2 (±7)	5 (±7)	1 (±5)	7 (±11)	4 (±10)	2 (±9)	4 (±8)
Pantothenate	44 (±40)	16 (±30)	0 (±0)	15 (±27)	2 (±9)	9 (±26)	5 (±18)	0 (±0)	10 (±17)
Biotin	32 (±39)	13 (±27)	0 (±0)	19 (±32)	2 (±14)	0 (±0)	5 (±16)	0 (±0)	29 (±39)
Vitamin C	91 (±21)	1 (±2)	0 (±2)	0 (±2)	0 (±0)	4 (±12)	4 (±12)	0 (±0)	0 (±0)

3.2.2.7 *Dietary diversity scores*

Mean dietary diversity score data for each study site and time point, along with the proportion of children considered to have "diverse" diets (i.e. DDS ≥4), and associated statistics are presented in Table 3.12. Note that diversity score data only counts intake from WHO food groups, and not the additional sweetened foods or 'other' groups added by the author in previous sections. In Mbale there is a significant increase in mean DDS from 2.79 (±1.19) on admission, to 3.92 (±1.18) on day 28 and 3.79 (±1.15) on day 180, p<0.001. The latter two time points do not differ significantly. This pattern is echoed in the proportion of children categorised as having diverse diets, increasing from 26.8% on admission, to 59.4% at day 28 and 60.0% at day 180, p<0.001. In Soroti, the mean DDS is also low at 2.87 (±1.18), which is not significantly different from Mbale, p=0.833. The DDS in Soroti increased to 3.53 (±1.03) by day 28, and 3.70 9 (±0.84) by day 180. In Soroti, only at day 180 was the DDS significantly higher than at admission, p=0.002. On day 28 the DDS of the diets reported by children in Mbale (3.92), was significantly greater than the diets of children in Soroti (3.53), p=0.031. The proportion of children in Soroti eating four or more foods groups increased from 25.8% on admission, to 51.6% at day 28 and 58.9% at day 180, p<0.001. No differences were detected in the proportion of children with diverse diets between the two study sites, p>0.05 in all instances.

Table 3.12 Dietary diversity scores and percentage of diets classified as diverse

	Mbale			Soroti			Between site	
	n	Mean (SD)	% ≥ 4	n	Mean (SD)	% ≥ 4	*p-value	χ^2 p-value
Day 0	162	2.79 (1.19)^{D28,D180}	26.8	152	2.87 (1.18)^{D180}	25.8	0.833	0.848
Day 28	152	3.92 (1.18)^{D0}	59.4	92	3.53 (1.03)	51.6	0.031	0.251
Day 180	142	3.79 (1.15)^{D0}	60.0	80	3.70 (0.84)^{D0}	58.9	0.626	0.882
Within Site		** p<0.001	χ^2 p<0.001		**p = 0.002	χ^2 p<0.001		

* Independent Samples Mann-Whitney U Test compares continuous variables between sites
** Related-Samples Friedman's Two-Way Analysis of Variance by Ranks compares continuous variables between time points; difference from Day 0, Day 28, Day 180 indicated by superscript

3.2.2.8 *Dietary patterns*

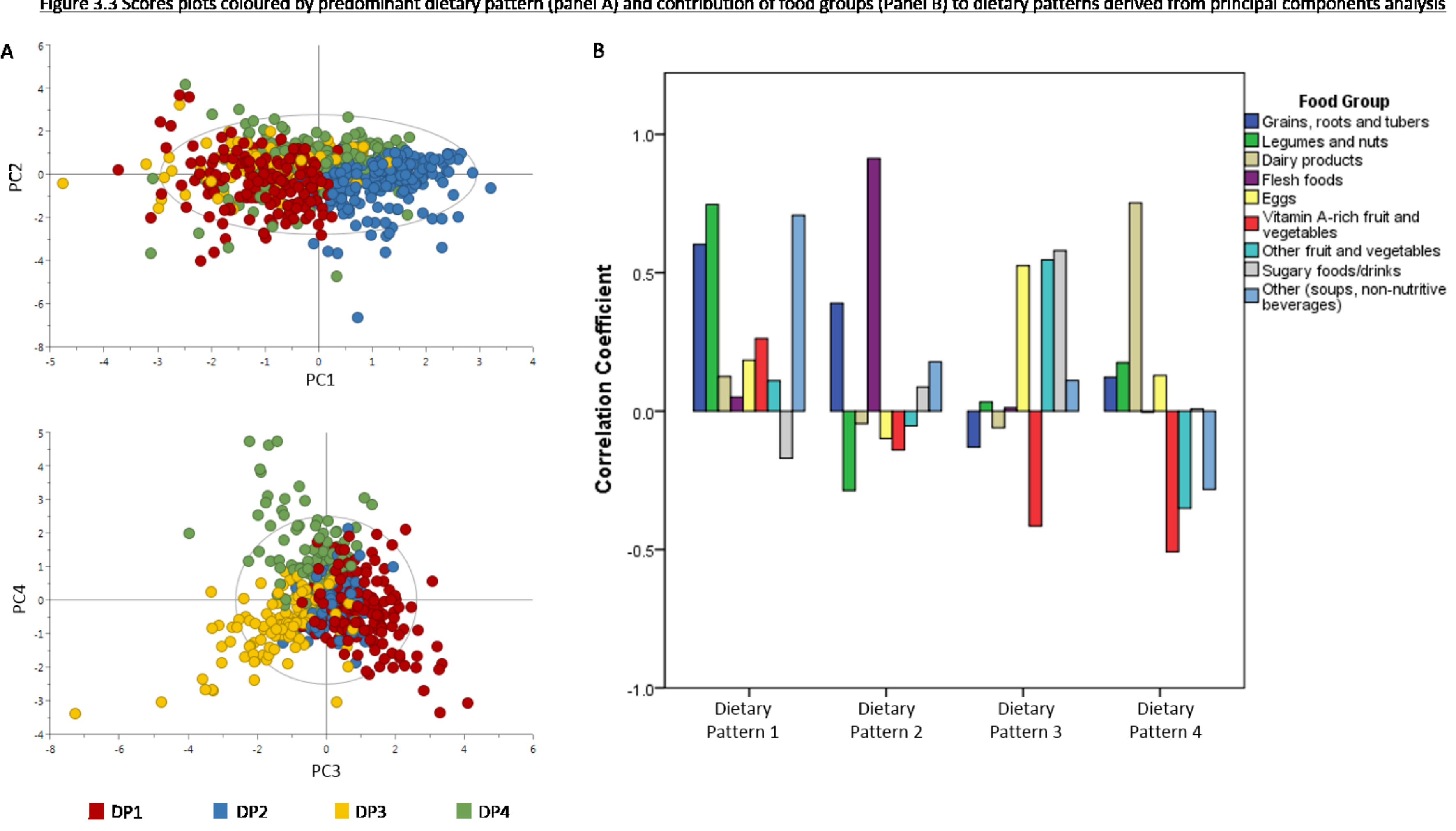
Factor analysis by Principal Components Analysis using individual food-level data (i.e. ungrouped foods such as maize and matooke) was deemed not to be appropriate, based on unacceptable Kaiser-Meyer-Olkin (KMO) sampling adequacy index value of 0.465, although results of Bartlett's Test of Sphericity (χ^2 = 6343, p<0.001) indicated that the correlation matrix was not an identity matrix (i.e. was not restricted to values of 0 or 1) and in that respect would be suitable for factor analysis. Factor analysis using food group-level data from all 24hr DRs collected (n=782) was determined to be suitable based on acceptable sampling adequacy assessment by Kaiser-Meyer-Olkin (KMO) index value of 0.525. Bartlett's Test of Sphericity also indicated that this data was suitable for factor analysis, with

approximate χ^2 of 238.3, $p<0.001$. Using a cut-off of eigen-values greater than 1, a PCA model was constructed with 4 components which explained 54% of the variance (PC1 = 18%, PC2 = 13%, PC3 = 12%, PC4 = 11%). Figure 3.3 panel A displays the scores plots for the PCA model coloured by the predominant dietary pattern. Following Varimax rotation of the component matrix to maximise component orthogonality and interpretability, the contribution of food groups to each of the four components was visualised, shown in Figure 3.3 panel B. From the PCs, four dietary patterns (DPs) were identified:

- Dietary pattern 1 “Carbohydrate-rich staples, legumes and beverages”
- Dietary pattern 2 “Meat and carbohydrate-rich staples”
- Dietary pattern 3 “Sweetened foods/drinks, fruit, eggs”
- Dietary pattern 4 “Milk-based, with low fruit and vegetable intake”

Each dietary pattern identified varied substantially in the dominant food groups displayed, with minimal overlap. Dietary pattern 1 is positively associated with all food groups except sugary foods, but is particularly associated with high intake of grains, roots and tubers, legumes and soups and non-nutritive drinks such as black tea and coffee. Dietary pattern 2 also relied on grains, roots and tubers, but combined with a high intake of meat, poultry or fish. Dietary pattern 3 was comprised of roughly equal contributions from eggs, fruit and vegetables and sugary foods and beverages. Dietary pattern 4 was principally defined by high intake of dairy (milk), but low fruit and vegetable intake, and low intake of soups and other beverages.

Figure 3.3 Scores plots coloured by predominant dietary pattern (panel A) and contribution of food groups (Panel B) to dietary patterns derived from principal components analysis



Differences in subject characteristics and nutrient profiles of each dietary pattern were compared between diets scoring in the lowest and highest tertiles for each component, summarised in Table 3.13. As a total of n=749 dietary recalls were used to conduct dietary pattern analysis, the lowest tertile comprises n=249 recalls, while the highest comprises n=251.

It is worthwhile to initially address how the dietary patterns are associated with location, time point and subject characteristics. Due to the loss to follow up found in the Soroti site, there is a larger amount of dietary data overall from Mbale. Although DP1, DP2, and DP3 all exhibit significant variation in the proportion of children from Mbale vs. Soroti between the highest and lowest tertiles, in most cases the majority of children are still from Mbale, χ^2 $p<0.001$. Only DP4 does not show significant variation with regards to the study site, $p>0.05$. No dietary pattern shows differences in the proportion of children who are male or female between the highest and lowest tertiles, with all being between 52.0-59.4% male.

There was a significant difference in the number of diets coming from admission, day 28 and day 180 in the highest and lowest tertiles of DP1, DP2 and DP3. The highest tertile of dietary pattern 1 was associated with greater proportions of recalls coming from day 28 (n=108/251) and day 180 (n=85/251), while the lowest tertile displayed greater numbers from admission samples (n=151/251), $p<0.001$. Diet recalls scoring highest in dietary pattern 2 had broadly equal distribution among each time point (Day 0=86/251: Day 28=94/251: Day 80=70/251), significantly different from the lowest tertile in which almost half of recalls were from admission (n=121/249), $p<0.01$. Dietary pattern 3 also exhibited differences in the proportions of diets coming from different time points. In the highest tertile of DP3 61% (n=152/251) of recalls were from admission, with the remainder equally divided between day 28 and day 180, compared to only 26% (n=64/249) from admission in the lowest tertile of DP3, $p<0.001$.

Age differences were a significant factor in dietary patterns 1 and 2: those scoring in the highest tertile for DP1 were aged 59.8 months on average compared to 51.0 months in those in the lowest tertile ($p<0.01$), while those who scored highest in DP2 were aged 61months compared to 49.1months in those who scored lowest ($p<0.001$). Less children under 24months and more children aged over 60 months reported diets which aligned with DP1 ($p<0.01$) and DP2 ($p<0.001$).

Anthropometric indices varied significantly only in comparison between the highest and lowest tertile of scores within dietary pattern 1. Children who scored highly in DP1 were less likely to be underweight (16.5 vs. 25.8%, $p<0.05$), stunted (2.4 vs. 9.9%, $p<0.01$) and wasted (5.4 vs. 23.7%, $p<0.001$) than those in the lowest tertile.

Diets in the highest tertile of DP1 are characterised by significantly higher quantities of energy, protein, fat, carbohydrate, sugar, fibre and all micronutrients than those in the lowest tertile, p<0.01 in all cases. As this dietary pattern is associated with greater intakes of starchy staples, legumes and most other foods groups, the increased intakes are expected. Those scoring highest in dietary pattern 1 take 129% of their estimated energy requirements while those in the lowest tertile are taking just over half (53.5%), p<0.001. Average protein intakes are close to requirements in the lowest tertile of DP1 (93.8% of requirement), but they are almost 3 times (276%) requirements in the highest scoring tertile. The discrepancies in micronutrient intakes are more nutritionally relevant, with those scoring lowest on DP1 having inadequate intakes, while those in the highest tertile have adequate or excessive intakes. For example iron intake in tertile one of DP1 is 1.9mg or 30.6% of requirement, while those in tertile 3 are on average meeting their requirement with intake of 6.5mg (99.6% requirement). Folate also displays wide differences between intake adequacy in the highest (219.6µg, 117.9%) and lowest tertiles (51.0µg, 29.2%), p<0.001. DP1 is also characterised by greater dietary diversity, with on average 3.9 food groups being eaten, compared to 2.8 in the lowest tertile, p<0.001.

In dietary pattern 2, there is no significant difference between the highest and lowest tertiles with respect to dietary diversity (DDS 3.62 vs. 3.26), fat (23.7g vs. 21.9g), sucrose (31.1g vs. 32.9g), fibre (5.6g vs. 5.7g), vitamin A (641µg RE vs. 387µg RE), riboflavin (0.6mg vs. 0.6mg, folate (124.8µg vs. 130.9µg), pantothenate (0.9mg vs. 0.9mg) or biotin (8.73µg vs. 10.5µg), p>0.05 in all cases. Similar to DP1, the highest tertile of DP2 is characterised by higher energy intake (5076kJ vs. 3759kJ, p<0.001), and higher protein intake (238% vs. 171% of requirements, p<0.001). The average intake of iron in the highest tertile is still only 76.7% of requirements (5.0mg ±3.2), but this is significantly greater than the lowest tertile (57% requirements or 3.7mg ±2.8), p<0.01. Vitamin B12 intake is positively associated with DP2, where the highest tertile averages 284% of requirement, compared to on 33% of requirements met in the lowest tertile, p<0.001. Folate intake is poor regardless of the tertile of DP2, containing 67-73% of requirements, p>0.05.

Dietary pattern 3 is largely defined by the sources of dietary energy, with the total amount of energy and protein being similar. The highest tertile of DP3 contains 27.3g (± 23.2) of fat and 39.2g (± 26.7) of sucrose, compared to 17.4g (±22.8) fat and 28.7g (±23.4) sucrose in the lowest tertile, p<0.001 in both cases. While there are significant differences in several micronutrient intakes (thiamine, folate, pantothenate and vitamin C), in most of these cases the intakes differ only slightly in the degree of sufficiency or deficiency. For example, although thiamine intake in the highest tertile (0.6mg/116% of requirement) is significantly greater than the lowest tertile (0.8mg/135% of requirement), p<0.01, both tertiles have adequate intakes. An exception to this is for biotin, where the average intake of the

highest tertile is sufficient (106.5% of requirement), while the intake in the lowest tertile is insufficient (44.0% of requirement), p<0.001.

In the final dietary pattern (DP4), energy and protein intakes differ significantly between the highest and lowest tertiles. Mean energy intake in the highest tertile is in excess of requirements (114.8%), while in the lowest the mean intake is only 89% of requirements, p<0.001. Protein in both tertiles is above requirements (263.8% vs. 161.3%, p<0.001). The content of fat within T3 of DP4 is significantly higher than T1, 32.1g vs. 17.6g (p<0.001), while the opposite trend is true for sucrose (31.4g in T3 vs. 40.0g in T1, p<0.001). Dietary fibre content is also significantly lower in the highest tertile of DP4 (5.2g ±6.3) compared to the lowest (8.2g ±7.7g), p<0.001. Riboflavin intake is highly discriminant within DP4, with the highest tertile reporting an average intake totalling 166.0% (±184.6) of requirements, compared to only 75.4% (±55.1) in the lowest tertile. Zinc is also discordant for the sufficiency of the average intake, with T3 containing on average 100.4% of the required intake, compared to 70.9% in T1, p<0.001. Mean intakes of thiamine and vitamin C also differ significantly, however both the highest and lowest tertiles report sufficient intakes: 111-142% of thiamine intake and 216-346% of vitamin C intake.

Table 3.13 Meal pattern association with differences in subject characteristics and contribution to differences in nutrient intakes (T1 n = 249, T3 n=251)

	Pattern 1 Mean (SD)			Pattern 2 Mean (SD)			Pattern 3 Mean (SD)			Pattern 4 Mean (SD)		
	T1	T3	p	T1	T3	p	T1	T3	p	T1	T3	p
Age (months)†	51.0 (28.5)	59.8 (30.0)	**	49.1 (28.4)	61.0 (30.5)	***	57.0 (31.2)	55.9 (28.6)	-	57.5 (29.7)	54.9 (30.6)	-
% Under 24‡	13.8	6.8		16.9	7.6		12.5	7.6		9.3	14.3	
% 24-60‡	56.2	52.2	**	57.3	49.2	***	49.2	58.4	-	55.2	49.0	-
% Over 60‡	30.0	41.0		25.8	43.2		38.3	34.0		35.5	36.7	
Gender (% male)‡	54.1	53.4	-	57.3	54.4	-	52.0	59.8	-	58.5	59.4	-
Location (% Mbale)‡	70.4	88.0	***	69.0	53.2	***	48.4	67.6	***	70.2	63.3	-
WAZ†	-1.2 (1.1)	-0.9 (0.9)	*	-1.1 (1.1)	-1.0 (1.1)	-	1.1 (1.1)	-1.1 (1.0)	-	-1.0 (1.0)	-1.2 (1.1)	-
% underweight (<-2)‡	25.8	16.5	*	23.5	21.4	-	21.3	15.9	-	17.3	22.0	-
HAZ†	-0.3 (1.0)	-0.1 (0.7)	**	-0.2 (0.9)	-0.2 (1.0)	-	-0.2 (0.7)	-0.3 (1.1)	-	-0.2 (0.9)	-0.2 (1.0)	-
% stunted (<-2)‡	9.9	2.4	**	7.1	5.8	-	4.2	8.6	-	5.3	6.9	-
WHZ†	-1.0 (1.4)	-0.2 (1.1)	***	-0.7 (1.3)	-0.5 (1.3)	-	-0.7 (1.4)	-0.6 (1.1)	-	-0.5 (1.2)	-0.6 (1.2)	-
% wasted (<-2)‡	23.7	5.4	***	17.7	12.0	-	16.8	11.5	-	10.4	13.1	-
MUAC (cm)†	15.1 (1.4)	15.3 (1.8)	-	14.7 (1.2)	14.9 (1.3)	-	15.0 (2.3	15.2 (1.3)	-	15.2 (2.3)	15.0 (1.4)	-
% <11.5cm‡	0	0	-	0	0.4	-	0.4	0	-	0	0.4	-
Time point (D0:D28:D180)‡	151:45:51	58:108:85	***	121:63:64	86:94:70	**	64:89:95	152:49:49	***	103:63:82	97:84:70	-
DDS†	2.8 (1.2)	3.9 (1.1)	***	3.26 (1.3)	3.62 (1.1)	-	3.54 (1.1)	3.37 (1.3)	-	3.66 (1.2)	3.47 (1.2)	-
% <4‡	69.2	42.2	***	58.9	50.4	-	46.8	57.6	*	44.4	55.0	*
Energy (kJ)†	2406 (1461)	6196 (2170)	***	3759 (2406)	5076 (2280)	***	4332 (2514)	4758 (2386)	-	4280 (1947)	5219 (2673)	***
% of reqt met	53.5 (33.9)	129.1 (51.8)	***	84.4 (57.0)	106.3 (54.2)	***	94.6 (61.8)	100.0 (51.8)	-	89.5 (42.3)	114.8 (65.1)	***
Protein(g)†	12.8 (10.4)	38.6 (19.5)	***	22.8 (19.4)	33.4 (19.6)	***	26.0 (21.9)	27.3 (17.2)	-	22.8 (13.1)	35.5 (23.5)	***
% of reqt met	93.8 (82.1)	276.9 (159.3)	***	170.7 (154.5)	237.6 (167.4)	***	190.9 (179.4)	193.3 (132.4)	-	161.3 (97.6)	263.8 (197.6)	***
Fat (g)†	11.0 (13.5)	30.6 (24.9)	***	21.9 (23.5)	23.7 (21.5)	-	17.4 (22.8)	27.3 (23.2)	***	17.6 (15.7)	32.1 (2.2)	***
Carbohydrate (g)†	106.6 (53.3)	258.8 (91.5)	***	153.3 (92)	217.7 (94.1)	***	193.3 (100.00)	196.2 (95.4)	-	192.3 (88.3)	205.4 (98.5)	-
Sucrose (g)†	22.4 (17.2)	38.1 (27.2)	***	32.9 (25.6)	31.1 (21.5)	-	28.7 (23.4)	39.2 (26.7)	***	40.0 (28.1)	31.4 (22.9)	***
Fibre (g)†	2.1 (3.6)	9.2 (8.1)	***	5.7 (7.0)	5.6 (6.1)	-	6.3 (7.8)	6.1 (6.3)	-	8.2 (7.7)	5.2 (6.3)	***
Calcium (mg)†	161.1 (295.1)	416.2 (529.7)	***	381.3 (562.4)	279.3 (457.1)	*	348.7 (589.4)	265.6 (379.7)	-	151.4 (116.0)	596.1 (675.4)	***
% of reqt met	29.4 (54.7)	75.7 (98.6)	***	70.8 (107.2)	50.7 (87.0)	*	64.0 (112.5)	48.5 (71.3)	-	27.1 (21.7)	109.9 (129.5)	***

Table 3.13 continued	Pattern 1 Mean (SD)			Pattern 2 Mean (SD)			Pattern 3 Mean (SD)			Pattern 4 Mean (SD)		
	T1	T3	p	T1	T3	p	T1	T3	p	T1	T3	p
Iron (mg)†	1.9 (1.4)	6.5 (3.0)	***	3.7 (2.8)	5.0 (3.2)	**	4.4 (3.0)	4.3 (2.7)	-	4.6 (2.5)	4.6 (3.4)	-
% of reqt met	30.6 (23.1)	99.6 (50.9)	***	57.3 (43.5)	76.7 (52.7)	**	66.3 (48.9)	67.1 (42.5)	-	69.4 (39.6)	71.7 (55.9)	-
Zinc (mg)†	1.6 (1.5)	5.4 (3.7)	***	3.1 (2.5)	4.6 (4.1)	***	3.7 (3.4)	3.6 (2.6)	-	3.3 (2.4)	4.6 (4.1)	***
% of reqt met	36.1 (34.0)	115.1 (84.1)	***	67.8 (55.0)	99.3 (4.9)	***	79.2 (74.2)	77.7 (57.7)	-	70.9 (51.8)	100.4 (92.5)	***
Vitamin A (µg RE)†	146 (565)	740 (3434)	**	387 (714)	640.7 (3578)	-	506.8 (1495)	305 (1225)	-	547 (1377)	523 (3294)	-
% of reqt met	68.8(266.2)	354.5 (1652.0)	**	183.4 (341.6)	282.9 (1698.9)	-	233.8 (727.8)	136.3 (548.0)	-	251.9 (631.5)	244.4 (1608.0)	-
Thiamine (mg)†	0.4 (0.3)	1.0 (0.6)	***	0.5 (0.4)	0.9 (0.5)	***	0.8 (0.5)	0.6 (0.4)	**	0.6 (0.4)	0.8 (0.5)	**
% of reqt met	75.4 (47.1)	175.6 (111.5)	***	94.4 (76.9)	153.5 (104.8)	***	134.7 (91.3)	116.2 (91.8)		110.7 (79.6)	141.6 (96.6)	***
Riboflavin (mg)†	0.3 (0.3)	0.8 (0.7)	***	0.6 (0.6)	0.6 (0.7)	-	0.6 (0.7)	0.5 (0.5)	-	0.4 (0.3)	0.9 (0.9)	***
% of reqt met	50.0 (58.7)	142.0 (151.6)	***	110.6 (131.0)	109.4 (150.1)	-	107.9 (144.4)	101.4 (99.3)	-	75.4 (55.1)	166.0 (184.6)	***
Niacin (mg)†	1.8 (1.8)	4.8 (4.8)	***	2.4 (3.0)	5.1 (4.6)	***	4.1 (4.0)	3.0 (3.1)	-	3.8 (4.0)	3.5 (4.1)	-
% of reqt met	40.7 (30.1)	112.1 (70.7)	***	61.5 (52.5)	101. (64.5)	***	77.8 (55.7)	81.6 (61.6)	-	87.1 (59.5)	81.3 (64.2)	-
Vitamin B6 (mg)†	0.5 (0.4)	1.5 (1.3)	***	0.7 (0.6)	1.2 (0.8)	***	1.0 (0.7)	1.0 (0.8)	-	1.0 (0.7)	1.0 (0.8)	-
% of reqt met	84.3 (74.9)	246.0 (174.5)	***	123.2 (112.7)	208.0 (156.9)	***	163.8 (136.4)	175.8 (158.9)	-	178.6 (139.6)	178.1 (154.3)	-
Vitamin B12 (µg)†	0.9 (1.5)	2.2 (8.1)	**	0.3 (0.4)	3.1 (8.2)	***	1.1 (3.3)	1.3 (3.1)	-	1.1 (3.1)	1.8 (7.7)	-
% of reqt met	82.0 (142.7)	203.5 (854.6)	**	32.6 (46.8)	284.4 (865.8)	***	97.3 (287.2)	115.8 (274.0)	-	102.4 (281.8)	175.5 (828.0)	-
Folate (µg)†	51.0 (39.1)	219.6 (125.4)	***	130.9 (124.7)	124.8 (93.9)	-	109.9 (97.9)	147.5 (113.4)	***	128.5 (85.0)	151.0 (136.3)	*
% of reqt met	29.2 (23.6)	117.9 (77.4)	***	73.4 (72.6)	66.7 (54.5)	-	59.1 (57.4)	79.8 (63.9)	**	68.8 (48.7)	83.6 (82.3)	*
Pantothenate (mg)†	0.4 (0.6)	1.4 (1.1)	***	0.9 (1.0)	0.9 (0.9)	-	0.8 (0.8)	1.1 (0.9)	***	1.1 (1.0)	0.8 (0.9)	**
% of reqt met	16.8 (25.8)	51.5 (43.8)	***	35.8 (39.8)	34.1 (40.0)	-	27.4 (33.7)	42.0 (38.3)	***	42.9 (39.8)	32.6 (38.8)	**
Biotin (µg)†	2.9 (4.2)	14.9 (24.4)	***	10.5 (22.1)	8.73 (13.0)	-	5.81 (13.6)	12.5 (21.5)	***	10.5 (17.3)	10.6 (22.0)	-
% of reqt met	30.7 (46.9)	136.2 (219.1)	***	86.7 (160.)	76.7 (135.1)	-	44.0 (98.4)	106.5 (154.6)	***	93.5 (155.4)	87.5 (171.8)	-
Vitamin C (mg)†	46.9 (45.5)	109.0 (95.9)	***	63.8 (78.6)	95.1 (73.0)	***	98.7 (84.5)	78.3 (76.2)	**	107.9 (90.1)	67.1 (61.7)	***
% of reqt met	152.0 (148.8)	348.1 (307.2)	***	205.6 (251.7)	305.5 (239.7)	***	314.7 (271.7)	253.2 (249.4)	*	345.6 (291.8)	216.4 (198.8)	***

Abbreviations: T1, first tertile; T3, third tertile; SD, standard deviation; WAZ, weight-for-age z-score; HAZ, height-for-age z-score ; WHZ, weight-for-height z-score; MUAC, mid-upper arm circumference; DDS, dietary diversity score; reqt, requirement

†p-values from Independent Samples Mann-Whitney U Test, Bonferroni-corrected for multiple testing

‡p-values from Pearson Chi-Square test, or Likelihood Ratio where assumptions are violated, Bonferroni-corrected for multiple testing

p-value significance level: >0.05/non-significance (-),<0.05 (*), <0.01 (**), <0.001(***)

3.2.2.9 OUTCOME PREDICTION

Using binomial regression which was not adjusted for additional confounders there was no difference in the haemoglobin recovery rates by day 180 between the children who received iron and folate and those who received multi-vitamin multi-mineral supplementation, χ^2 p=0.123. The odds ratio for recovery by day 180 in those treated with IF compared to MVMM was 1.55, (95% CI 0.89-2.70, p=0.124).

Table 3.14 displays associations between dietary diversity scores and dietary patterns identified through PCA, and anthropometric and haematological measures (haemoglobin), unadjusted for additional confounders. Higher dietary diversity scores were associated with higher HAZ scores (each additional food group was associated with 0.11 rise in HAZ, p=0.001), and haemoglobin (1 food group associated with 0.71g/dl increase in Hb, p<0.001). Dietary pattern 1 (rich in carbohydrate staples, legumes and beverages) was positively associated with all anthropometric measures and haemoglobin. Dietary pattern 3 (high in sweetened foods/drinks, fruit and vegetables, and eggs), was negatively associated with haemoglobin (each higher tertile was associated with -0.92g/dl Hb, p<0.001). DP2 and DP4 were not associated with differences in any anthropometric measure or haemoglobin.

Table 3.15 shows the association between any intake of individual food groups (FG) and anthropometric outcomes and haemoglobin recovery by day 180, unadjusted for additional confounders. Intake of FG1 (grains, roots, tubers) was negatively associated with haemoglobin recovery (OR 0.98, 95% CI 0.96-0.99, p=0.036). Legume and nut intake (FG2) was inversely associated with becoming underweight (OR 0.92, 95% CI 0.86-0.98, p<0.017), but positively associated with haemoglobin recovery (OR 1.13, 95% CI 1.00-1.29, p=0.047). Dairy foods (FG3) were associated with underweight (OR 1.02, 95% CI 1.00-1.03, p=0.010); a positive association with wasting was also detected which bordered on statistical significance (OR 1.02, 95% CI 1.00-1.04 p=0.050). Of the remaining food groups, sugary foods and drinks (FG8) were positively associated with stunting by day 180 (OR 1.03, 95% CI 1.01-1.05, p=0.003).

Table 3.14. Association between dietary diversity scores and dietary patterns, and anthropometric and haematological measures†

	DDS*			DP1**			DP2**			DP3**			DP4**		
n=442†	β	SE	p	β	SE	p	β	SE	p	β	SE	p	β	SE	p
WAZ	0.07	0.04	0.077	**0.11**	**0.06**	**0.041**	-0.05	0.05	0.313	0.01	0.05	0.881	-0.05	0.05	0.324
HAZ	**0.11**	**0.03**	**0.001**	**0.12**	**0.05**	**0.016**	0.04	0.05	0.415	-0.07	0.05	0.137	0.02	0.05	0.673
WHZ	0.08	0.05	0.148	**0.37**	**0.08**	**<0.001**	0.02	0.08	0.849	0.01	0.08	0.926	-0.03	0.08	0.682
Hb (g/dl)	**0.71**	**0.11**	**<0.001**	**0.63**	**0.16**	**<0.001**	0.10	0.15	0.524	**-0.92**	**0.15**	**<0.001**	-0.06	0.15	0.674

Abbreviations: DDS, dietary diversity score; DP#, dietary pattern 1-4;, WAZ, weight for age z-score; HAZ, height for age z-score; WHZ, weight for height z-score; Hb, haemoglobin

†Associations were calculated using day 28 and day 180 review data

*Univariate linear regression models report regression coefficients (β) in outcome variables for 1 point increase in DDS, and models are unadjusted

**Regression coefficients (β) reported per 1 tertile increase in each dietary pattern

Table 3.15. Association* of food group intakes with anthropometric and haematological outcomes at day 180

	Underweight (WAZ<-2)			Stunted (HAZ<-2)			Wasted (WHZ<2)			Hb Recovery (>9g/dl)		
n=222	OR	95% CI	p	OR	95% CI	p	OR	95% CI	p	OR	95% CI	p
FG1	1.00	0.98 - 1.01	0.989	1.00	0.98 - 1.02	0.874	1.01	0.99 - 1.03	0.061	**0.98**	**0.96 - 0.99**	**0.036**
FG2	**0.92**	**0.86 - 0.98**	**0.017**	1.02	0.93 - 1.11	0.638	1.01	0.94 - 1.09	0.636	**1.13**	**1.00 - 1.29**	**0.047**
FG3	**1.02**	**1.00 - 1.03**	**0.010**	1.01	0.99 - 1.04	0.150	**1.02**	**1.00 - 1.04**	**0.050**	1.00	0.97 - 1.04	0.609
FG4	1.02	0.94 - 1.10	0.597	1.11	0.99 - 1.25	0.053	1.04	0.92 - 1.17	0.476	1.00	0.84 - 1.18	0.979
FG5	0.98	0.87 - 1.09	0.687	1.01	0.83 - 1.23	0.892	1.08	0.95 - 1.22	0.217	0.82	0.56 - 1.19	0.309
FG6	0.99	0.96 - 1.01	0.388	0.98	0.94 - 1.03	0.590	1.01	0.98 - 1.04	0.391	1.00	0.97 - 1.04	0.616
FG7	1.01	0.98 - 1.03	0.565	0.99	0.94 - 1.04	0.815	1.00	0.96 - 1.04	0.853	0.98	0.94 - 1.02	0.479
FG8	0.99	0.96 - 1.00	0.186	**1.03**	**1.01 - 1.05**	**0.003**	1.00	0.98 - 1.03	0.520	0.95	0.89 - 1.02	0.198
FG9	1.01	0.99 - 1.01	0.321	1.01	0.99 - 1.03	0.077	1.00	0.98 - 1.01	0.688	0.99	0.97 - 1.00	0.346

Abbreviations: DDS, dietary diversity score; DP#, dietary pattern 1-4;, WAZ, weight for age z-score; HAZ, height for age z-score; WHZ, weight for height z-score; Hb, haemoglobin; OR, odds ratio

FG1: Grains, roots, tubers & starchy banana; FG2: Legumes and nuts; FG3: Dairy; FG4: Flesh foods; FG5: Eggs; FG6: Vitamin A-rich fruit and vegetables; FG7: Other fruit and vegetables; FG8: Sugar and highly sweetened foods; FG9: Other

*Analysed by binomial regression, unadjusted

Table 3.16 summarises unadjusted associations between intake of specific nutrients at day 180 and haemoglobin recovery. Higher intakes of iron and pantothenate were positively associated with haemoglobin recovery, OR 1.05 (95% CI 1.01-1.08, p=0.006) and 1.03 (95% CI 1.01-1.04, p=0.010) respectively. In contrast vitamin A and thiamine were negatively associated with Hb recovery, both having OR of 0.99 (95% CI 0.99-1.00 for vitamin A and 0.98-0.99 for thiamine).

Table 3.16. Association of specific nutrients with haemoglobin recovery at day 180 (n=222)

	Hb Recovery*		
Nutrient (% of requirement)	Odds Ratio	95% CI	p
Energy	0.98	0.96-1.00	0.115
Protein	0.99	0.98-1.01	0.289
Calcium	1.02	1.00-1.04	0.082
Iron	**1.05**	**1.01-1.08**	**0.006**
Zinc	0.98	0.97-1.00	0.071
Vitamin A	**0.99**	**0.99-1.00**	**0.004**
Thiamine	**0.99**	**0.98-0.99**	**0.032**
Riboflavin	1.00	0.98-1.02	0.871
Niacin	1.01	0.99-1.03	0.221
Vitamin B6	1.00	0.99-1.01	0.832
Vitamin B12	1.00	1.00-1.01	0.142
Folate	1.00	0.99-1.01	0.944
Pantothenate	**1.03**	**1.01-1.04**	**0.010**
Biotin	1.00	1.00-1.00	0.396
Vitamin C	1.00	0.99-1.00	0.429

*Haemoglobin recovery at day 180 defined as Hb >9g/dl

3.3 Discussion

3.3.1 Validation of 24hr dietary recall method

Summary of main findings:

- The 24hrDR tool provided similar estimates of energy, protein and iron when used by different interviewers
- Estimates of energy, protein and iron intake from the 24hrDR tool were similar to a gold standard method, but with moderately wide limits of agreement

The primary objective of this portion of the study was to develop and validate a quantitative dietary recall method that was sufficiently reproducible and accurate to ensure confidence in its application for larger studies. The method adapted from Gibson and Ferguson (2008), with the inclusion of volumetric portion size estimation, was found to produce comparable estimates of energy, protein and iron intakes when used by two different assessors. A mean difference of less than 5% for estimates of energy, protein and iron intake was found between the two independent assessors. In conjunction with good intraclass correlation coefficients over 0.8 for energy and iron, and excellent ICC of 0.925 for protein, this suggests that the tool produces estimates of dietary intake that are highly reproducible between different interviewers. Intra-interviewer repeatability was not assessed in this study, however this is usually poorer than inter-interviewer agreement (McAvay and Rodin, 1988).

The recall method performed favourably compared to weighed food records completed by an independent researcher, indicating that it is sufficiently valid to employ in other studies in similar settings. On an individual nutrient level, Bland-Altman analysis showed a mean difference of only -2.8% for energy, -9.4% for protein and -8.3% for iron, suggesting mild but systemic over-estimation of intake in the 24hrDR. Other similar tools have reported a similar trend to over-reporting intake (Reilly et al., 2001; Montgomery et al., 2005). Other measures of agreement including intraclass correlation, and quartile cross classification support the conclusion that this tool provides an accurate representation of dietary intake. It is prudent to also examine the limits of agreement of the Bland-Altman analysis, which were acceptable for energy (-1620 to 1322kJ) and protein (-12.6 to 6.7g), but were broader for iron (-3.1mg to 2.3mg). One significant outlier was detected for estimates of iron intake, whose intake was underestimated by 4.99mg. Upon examining this particular child the reason for the discrepancy was uncovered: the child was taking a fortified ready-to-eat nutritional supplement (PlumpyNut), the portion size of which was poorly estimated as it was not decanted from the pack prior to consumption. Highly concentrated sources of nutrients such as PlumpyNut were not

commonly taken in either the validation study (n=1) or the TRACT study (n=0), therefore this extreme source of error is unlikely.

Compared to other similar tools validated in similar populations, the current 24hrDR method performed better in some respects but worse in others. Thakwalakwa et al. (2012) assessed the validity of a 24hrDR tool in Malawian children, however these children were significantly younger than those in the current study, with a mean age of 13.7 months (n=169), compared to roughly 40 months. Therefore some of the issues that affect accuracy are likely to be different, for example younger children cannot provide supporting information but all food and drink is likely to be given by the parent, while older children may provide additional detail but also can obtain food of which the parent is not aware. Thakwalakwa et al. (2012) report a mean over reporting in 24hrDRs compared to weighed food records in energy of 13% (95% CI 7-19%), protein by 20% (95% CI 12-26%), and iron by 34% (95% CI 26-43%), far higher than those in the current study. The limits of agreement reported in the Malawian study, however, are narrower than the current study, likely due to greater numbers of subjects. Intraclass correlation coefficients were better in the current study, consistently achieving excellent reliability (ICC>0.9), compared to moderate (ICC 0.5-0.75) or poor (ICC <0.5) reliability in the study by Thakwalakwa et al. Gewa et al. (2009) compared 24hrDR to WFR in 44 Kenyan school children aged 6-8 years, but Bland-Altman analyses presented by this group are based on normalised data per 4184kJ which is not comparable to the current study. Despite this one can infer from other reported data that 24hrDR underreported on average by 9% energy, 7% protein and 9% iron. The reported iron intakes in this group are notably higher than in the current study (11.0mg vs. 5.5mg), which may reflect regional differences in intake. Gewa et al. also conducted quartile cross-classification analysis, reporting lower levels of correct classification than the current study for energy (29% vs. 79%), protein (48% vs. 89%), and iron (45% vs. 89%). Consequently the reported weighted kappa statistics reported by Gewa et al. show fair to moderate agreement (κ<0.6 in all cases) in contrast to substantial or almost perfect agreement in the current study (κ>0.7 in all cases). Although Gewa et al. (2009) conducted their validation study in school-age children, it appears that it was conducted on days when the child was at home, and recall was based on the mother's recollection of intake.

Due to widely recognised limitations in recall methods 24hrDR can never have total fidelity to actual food eaten, but despite broad limits of agreement this 24hrDR tool performs comparably to weighed food records and favourably to other similar tools reported in the literature.

3.3.2 TRACT DIETARY SUB-STUDY

Summary of main findings:

- Diets of children in Mbale and Soroti lack diversity and are micronutrient poor even when energy and protein intake are adequate
- Regional differences in diet within Eastern Uganda are significant
- Four dietary patterns were identified
- Intakes of haematinic micronutrients (iron, folate, vitamin B12, vitamin A) are poor, but only iron and pantothenate intake appear are associated with haemoglobin recovery
- Dietary intake changes significantly during recovery from acute illness

The primary aims of the sub-study to the TRACT trial were to describe the dietary traits of children in the Eastern Region of Uganda, and to investigate how diet may be associated with recovery from severe anaemia. Prior to this study, information on the diet of children in this region and how this related to health was limited.

The results from the main TRACT trial were not published at the time of preparation of this manuscript, however these were made available to the author and are briefly outlined below for context. In the 30ml/kg (n=1598) vs. 20ml/kg (n=1598) whole blood transfusion comparison, no difference in mortality by day 28 or day 180 was found, although those receiving 30ml/kg recovered faster ($p<0.001$). Compared with 20ml/kg, children with fever who received 30ml/kg transfusions were found to have increased mortality (hazard ratio 1.91, 95% CI 1.04-3.49, p=0.040), while those who had no fever had lower risk of mortality (hazard ratio 0.43, 95% CI 0.27-0.69, p=0.001). In the second transfusion comparison, i.e. immediate transfusion (n=778) vs. triggered transfusion (n=787) in children with less severe anaemia, no significant difference in mortality was found (hazard ratio 0.54, 95% CI 0.22-1.36, p=0.190). 49% of children in the triggered transfusion arm ultimately received some transfusion during the course of the study. Lastly TRACT attempted to identify if post-discharge provision of iron and folate (n=1901) vs. multi micronutrient supplementation (n=1911), and cotrimoxazole prophylaxis (n=1922) vs. none (n=2061) improved outcome. No difference was found in mortality by day 180 in the nutrient intervention arm (hazard ratio 0.97, 95% CI 0.79-1.21, p=0.810), nor in the antibiotic prophylaxis arm (hazard ratio 1.07, 95% CI 0.86-1.32, p=0.560). Rates of hospital readmission were also similar between the post-discharge treatment comparisons.

A large majority (71%) of children recruited to TRACT were diagnosed with malaria on admission, and a significant minority were confirmed by genotyping to be homozygous for sickle cell disease (32% in Mbale, and 25% in Soroti). As there is a background rate of sickle cell disease of only 1-1.2% in the Eastern Region of Uganda (Ndeezi et al., 2016), it is clear that it is an important factor in the

development of severe anaemia for children in this area. Malaria and sickle cell disease were therefore the major causes of severe anaemia in children who were recruited to the TRACT sub-study. Death occurred in 7.7% (n=26/339) of children in this study by day 180, which is far lower than in previous studies of severely anaemia children which report 19% mortality within 6 months (Phiri et al., 2008). While it is outside the scope of this thesis to explain the lower-than-expected mortality rate, the rigorous provision of high standard medical care provided during the TRACT study may partially explain this finding. The low mortality rate also reduced the ability of this sub-study to address differences in mortality arising from diet, however this was not an aim of the sub-study and nor would it be adequately powered to detect such differences. This sub-study makes clear, however, that the infectious and genetic origins of severe anaemia occur on a background of inadequate nutrition.

3.3.2.1 Anthropometry and nutritional risk

The proportion of children in the TRACT sub-study who were considered underweight was greater at all time points than the regional average of 10.0% (Uganda Bureau of Statistics, 2011). On admission the prevalence of underweight was roughly double the background rate (19.8-22.4%), however various factors could contribute to this observation. Published information on nutritional status in Ugandan children is only available for those under 5 years of age and most recently from 2011, while in TRACT a significant proportion of children were school-age (26% in Mbale and 44% in Soroti). The regional information is also appropriately based on a random sampling of healthy and unwell children in Eastern Uganda, whereas the children recruited to TRACT were drawn from a population of acutely unwell children in two specific townships. Stunting was more prevalent in children from Mbale (25.8-32.2%) than those from Soroti (12.6-15.9%) at all time points, and the prevalence in Mbale was also above the local background rate for Eastern Uganda (25.3%). Stunting is reflective of longer term poor access to adequate energy and protein required for growth, whereas underweight and wasting reflect more recent intake. Over time the rates of both underweight and stunting decreased, suggesting improved access to nutrition, and/or the removal of some nutritional stresses such as infection by malaria or helminths. The rate of wasting was observed to increase over time in both sites, rising to 30.5% and 58.4% in Mbale and Soroti by the day 180 review. Wasting in Eastern Uganda is prevalent in 10.0% of the more general population of under 5's. One simple reason for this observation is the improved linear growth observed, meaning that while linear growth is optimised, weight does not appear to approve in line with this.

3.3.2.2 OVERVIEW OF NUTRIENT INTAKE AND REQUIREMENTS

Although median values for energy and protein intake in Mbale appeared adequate, a large proportion of children were not meeting energy requirements (41-58%). Those who were meeting requirements were taking 150% of requirements, while those who were not were taking only 66%. Fewer children were not meeting protein requirements (17-26%), and the difference in intake between those who did meet requirement (267%) and those who did not (59%) was even starker. Far greater numbers of children in Soroti consumed inadequate amounts of energy (up to 93%) and protein (up to 76%), but the difference in intake between those meeting vs. not meeting requirements was not as great. These dietary data partially support the anthropometric data, in that although there were improvements in weight and height, large numbers remained underweight or stunted. On day 28, intakes of energy, protein and all micronutrients were at their highest, but this did not immediately correspond to anthropometric improvements. It is likely that during this period, while the child was still recovering from acute illness, limited growth took place, but catch up growth occurred later as shown by improvement in HAZ by day 180 in both sites. One area of discrepancy is in the lower rate of stunting in Soroti, considering far greater numbers reporting inadequate intakes of energy and protein, which may be due to underreporting in this relatively older group of children. This is not entirely unexplained, as the prevalence of wasting balloons in Soroti up to 58.4% by day 180, suggesting preservation of linear growth at the expense of weight recovery.

A significant contribution of this study was in the generation of a detailed analysis of the dietary features of children in the Eastern Region of Uganda. Median nutritional data and information on the numbers of children with adequate intakes of various nutrients indicate that even when energy and protein intake are adequate, the diet of children in this area is micronutrient poor. Very large variations in intake were observed between children with adequate intakes, and those without. Jariseta et al. (2012) provide the only comparable data on nutrient density for children in Uganda, but not for the Eastern Region. Comparing to the Kampala region, they report similar nutrient densities (within 10%) to the current study for protein and zinc. For iron, vitamin A and folate, they report higher nutrient densities (18%, 74%, and 25%, respectively), while for thiamine, vitamin B6, vitamin B12 and vitamin C they report lower nutrient densities (-63%, -27%, -30%, and -115% respectively). Despite median energy intake in Mbale suggesting adequacy, median micronutrient intake in all but a few cases (thiamine, vitamin B6 and vitamin C) are inadequate. Supplementation from the TRACT study improves micronutrient intakes by day 28 but micronutrient density of the diet remains poor. For a child in Soroti eating a diet with the average local nutrient density meeting the day 180 energy

requirement of 5.1MJ (1219kcal), they would receive 83% of their iron requirement, 58% of zinc requirement, 7% of vitamin A requirement, 102% of vitamin B12 requirement and 58% of folate requirement. It is therefore impossible for children to obtain adequate intakes of many micronutrients even with adequate energy and protein intakes, which few children in this area have.

3.3.2.3 Dietary diversity, food groups and dietary patterns

Food group, diversity and dietary pattern analysis provide further insight. The diets of children in both regions are dominated by a single carbohydrate-rich, micronutrient poor food group 1: grains, roots, tubers and starchy bananas. This food group provides 55% of total energy at day 180 in Mbale and 73% in Soroti. It also provides disproportionately large amounts of micronutrients, in which these foods are relatively poor, e.g. by day 180 51-72% of dietary iron comes from these foods. The dietary data collected also makes clear that parents provide high energy food and drink when children are unwell. In both sites, sweetened and sugary foods make up a larger portion of the diet on admission than at later time points. Although dietary diversity improves as children recover, still approximately 40% of children have diets classified as not diverse (<3 food groups) by day 180. Principal components analysis also helped to qualitatively describe the major dietary patterns in this population group. The dietary pattern describing most variation was DP1, which was mostly associated with intake of food group 1, but also legumes and nuts, and beverages.

An impactful finding of this study was to show that greater dietary diversity was associated with higher haemoglobin concentrations. Each additional food group was associated with 0.71g/dl greater concentration of haemoglobin. While the effect of dietary diversity on mortality cannot be directly addressed in the current study, some comment can be made on the basis of previous research. As previously outlined, Scott et al. (2014) report that in childhood anaemia each 1.0g/dl rise in Hb concentration is associated with 24% reduced risk of mortality. Considering both findings together, a diverse diet consisting of foods from many food groups may be an important factor in reducing the burden of childhood anaemia. Results from the dietary pattern analysis suggest that DP1 (high in starchy staples, legumes, and beverages) was also associated with increased anthropometric measures and haemoglobin (0.63g/dl increase per tertile), however since the dietary pattern was positively associated with nearly all food groups, this is less surprising. One outcome from dietary pattern analysis that is more concerning is that DP3 (high in sweetened foods, fruit and eggs) was negatively associated with Hb (-0.93g/dl Hb per tertile of DP3). To maximise the power to identify associations, all available dietary data was used in these analyses, which does raise some issues. The

inclusion of pre-and post-treatment data will result in some confounding, i.e. the effect may be due to a combination of time, clinical treatment and diet. The statistical significance of the associations with dietary diversity was lost when repeated with latter time points only, but the association remained positive. The dietary patterns found were in some cases associated with a specific time point; for example DP3 was strongly associated with the admission time point: 61% of diets in the highest tertile of DP3 were from admission, compared to 26% from admission in the lowest tertile, χ^2 $p<0.001$.

Although unadjusted for confounders, food group level association with anthropometric status and Hb recovery by day 180 supports the conclusion that certain foods affected outcome. The strongest associations were observed with FG2, legumes and nuts: FG2 was positively associated with Hb recovery by day 180 (OR 1.13, p=0.047), and negatively associated with being underweight (OR 0.92, p=0.017). Legumes and nuts are known to be nutrient dense, providing protein, carbohydrate, dietary fibre, fats, and micronutrients (Polak et al., 2015), and they were the focus of a large WHO programme of research in 2016, the so called "International Year of the Pulse" (Global Pulse Confederation, 2016; Food and Agriculture Organization of the United Nations, 2019). Despite being known to contain anti-nutrients such as phytates which prevent the absorption of some micronutrients, they can be treated to reduce this effect, and indeed are themselves a valuable source of iron. Other associations between outcomes and specific food groups were observed but they were generally weaker. Surprisingly dairy food consumption was associated with greater risk of underweight and wasting (OR 1.02, p<0.05 for both). High intake of food group 1 (grains, roots, tubers and starchy banana) was negatively associated with haemoglobin recovery (OR 0.98, p=0.036). It is possible that this reflects the presence of anti-nutrients in these foods. Nutrient level interactions with haemoglobin recovery were also explored. In the context of the TRACT study, supplementary iron at different doses (10mg vs. 25/60mg) did not appear to affect outcome, but having adequate dietary iron intake did (OR 1.05, 95% CI 1.01-1.08, p=0.006), even if the effect was mild. Although recent studies (van Hensbroek et al., 2010) question the role of iron and folate in the development of severe anaemia, this study reinforces that diet is an important factor in the recovery from severe anaemia. As dietary iron absorption and homeostasis are highly dependent on presence of inflammation, additional parameters could be measured in future including C-reactive protein, interleukins, and TNF-α, and iron regulatory proteins hepcidin, ferritin, transferrin and ferroportin. Some simple dietary information could be provided to the parents of children with severe anaemia to support recovery in the future such as including legumes and nuts.

3.3.2.4 LIMITATIONS

While the 24hr recall method is valid at the population level, it is less accurate on an individual level due to wide limits of agreement. Conducting a larger validation study would improve the confidence in the information generated using this method. The lack of local food composition data also makes precise estimation of dietary intake of many nutrients challenging, since currently almost all composition data is derived from UK or US datasets.

Significant demographic and clinical differences were noted between children from Mbale and Soroti in the TRACT sub-study including age and severity of anaemia. Within the n=339 children recruited to this sub-study of the TRACT trial, it was found that children who presented to Mbale tended to be younger than those in Soroti. Many more children in Soroti were of school age (44.1% vs. 25.9% in Mbale), which was partially responsible for the significant loss of dietary recalls collected at day 28 and day 180, since the attending adult was not with the child during school hours. Along with clear dietary differences, this necessitated analysis of dietary data from each region separately, which consequently reduces power of some analyses. A further consideration relevant for outcome predication is that the potential impact of confounders such as socioeconomic status, mother's education level, local sanitation and availability of clean water sources were not actively adjusted for within regression models. Previous studies in an LMIC setting suggest that **w**ater/sanitation, **a**ssets, **m**aternal education and **i**ncome (or the WAMI index) are strongly associated with overall dietary quality and diversity (Morseth et al., 2017). Dietary quality was thus considered to be a product of these underlying factors in the current study; although this leaves open the possibility that dietary factors found to be associated with anthropometric or haematological outcomes were modifiable by these factors, a simple easy-to-measure assessment (such as dietary assessment) which sums the underlying aspects was deemed to be favourable.

It is also important to consider the temporal relationship between dietary exposure and outcome. This study used a 24hr dietary recall approach to obtain dietary data, which generated information from three specific days during a 6-month recovery period. Inferences drawn on the effect of diet during this period are therefore made with caution. Alternative methods may be used to obtain dietary information pertaining to larger time frames (food frequency questionnaires), but they are also subject to extensive issues with recall and portion size estimation, partially addressed by the approach taken here. A fourth possible occasion at which it would have been possible to collect dietary information was day 90 post discharge, when children finished the nutritional and antibiotic prophylaxis randomisations of TRACT, but this was not undertaken. As a result the period at the end of supplementation was not captured in the sub-study.

Fluid intake was not correctly systematically recorded by all interviewers, therefore it has been omitted as it was not possible to identify whether fluid was not taken, or not recorded.

Due to the particular characteristics of this study population, it was not possible to adequately account for under- or over-reporting, which is a significant limitation in dietary studies. The available methods were not appropriate here, as they are predicated on stable weight adults. Examination of the anthropometric results show that the children were not weight stable, nor were they experiencing stable growth.

3.3.2.5 Strengths

Several resources were developed during the validation study and TRACT sub-study that can facilitate future dietary research in children in sub-Saharan Africa. These resources include the validated 24hr recall method, a Dietplan coding protocol, local staff trained in nutrition/dietary research, collection of regional recipes, and weight/volume conversion method. Many local foods and compound dishes have been fully described.

The TRACT dietary sub-study represents a novel dietary dataset with geographical specificity, in a population whose diet has previously not been described in detail. The large number of children included, in addition to the repeated collection of dietary data, ensures that this is a representative sample of children's diets both when critically ill (admission), in recovery (day 28) and a more typical diet (day 180). A number of approaches were taken to characterise the diets fully, including nutrient level analysis, nutrient density, food group level analysis, diversity scores, and dietary pattern analysis. Food group and dietary patterns revealed dietary changes associated with critical illness that were previously anecdotal e.g. the provision of high quantities of sugar and sugary beverages when children were ill. As will be discussed later, this may be a counterproductive dietary intervention in the context of metabolic and gut microbial changes in critical illness. This study provides an important, previously unavailable, reference for future studies of children in this region. It can also provide the basis for comparative analyses of diets between children from different sub-regions within sub-Saharan Africa.

3.3.2.6 Conclusion and future work

This study generated significant understanding about the diets of children in Eastern Uganda, and suggested that poor quality diets lacking in diversity lead to poorer recovery from severe anaemia. Although interactions with medical treatments are likely, it is important to note that the background

nutritional status will affect response to all other treatments. Targeting nutritional supplementation to children with confirmed dietary inadequacy or insufficiency, and providing information and education on locally acceptable dietary approaches may result in a clearer benefit. Future studies should include diet as an important indicator and correlate of overall health in children in the context of critical illness.

4. Chapter 4 – Metabolic Profiling and Gut Microbiota in Severe Anaemia

4.1 Introduction

The thematic background and relevant overview of scientific literature to support this chapter can be found in Chapter 1, sections 1.3 and 1.4, while the full description of the methods used can be found in Chapter 2, sections 2.4 and 2.5.

Chapter 4 reports the results of ^{1}H NMR spectrometry-based urinary and plasma metabolic profiling, and 16s rRNA metataxonomic profiling of gut microbiota in children being treated for severe anaemia recruited to the TRACT study. Results of multivariate statistical models of urine and plasma ^{1}H NMR spectroscopy experiments are presented which summarise model statistics and associated metabolites. Metabolites which could be identified using statistical methods as described in Chapter 2, i.e. 2D J-resolved spectra, subset optimisation by reference matching (STORM) and statistical correlation spectroscopy (STOCSY) and internal and internet (Human Metabolome Database) reference databases are listed as: name of metabolite (chemical shift and multiplicity from multivariate model followed by correlated chemical shifts from STORM/STOCSY). The first chemical shift quoted has a minimum correlation coefficient (r) of greater than 0.5, unless otherwise stated, and this is reported. For example isoleucine (δ0.96t, δ1.03d) r=0.71.

Faecal metataxonomics analyses are presented as non-metric dimensional scaling (NMDS) plots to visually examine the similarity of samples with regards to the taxa of bacteria present in different proportions. NMDS plots are also presented with permutational analysis of variance (PERMANOVA) statistical test results. Where >2 groups were analysed within the same model e.g. admission, day 28 and day 180, the p-value is corrected for multiple testing by Benjamini-Hochberg false discovery rate (FDR). Where NMDS and PERMANOVA reveal significant differences between groups of samples, the taxa responsible for the differences are presented in White's non-parametric t-test extended error bar charts at different taxonomic levels where appropriate. Lastly richness and diversity comparisons are presented. Methods for this Chapter are outlined in Chapter 2, section 2.4 (^{1}H NMR spectroscopy) and section 2.5 (16S rRNA profiling).

Aims

- To describe the changes in the urine and plasma metabolomes of children recovering from severe anaemia
- To assess the association between diet and metabolic profiles

- To describe the gut microbiota and changes in gut microbial population associated with children recovering from severe anaemia
- To assess the association between diet and gut microbial profiles

Hypotheses

- Children with severe anaemia have distinct metabolic signatures related to the aetiology of their severe anaemia, which resolve with treatment
- Children with severe anaemia have altered gut microbial populations favouring pathogenic species
- Diet has a significant effect on both metabolism and the gut microbiota in children with severe anaemia

4.2 Results

For reference, Table 4.1 provides a brief summary of admission characteristics for children recruited to the TRACT sub-study. A more detailed overview of characteristics are provided in Chapter 3. A minority of children were aged under 24 months (13.3%), while the majority were aged 2-5 years (52.5%) with the remainder being above 5 years of age. Malaria was highly prevalent on admission with 71.7% of children being diagnosed. A large proportion of children were also confirmed by genotyping as having sickle cell disease (28.9%). Randomisation to the TRACT treatment arms was well balanced in all cases, however it is noted that during the period of recruitment to the sub-study less children with milder or uncomplicated anaemia were recruited and randomised to TRACT B.

Table 4.1 Admission characteristics and randomisations for TRACT study (n = 339)

Characteristic	
Site (Mbale:Soroti)	182:157
Age (months)	
% Under 24	13.3
% 24-60	52.5
% Over 60	34.2
Gender (% male)	57.2
Malaria (% Present)	71.7
Sickle Cell Disease (% Present)	28.9
TRACT Randomisation	
TRACT A (20ml/kg:30ml/kg)	135:133
TRACT B (Transfusion:None)	33:38
Nutritional strategy (IF:MVMM)	175:164
Cotrimoxazole (No:Yes)	160:179

4.2.1 Urine metabolomics

^1H NMR spectra were acquired for 625 urine samples from n=256 children (n=130 from Mbale and n=126 from Soroti), in addition to n=32 pooled quality control samples. A total of n=26/625 spectra were determined to be extreme outliers in initial PCA analysis, falling outside the Hotelling's T^2 95% CI ellipse, therefore these were excluded from further analysis, leaving 599 remaining spectra. A PCA plot of all urine samples displaying tight clustering of the quality control samples is presented in Appendix X, implying good instrumental stability throughout the experiment.

4.2.1.1 Site-related differences

Table 4.2 shows OPLS-DA models assessing urine metabolome differences between children from Mbale and Soroti. All models generated had low-moderate Q^2 scores (0.255-0.308), but significant CV-ANOVA p-values (<0.001). An unknown metabolite (δ1.14s, δ3.49d, δ3.60s, δ3.61d, δ3.69dd, δ3.85dd, *r*=0.57) was responsible for class separation particularly at day 28, being strongly associated with day 28 urine samples from children from Soroti when compared to those from Mbale (*r*=0.57). 4-hydroxyphenylacetic acid (δ6.87d, δ6.98d), was also associated with Soroti urine samples at day 28, *r*=0.51. Creatinine (δ4.06s) was associated with samples from Mbale when all time points were combined, however the model had low Q^2 (0.262) and the correlation coefficient was also low (*r*=0.31). At day 180, hippuric acid (δ7.45t, δ7.55t, δ7.65d, δ8.53s, δ3.97d) was associated with urine samples from children from Soroti, *r*=0.51.

Table 4.2 Site differences in urine metabolome of children in TRACT (n=599)

	OPLS-DA			
	All time points	Day 0	Day 28	Day 180
n (Mbale:Soroti)	326:273	130:126	110:72	86:75
Components	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth
R^2Y	0.466	0.620	0.711	0.645
Q^2Y	0.262	0.266	0.308	0.255
CV-ANOVA p-value	<0.001	<0.001	<0.001	<0.001
Metabolite	Associated Site (*r*)			
Unknown 1 (δ1.14s, δ3.49d, δ3.60s, δ3.61d, δ3.69dd, δ3.85dd)	Soroti ↑ (0.36)		Soroti ↑ (0.57)	
Creatinine (δ4.06s)	Mbale ↑ (0.31)			
4-hydroxyphenylacetic acid (δ 6.87, δ6.98d)			Soroti ↑ (0.51)	
Hippuric acid (δ7.45t, δ7.55t, δ7.65d, δ8.53s, δ3.97d)				Soroti ↑ (0.51)

Abbreviations: OPLS-DA, orthogonal partial least squares discriminant analysis; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance

4.2.1.2 Baseline characteristics' effects on urine metabolome

Associations between baseline characteristics and admission urine metabolome are summarised in Table 4.3. Models investigating gender, underweight and WAZ, stunting, wasting and WHZ, temperature and fever, white blood cell count and leucocytosis, and malaria all produced negative Q^2 scores so were determined not to be valid. Models investigating age and age group, height-for-age z-score and presence of sickle cell anaemia were all of low predictive ability (Q^2 0.095, 0.105, 0.040, and 0.165 respectively), therefore discriminant metabolites were not explored.

Table 4.3 Summary of OPLS and OPLS-DA models assessing association between baseline characteristics with admission urine metabolome (n=256)

	Details of Model					
Characteristic	OPLS or OPLS-DA	n	Components	R^2	Q^2	CV-ANOVA p-value
Age (months)	OPLS	256	1Pred + 1Orth	0.601	0.105	0.040
Age Group (<60 months vs >60 months)	OPLS-DA	162:94	1Pred + 1Orth	0.585	0.095	0.051
Gender (male : female)	OPLS-DA	146:110	1Pred + 1Orth	0.563	-0.173	1.000
Weight-for-age z-score (WAZ)	OPLS	248	1Pred + 1Orth	0.544	-0.102	1.000
Underweight : Not underweight	OPLS-DA	69:179	1Pred + 1Orth	0.585	-0.090	1.000
Height-for-age z-score (HAZ)	OPLS	254	1Pred + 1Orth	0.553	0.040	0.057
Stunted : Not stunted	OPLS-DA	58:196	1Pred + 1Orth	0.602	-0.090	1.000
Weight-for-height z-score (WHZ)	OPLS	164	1Pred + 1Orth	0.617	-0.265	1.000
Wasted : Not wasted	OPLS-DA	36:128	1Pred + 1Orth	0.641	-0.181	1.000
Temperature (^{0}C)	OPLS	256	1Pred + 1Orth	0.560	-0.240	1.000
Fever : No fever	OPLS-DA	97:159	1Pred + 1Orth	0.569	-0.197	1.000
White blood cell count (g/dl) (WBC)	OPLS	248	1Pred + 1Orth	0.530	-0.267	1.000
Leucocytosis : Normal WBC	OPLS-DA	141:107	1Pred + 1Orth	0.552	-0.134	1.000
Malaria : No malaria	OPLS-DA	172:84	1Pred + 1Orth	0.577	-0.002	1.000
Sickle cell anaemia : no sickle cell anaemia	OPLS-DA	89:167	1Pred + 1Orth	0.609	0.165	<0.001

Abbreviations: OPLS-DA, orthogonal partial least squares discriminant analysis; MVMM, multivitamin multi-mineral; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance

Definitions: Underweight, WAZ <-2; Stunted, HAZ <-2; Wasted, WHZ <-2; Fever, axillary temperature >37.5^{0}C; Leucocytosis, WBC>12x10^9/L; Malaria diagnosis confirmed by malaria parasite presence on microscopy; Sickle cell anaemia diagnosis confirmed by genotyping AS or SS; No sickle cell anaemia confirmed by genotyping AA

4.2.1.3 Urine metabolome change over time

As site differences in urine metabolome were uncovered (section 4.2.1.2 above) changes occurring in each site over time were explored separately. These results are summarised in Table 4.4 (Mbale) and Table 4.5 (Soroti) below. In children from Mbale, only N-acetylcysteine (δ2.01s, δ4.40t), *r*=0.65, was found to be associated with later time points in the OPLS regression model (R^2 0.598, Q^2 0.317, CV-ANOVA p<0.001). In the OPLS-DA model directly comparing admission and day 28 samples in Mbale (R^2 0.747, Q^2 0.522, CV-ANOVA <0.001), L-lysine (δ1.98m), N-acetylcysteine (δ2.01s), and glutamine (δ2.1m) were associated with day 28 samples, while only glucose (δ5.22) was associated with admission samples. An OPLS-DA model comparing admission urine samples to those from day 180 corroborated the above findings, but also indicated that N-acetylaspartic acid (δ2.04s), citrate (δ2.55d, δ2.66d), glycolic acid (δ3.95s), and hippuric acid (δ 3.97d, δ7.45t, δ7.55t, δ7.65d, δ8.53s) were discriminant for day 180, while 3-hydroxybutyric acid (δ0.90m, δ1.33d, δ4.12m) was associated with admission samples. No metabolites were found to be discriminant between day 28 and day 180 samples.

Similar trends were found in samples from Soroti (Table 4.5). 3-hydroxybutyric acid was associated with admission samples in all valid models, however glucose was not associated with admission samples in any model. In the OPLS regression model comparing samples from all three time points, L-lysine (*r*=0.62), N-acetylaspartic acid (*r*=0.57), N-acetylcysteine (*r*=0.57) and hippuric acid (*r*=0.54) were associated with later time points. In addition to the above metabolites, when compared directly with admission samples, day 28 samples were also associated with the unknown compound noted in 4.2.1.2 above (*r*=0.53), trimethylamine (δ2.93s; *r*=0.56), and trigonelline (δ4.44s, δ8.08m, δ8.84m, δ9.13s; *r*=0.56). When directly compared with admission samples, day 180 samples were associated with all of the above metabolites associated with later time points in OPLS regression and with day 28 in OPLS-DA, and also leucine (δ0.99t; *r*=0.58).

Table 4.4 Urine metabolome changes over time in children from Mbale (n=325)

	OPLS Regression	OPLS-DA		
	All Time Points	Day 0 vs Day 28	Day 0 vs Day 180	Day 28 vs Day 180
Time point (n:n)	325	129:111	129:85	111:85
Components	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth
R^2	0.598	0.747	0.785	0.602
Q^2	0.317	0.522	0.588	0.156
CV-ANOVA p-value	<0.001	<0.001	<0.001	0.002
Metabolite	Earlier/Later (*r*)	Time Point (*r*)	Time Point (*r*)	Time Point (*r*)
3-hydroxybutryic acid (δ0.90m, δ1.33d, δ4.12m)			Day 0 ↑ (0.52)	-
L-lysine (δ1.98m)		Day 28 ↑ (0.64)	Day 180 ↑ (0.70)	-
N-acetylaspartic acid (δ2.04s)			Day 180 ↑ (0.56)	-
N-acetylcysteine (δ2.01, δ4.40t)	Later ↑ (0.65)	Day 28 ↑ (0.62)	Day 180 ↑ (0.72)	-
Glutamine (δ2.1m)		Day 28 ↑ (0.51)	Day 180 ↑ (0.70)	-
Citrate (δ2.55, δ2.66)			Day 180 ↑ (0.51)	-
Glycolic acid (δ3.95s)			Day 180 ↑ (0.55)	-
Glucose (δ5.22d)		Day 0 ↑ (0.52)	Day 0 ↑ (0.53)	-
Hippuric acid (δ7.45t, δ7.55t, δ7.65d, δ8.53s, δ3.97d)			Day 180 ↑ (0.55)	-

Abbreviations: OPLS, orthogonal partial least squares; OPLS-DA, orthogonal partial least squares discriminant analysis; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance; *r*, correlation coefficient

Table 4.5 Urine metabolome changes over time in children from Soroti (n=274)

	OPLS Regression	OPLS-DA		
	All Time Points	Day 0 vs Day 28	Day 0 vs Day 180	Day 28 vs Day 180
Time point (n:n)	274	129:71	129:74	71:74
Components	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth
R^2	0.575	0.751	0.766	0.706
Q^2	0.312	0.579	0.637	-0.001
CV-ANOVA p-value	<0.001	<0.001	<0.001	1.000
Metabolite	Earlier/Later (*r*)	Time Point (*r*)	Time Point (*r*)	Time Point (*r*)
3-hydroxybutryic acid (δ0.90m, δ1.33d, δ4.12m)	Earlier ↑ (0.55)	Day 0 ↑ (0.54)	Day 0 ↑ (0.57)	-
Leucine (δ0.99t)			Day 180 ↑ (0.58)	-
Unknown 1 (δ1.14s, δ3.49d, δ3.60s, δ3.61d, δ3.69dd, δ3.85dd)		Day 28 ↑ (0.53)	Day 180 ↑ (0.54)	-
L-lysine (δ1.98m)	Later ↑ (0.62)	Day 28 ↑ (0.56)	Day 180 ↑ (0.68)	-
N-acetylaspartic acid (δ2.04s)	Later ↑ (0.57)	Day 28 ↑ (0.60)	Day 180 ↑ (0.56)	-
N-acetylcysteine (δ2.01, δ4.40t)	Later ↑ (0.57)		Day 180 ↑ (0.64)	-
Dimethylamine (δ2.75s)			Day 180 ↑ (0.54)	-
Trimethylamine (δ2.93s)		Day 28 ↑ (0.56)	Day 180 ↑ (0.52)	-
Trigonelline (δ4.44s, δ8.08m, δ8.84m, δ9.13s)		Day 28↑ (0.56)	Day 180 ↑ (0.52)	-
Hippuric acid (δ7.45t, δ7.55t, δ7.65d, δ8.53s, δ3.97d)	Later ↑ (0.54)	Day 28 ↑ (0.55)	Day 180 ↑ (0.65)	-

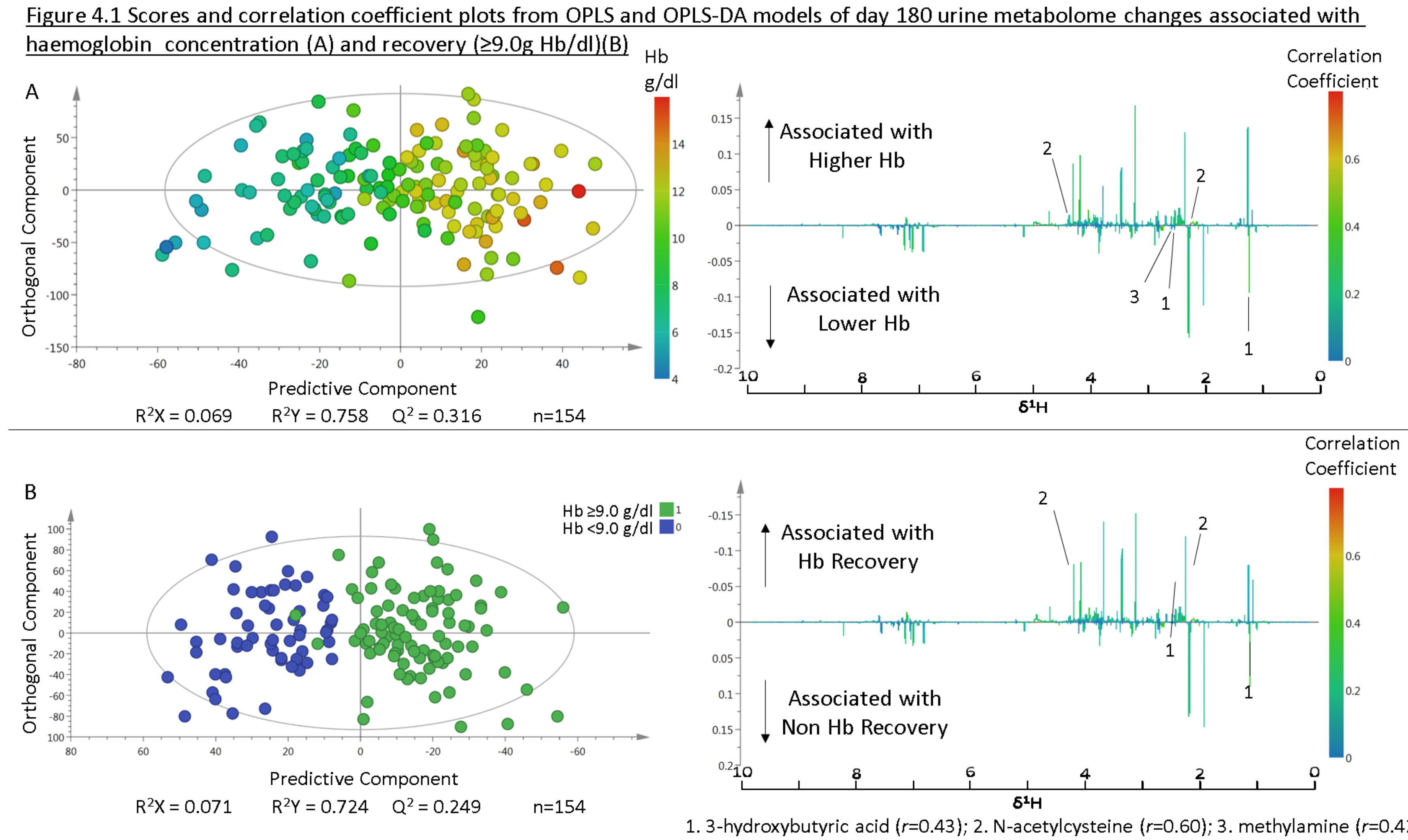
Abbreviations: OPLS, orthogonal partial least squares; OPLS-DA, orthogonal partial least squares discriminant analysis; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance

4.2.1.4 *TRACT INTERVENTIONS AND HAEMOGLOBIN RECOVERY*

Table 4.6 summarises OPLS-DA models comparing the effect of TRACT randomisations on urine metabolome at day 28. Transfusion strategies (30ml/kg vs. 20ml/kg in TRACT arm A, and 30/20ml/kg vs. no transfusion in TRACT arm B), cotrimoxazole prophylaxis vs. none, and iron-folate vs. multivitamin multi-mineral supplementation had no discernible effect on urine metabolome.

While no direct effects of TRACT interventions were observed, OPLS regression of haemoglobin concentration at day 180 was associated with changes in urine metabolome (R^2 0.758, Q^2 0.316, CV-ANOVA $p<0.001$), as was OPLS-DA comparing children whose haemoglobin had recovered sufficiently to those who had not (R^2 0.724, Q^2 0.249, CV-ANOVA $p<0.001$), though this model had less predictive ability than the regression model (Table 4.7). This was further investigated (Figure 4.1), and elevated 3-hydroxybutyric acid was associated with failure to recover haemoglobin concentration by day 180 (r=0.43), while N-acetylcysteine was positively associated with recovery (r=0.60). In the OPLS regression model (Figure 4.1 panel A) methylamine was also associated (r=0.42) with non-recovery at day 180. Models were constructed evaluating whether admission urine metabolome had any relation with subsequent mortality, however since all models generated were invalid (low or negative Q^2), they are omitted for brevity.

Figure 4.1 Scores and correlation coefficient plots from OPLS and OPLS-DA models of day 180 urine metabolome changes associated with haemoglobin concentration (A) and recovery (≥9.0g Hb/dl)(B)



1. 3-hydroxybutyric acid (*r*=0.43); 2. N-acetylcysteine (*r*=0.60); 3. methylamine (*r*=0.42)

Table 4.6 Summary of OPLS-DA models comparing TRACT interventions' effects on urine metabolome at day 28 (n=179)

	OPLS-DA Model			
	TRACT A 30ml vs 20ml transfusion	TRACT B 30/20ml transfusion vs none	Cotrimoxazole vs None	Iron-Folate vs MVMM Supplementation
n:n	81:67	15:16	82:97	97:82
Components	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth
R^2	0.699	0.949	0.614	0.684
Q^2	-0.082	0.068	0.051	-0.111
CV-ANOVA p-value	1.000	0.766	0.057	1.000

Abbreviations: OPLS-DA, orthogonal partial least squares discriminant analysis; MVMM, multivitamin multi-mineral; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance

Table 4.7 Summary of OPLS and OPLS-DA models examining relationship between haemoglobin and urine metabolome across all time points (n=599)

	Details of Model					
	OPLS or OPLS-DA	n	Components	R^2	Q^2	CV-ANOVA p-value
Admission Haemoglobin	OPLS	264	1 Pred + 1 Orth	0.525	-0.179	1.000
Day 28 Haemoglobin	OPLS	181	1 Pred + 1 Orth	0.689	0.183	<0.001
Not recovered : Recovered	OPLS-DA	77:104	1 Pred + 1 Orth	0.673	0.109	<0.001
Day 180 Haemoglobin	OPLS	154	1 Pred + 1 Orth	0.758	0.316	<0.001
Not recovered : Recovered	OPLS-DA	61:101	1 Pred + 1 Orth	0.724	0.249	<0.001

Abbreviations: OPLS-DA, orthogonal partial least squares discriminant analysis; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance; Recovery defined as haemoglobin ≥ 9.0g/dl

4.2.1.5 DIETARY EFFECTS ON URINE METABOLOME

To investigate the association of dietary intake with urinary metabolome, dietary diversity score (DDS), dietary pattern scores (DP), and food group (FG) intake in grams all generated in Chapter 3 above were used to construct OPLS regression and discriminant analysis models. In order to detect dietary effects, urine samples from all time points and sites were included to maximise the available number of spectra. Neither OPLS regression with dietary diversity scores, nor OPLS discriminant analysis comparing diets classified as ‘diverse’ (DDS≥4) to ‘not diverse’ (DDS<4) resulted in invalid multivariate models (Q^2 0.001 and -0.062 respectively) suggesting no effect on urinary metabolome. Regression of scores in dietary pattern 1 (characterised by high intake of carbohydrate-rich staples, legumes and beverages) and discriminant analysis comparing those scoring in the highest and lowest tertiles resulted in models with low Q^2 scores (Q^2 0.124 in the regression model, and Q^2 0.174 in the discriminant model). Using scores from other dietary patterns to examine any effect of diet on urine metabolome also generated models with low or negative Q^2 scores (Q^2 <0.023 in all other cases), suggesting poor predictive ability and model invalidity. In summary the dietary patterns and the foods with which they were most strongly associated were: DP1 “Carbohydrate-rich staples, legumes and beverages”, DP2 “Meat and carbohydrate-rich staples”, DP3 “Sweetened foods/drinks, fruit, eggs”, DP4 “Milk-based, with low fruit and vegetable intake”.

Individual food group intakes in grams were used to assess if food group intake had any effect on urinary metabolome. Where sufficient numbers of children reported intake of a food, discriminant analyses compared those with the highest and lowest intakes, i.e. tertile 1 vs. tertile 3 (FG1 grains, roots, tubers and starchy banana, FG7 “other” fruit and vegetables, FG8 sugar and highly sweetened foods, and FG9 “other”). Where this was not possible due to high numbers of children reporting no intake of a food group (as for FG2 legumes and nuts, FG3 dairy products, FG4 flesh foods, FG5 eggs, and FG6 vitamin A-rich fruit and vegetables), discriminant analysis compared those who reported any intake to those who reported no intake of the foods. No multivariate model using food group data had sufficiently high predictive ability, or model validity, to warrant further investigation of discriminant metabolites.

Table 4.8 Summary of OPLS and OPLS-DA models examining the effect of diet on urine metabolome across all time points (n=519)

Dietary factor	Details of Model					
	OPLS or OPLS-DA	n	Components	R^2	Q^2	CV-ANOVA p-value
Dietary diversity score*	OPLS	519	1 Pred + 1 Orth	0.353	0.001	0.971
Diverse (DDS<4) : not diverse (DDS≥4)*	OPLS-DA	215:304	1 Pred + 1 Orth	0.366	-0.062	1.000
Dietary Pattern 1	OPLS	519	1 Pred + 1 Orth	0.409	0.124	<0.001
T1 : T3	OPLS-DA	175:178	1 Pred + 1 Orth	0.507	0.174	<0.001
Dietary Pattern 2	OPLS	519	1 Pred + 1 Orth	0.394	-0.150	1.000
T1 : T3	OPLS-DA	175:179	1 Pred + 1 Orth	0.462	-0.120	1.000
Dietary Pattern 3	OPLS	519	1 Pred + 1 Orth	0.386	-0.008	1.000
T1 : T3	OPLS-DA	161:185	1 Pred + 1 Orth	0.487	0.023	0.095
Dietary Pattern 4	OPLS	519	1 Pred + 1 Orth	0.404	-0.127	1.000
T1 : T3	OPLS-DA	169:171	1 Pred + 1 Orth	0.501	-0.119	1.000
Grains, roots and tubers (FG1)	OPLS	519	1 Pred + 1 Orth	0.409	-0.108	1.000
T1 : T3	OPLS-DA	164:163	1 Pred + 1 Orth	0.419	-0.025	1.000
Legumes and nuts (FG2)*	OPLS	519	1 Pred + 1 Orth	0.434	0.086	0.056
No intake : Any intake	OPLS-DA	332:187	1 Pred + 1 Orth	0.428	0.050	0.088
Dairy products (FG3)	OPLS	519	1 Pred + 1 Orth	0.444	-0.066	1.000
No intake : Any intake	OPLS-DA	428:91	1 Pred + 1 Orth	0.390	-0.010	1.000
Flesh foods (FG4)	OPLS	519	1 Pred + 1 Orth	0.418	-0.234	1.000
No intake : Any intake	OPLS-DA	245:274	1 Pred + 1 Orth	0.450	-0.272	1.000
Eggs (FG5)	OPLS	519	1 Pred + 1 Orth	0.390	-0.211	1.000
No intake : Any intake	OPLS-DA	479:40	1 Pred + 1 Orth	0.388	-0.163	1.000
Vitamin A-rich fruit and vegetables (FG6)	OPLS	519	1 Pred + 1 Orth	0.358	-0.070	1.000
No intake : Any intake	OPLS-DA	320:199	1 Pred + 1 Orth	0.373	-0.014	1.000
Other fruit and vegetables (FG7)*	OPLS	519	1 Pred + 1 Orth	0.350	-0.190	1.000
T1 : T3	OPLS-DA	348:171	1 Pred + 1 Orth	0.422	-0.154	1.000
Sugar and highly sweetened food (FG8)	OPLS	519	1 Pred + 1 Orth	0.428	-0.011	1.000
T1 : T3*	OPLS-DA	166:170	1 Pred + 1 Orth	0.466	0.043	0.060
Other (FG9)	OPLS	519	1 Pred + 1 Orth	0.422	0.045	0.097
T1 : T3*	OPLS-DA	168:170	1 Pred + 1 Orth	0.463	0.006	0.757

Abbreviations: OPLS-DA, orthogonal partial least squares discriminant analysis; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance; DDS, dietary diversity score; DP, dietary pattern; T#, tertile; FG, food group
* indicates models where the addition of the orthogonal component decreased model predictive ability (Q^2)

4.2.2 Plasma metabolomics

^{1}H NMR spectra were acquired for 574 plasma samples from n=212 children (n= 133 from Mbale and n=79 from Soroti), in addition to n=31 pooled quality control samples. A total of n=31/574 spectra were determined to be extreme outliers (outside Hotelling's T^2 95% CI limit) in initial PCA analysis, therefore these were excluded from further analysis, leaving 543 remaining spectra. The principal causes for exclusion were poor quality spectra (peak width at half height of >1.3Hz) and the presence of EDTA in 13 samples, indicating the incorrect tubes were used for sample collection. Appendix X displays a PCA plot of all samples plus tight clustering of quality controls, implying good instrumental stability throughout the experiment.

4.2.2.1 Site-related differences

Table 4.9 shows differences detected in the plasma metabolome of children from Mbale and Soroti sites at each time point and across all time points combined. All models generated were considered valid and had moderate to high Q^2 scores ranging from 0.469 to 0.776. At all time points and when all samples were considered together, children from Mbale were associated with greater concentrations of VLDL/LDL cholesterol (δ1.26 broad and δ2.02m; r>0.60) and phosphorylcholine (δ3.25s, δ5.27-5.34 broad, r>0.55). Admission samples from Mbale were also associated with glutamate (δ2.46m, r=0.53). Samples from all time points in Soroti were associated with higher sphingomyelin concentrations (r>0.65). At days 28 and 180, samples from Soroti were associated with higher glucose (r=0.73) and valine (r=0.56-0.72) concentrations, while phenylalanine (r=0.60) and 3-OH-butyric acid (r=0.73) concentrations were higher in Soroti at day 180 only.

Table 4.9 Site differences in plasma metabolome of children in TRACT (n=543)

	OPLS-DA			
	All time points	Day 0	Day 28	Day 180
n (Mbale:Soroti)	363:180	128:61	119:60	116:59
Components	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth
R^2Y	0.740	0.690	0.905	0.794
Q^2Y	0.661	0.469	0.776	0.713
CV-ANOVA p-value	<0.001	<0.001	<0.001	<0.001
Metabolite	Associated Site (*r*)			
Cholesterol (VLDL/LDL) (δ1.26 broad, δ2.02m)	Mbale ↑ (0.60)	Mbale ↑ (0.60)	Mbale↑ (0.66)	Mbale ↑ (0.76)
Acetate (δ1.94s)			Soroti ↑ (0.64)	
Glutamate (δ2.46m)		Mbale ↑ (0.53)		
Sphingomyelin (δ3.23s)	Soroti ↑ (0.72)	Soroti ↑ (0.65)	Soroti ↑ (0.78)	Soroti ↑ (0.66)
Glucose (δ3.55d, δ5.26d)	Soroti ↑ (0.69)		Soroti ↑ (0.73)	Soroti ↑ (0.73)
Valine (δ3.60d, δ1.01d, δ1.06d)	Soroti ↑ (0.65)		Soroti ↑ (0.72)	Soroti ↑ (0.56)
3-OH-butyric acid (δ4.10m, δ1.2d)	Soroti ↑ (0.66)			Soroti ↑ (0.73)
Phosphorylcholine (δ5.27-5.34 broad, δ3.25s)	Mbale ↑ (0.55)	Mbale↑ (0.58)	Mbale ↑ (0.69)	Mbale ↑ (0.78)
Phenylalanine (δ7.35d, δ7.37m, δ7.44m)				Soroti ↑ (0.60)

Abbreviations: OPLS-DA, orthogonal partial least squares discriminant analysis; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance; VLDL, very low density lipoprotein; LDL, low density lipoprotein

4.2.2.2 Baseline characteristics' effects on plasma metabolome

OPLS regression and OPLS-DA models exploring the effects of baseline differences in subject characteristics (age, gender), anthropometry (WAZ, HAZ, WHZ), clinical parameters (temperature and white blood cell count), malaria status, and sickle cell anaemia status on admission plasma metabolic profiles are summarised in Table 4.10. Low or negative Q^2 scores and insignificant CV-ANOVA p-values (>0.05) were found in all instances, except for sickle cell status. The OPLS-DA model comparing children with genotype-confirmed sickle cell disease (SS) to non-carriers (AA) was found to have a Q^2 value of 0.192 and CV-ANOVA p value <0.001, therefore assessment of discriminant metabolites was undertaken. These results are summarised in Figure 4.2, and show that higher plasma concentrations of the phosphorylcholine (r=0.60) were associated with sickle cell disease, while a negative sickle cell status was associated with higher concentrations of the branched chain amino acids (isoleucine, leucine, valine, r=0.41-0.59) and phenylalanine (r=0.54).

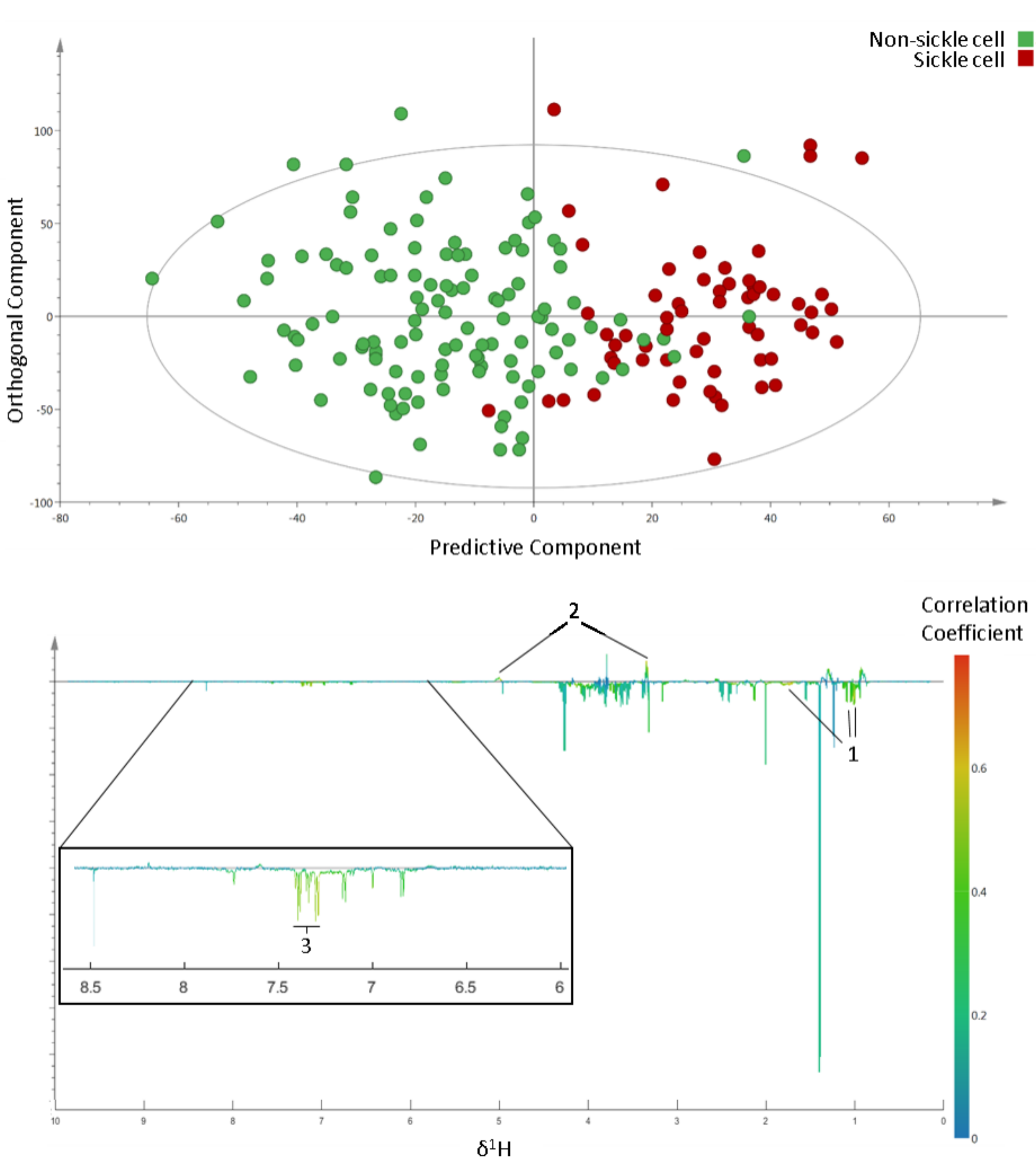
Table 4.10 Summary of OPLS and OPLS-DA models assessing association between baseline characteristics with plasma metabolome (n=189)

Characteristic	Details of Model					
	OPLS or OPLS-DA	n	Components	R^2	Q^2	CV-ANOVA p-value
Age (months)	OPLS	189	1 Pred + 1 Orth	0.644	0.058	0.887
Age Group (<60 months vs >60 months)	OPLS-DA	122:67	1 Pred + 1 Orth	0.642	-0.017	1.000
Gender (male : female)	OPLS-DA	97:92	1 Pred + 1 Orth	0.604	-0.171	1.000
Weight-for-age z-score (WAZ)	OPLS	183	1 Pred + 1 Orth	0.593	-0.086	1.000
Underweight : Not underweight	OPLS-DA	49:134	1 Pred + 1 Orth	0.667	-0.140	1.000
Height-for-age z-score (HAZ)	OPLS	186	1 Pred + 1 Orth	0.678	-0.158	1.000
Stunted : Not stunted	OPLS-DA	48:138	1 Pred + 1 Orth	0.683	-0.063	1.000
Weight-for-height z-score (WHZ)	OPLS	120	1 Pred + 1 Orth	0.763	0.039	0.317
Wasted : Not wasted	OPLS-DA	20:100	1 Pred + 1 Orth	0.817	0.031	0.445
Temperature (^{0}C)	OPLS	189	1 Pred + 1 Orth	0.823	-0.101	1.000
Fever : No fever	OPLS-DA	81:108	1 Pred + 1 Orth	0.708	-0.115	1.000
White blood cell count (g/dl) (WBC)	OPLS	183	1 Pred + 1 Orth	0.584	0.092	0.963
Leucocytosis : Normal WBC	OPLS-DA	96:87	1 Pred + 1 Orth	0.631	0.009	0.829
Malaria : No malaria	OPLS-DA	137:52	1 Pred + 1 Orth	0.590	0.076	0.972
Sickle cell anaemia : no sickle cell anaemia	OPLS-DA	59:116	1 Pred + 1 Orth	0.606	0.192	<0.001

Abbreviations: OPLS-DA, orthogonal partial least squares discriminant analysis; MVMM, multivitamin multi-mineral; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance

Definitions: Underweight, WAZ <-2; Stunted, HAZ <-2; Wasted, WHZ <-2; Fever, axillary temperature >37.5^{0}C; Leucocytosis, WBC >12x10^9/L; Malaria diagnosis confirmed by malaria parasite presence on microscopy; Sickle cell anaemia diagnosis confirmed by genotyping AS or SS; No sickle cell anaemia confirmed by genotyping AA

Figure 4.2 Scores and correlation coefficient loading plot from OPLS-DA model comparing admission ^{1}H NMR plasma metabolic profiles of children with sickle cell anaemia (n=59) vs. those without (n=116), R^2 0.606 Q^2 0.192, CV-ANOVA <0.001



Metabolites (chemical shift/s; correlation coefficient): **1** – Branched chain amino acids leucine (δ0.99t, δ1.76m; *r*=0.59), isoleucine (δ0.96t, δ1.03d; *r*=0.41) and valine (δ 1.01d, δ 1.06d; *r*=0.45), **2** – phosphorylcholine (δ3.25s, δ5.27-2.34 broad; *r*=0.60), **3** – phenylalanine (δ7.35d, δ7.37m, δ7.44m; *r*=0.54)

4.2.2.3 Plasma Metabolome Change Over Time

Tables 4.11 and 4.12 display changes in the plasma metabolome over time from OPLS regression and discriminant analysis models in Mbale and Soroti respectively. In Mbale, OPLS regression (R^2 0.501, Q^2 0.355, CV-ANOVA $p<0.001$) showed that later time points were associated with higher concentrations of lipid transport proteins including low density lipoprotein (LDL) cholesterol (r=0.57) and very low density lipoprotein (VLDL) cholesterol (r=0.54), in addition to an unknown lipid species (δ3.23-3.25 broad, r=0.61). Conversely triacylglyceride (δ1.55-1.61 broad, δ2.22-2.25 broad) was found to be associated with earlier time points (r=0.51). Both LDL and VLDL cholesterol were higher in day 28 and day 180 samples when directly compared to admission samples, but when the two later time points were compared, levels of these compounds were found to be higher at day 28 compared to day 180 (r=0.63 and r=0.62 for LDL and VLDL in OPLS-DA model). Phosphorylcholine (δ3.25s, δ 5.27-5.34 broad) also followed a pattern of relative elevation at day 28 compared to both admission and day 180 samples. Higher concentrations of the short chain fatty acid acetate (δ1.94s) were associated with admission and day 180 samples when each were compared to day 28. N-acetylneuraminic acid (δ2.06s) and phenylalanine (δ7.35d, δ7.37m, δ7.44m) associated with admission samples when compared to both day 28 and day 180 samples.

In Soroti (Table 4.12), admission samples contrasted strongly with later time points across many metabolites. The branched chain amino acids isoleucine (δ0.96t, δ1.03d), leucine (δ0.99t) and valine (δ1.01d, δ1.06d), were all associated with admission samples when compared to day 28 (r=0.56, r=0.56, r=0.57 respectively). Higher concentrations of other amino acids including tyrosine (δ6.91d, δ7.02d; $r>0.5$) and phenylalanine (δ7.30d, δ7.37m, δ7.44m; $r>0.5$) were associated with admission samples when compared either to day 28 (R^2 0.730, Q^2 0.474, CV-ANOVA <0.001) or day 180 (R^2 0.765, Q^2 0.524, CV-ANOVA $p<0.001$). N-acetylneuraminic acid was also associated with admission samples compared to day 28 or day 180 samples, similar to the case in Mbale, as was triacylglyceride. Lipid transport proteins of children in Soroti were only associated with admission plasma samples when compared to day 28 samples, and in OPLS regression of all time points, in contrast to the pattern seen in Mbale.

Table 4.11 Plasma metabolome changes over time in children from Mbale (n=363)

	OPLS Regression	OPLS-DA		
	All Time Points	Day 0 vs Day 28	Day 0 vs Day 180	Day 28 vs Day 180
Time point (n:n)	128:119:116	128:119	128:116	119:116
Components	1Pred + 1Orth	1Pred + 1Orth	1Pred + 1Orth	1Pred + 1Orth
R^2	0.501	0.718	0.678	0.633
Q^2	0.355	0.629	0.562	0.397
CV-ANOVA p-value	<0.001	<0.001	<0.001	<0.001
Metabolite	Earlier/Later (*r*)	Time Point (*r*)	Time Point (*r*)	Time Point (*r*)
Cholesterol (LDL) (δ 0.88broad, δ0.91 broad)	Later ↑ (0.57)	Day 28 ↑ (0.58)	Day 180 ↑ (0.68)	Day 28 ↑ (0.63)
Cholesterol (VLDL) (δ0.89 broad, δ0.92 broad)	Later ↑ (0.54)	Day 28 ↑ (0.79)	Day 180 ↑ (0.72)	Day 28 ↑ (0.62)
Cholesterol (VLDL/LDL) (δ1.26 broad)		Day 28 ↑ (0.67)	Day 180 ↑ (0.53)	Day 28 ↑ (0.65)
Triacylglyceride (δ1.55-1.61 broad, δ2.22-2.25 broad)	Earlier ↑ (0.51)		Day 0 ↑ (0.61)	
Acetate (δ1.94s)		Day 0 ↑ (0.66)		Day 180 ↑ (0.64)
N-acetyneuraminic acid (δ2.06s)		Day 0 ↑ (0.60)	Day 0 ↑ (0.62)	
Unknown lipid (δ3.23-3.25 broad)	Later ↑ (0.61)	Day 28 ↑ (0.80)	Day 180 ↑ (0.73)	
Phosphorylcholine (δ3.25s, δ5.27-5.34 broad)		Day 28 ↑(0.78)	Day 180 ↑ (0.70)	Day 28 ↑ (0.67)
Phenylalanine (δ7.35 d, δ7.37 m, δ7.44 m)		Day 0 ↑ (0.54)	Day 0 ↑ (0.55)	
Unknown 1 (δ7.63m)			Day 180 ↑ (0.52)	

Abbreviations: OPLS, orthogonal partial least squares; OPLS-DA, orthogonal partial least squares discriminant analysis; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance; *r*, correlation coefficient; VLDL, very low density lipoprotein; LDL, low density lipoprotein;

Table 4.12 Plasma metabolome changes over time in children from Soroti (n=180)

	OPLS Regression	OPLS-DA		
	All Time Points	Day 0 vs Day 28	Day 0 vs Day 180	Day 28 vs Day 180
Time point (n:n)	61:60:59	61:60	61:59	60:59
Components	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth
R^2	0.575	0.730	0.765	0.618
Q^2	0.292	0.474	0.524	0.206
CV-ANOVA p-value	<0.001	<0.001	<0.001	<0.001
Metabolite	Earlier/Later (*r*)	Time Point (*r*)	Time Point (*r*)	Time Point (*r*)
Isoleucine (δ0.96t, δ1.03d)		Day 0 ↑ (0.56)		
Leucine (δ0.99t)		Day 0 ↑ (0.57)		
Valine (δ1.01d, δ1.06d)	Earlier ↑ (0.55)	Day 0 ↑ (0.57)		
Dihydrothymine (δ1.09d)		Day 0 ↑ (0.55)	Day 0 ↑ (0.59)	Day 28 ↑ (0.52)
Cholesterol (LDL) (δ 0.88broad, δ0.91 broad)	Earlier ↑ (0.56)		Day 0 ↑ (0.74)	
Cholesterol (VLDL) (δ0.89 broad, δ0.92 broad)	Earlier ↑ (0.57)		Day 0 ↑ (0.73)	
Cholesterol (VLDL/LDL) (δ1.26 broad)	Earlier ↑ (0.73)		Day 0 ↑ (0.72)	
Triacylglyceride (δ1.55-1.61 broad, δ2.22-2.25 broad)	Earlier ↑ (0.75)	Day 0 ↑ (0.58)	Day 0 ↑ (0.79)	
N-acetylneuraminic acid (δ2.06s)		Day 0 ↑ (0.51)	Day 0 ↑ (0.68)	
Unknown lipid (δ3.23-3.25 broad)		Day 28 ↑ (0.74)	Day 180 ↑ (0.54)	
Glucose (δ5.26d, δ3.00-4.00 resonances)				Day 180 ↑ (0.51)
Phosphorylcholine (δ3.25s, δ5.27-5.34 broad)	Earlier ↑ (0.54)		Day 0 ↑ (0.52)	Day 28 ↑ (0.50)
Unknown 1 (δ5.2 broad)	Earlier ↑ (0.60)		Day 0 ↑ (0.68)	
Tyrosine (δ6.91d, δ7.20d)		Day 0 ↑ (0.65)	Day 0 ↑ (0.50)	
Phenylalanine (δ7.3 d, δ7.37m, δ7.44m)		Day 0 ↑ (0.74)	Day 0 ↑ (0.60)	

Abbreviations: OPLS, orthogonal partial least squares; OPLS-DA, orthogonal partial least squares discriminant analysis; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance; VLDL, very low density lipoprotein; LDL, low density lipoprotein;

4.2.2.4 *TRACT INTERVENTIONS AND HAEMOGLOBIN RECOVERY*

OPLS-DA models exploring the effects of TRACT interventions on plasma metabolome at day 28 are summarised in Table 4.13. All OPLS-DA models were invalid suggesting no detectable effects on ^{1}H NMR plasma profiles in comparisons between TRACT A 30ml vs. 20ml transfusion arms (Q^2 -0.116, CV-ANOVA p =1.000), TRACT B 30ml/20ml vs. no transfusion (Q^2 0.041, CV-ANOVA p =0.803), cotrimoxazole prophylaxis vs. none (Q^2 -0.141, CV-ANOVA p =1.000), and iron and folate vs. multivitamin multi-mineral supplementation (Q^2 -0.172, CV-ANOVA p =1.000). As all models were determined to be invalid due to low or negative Q^2 scores and non-significant CV-ANOVA p-values, identification of discriminant metabolites was not undertaken.

To more broadly assess any effect on plasma metabolome from changes in haemoglobin, this was assessed separately (Table 4.14). At no time point was blood haemoglobin found to have an effect on the broader plasma metabolome (at admission Q^2 of OPLS regression with haemoglobin concentration was 0.118, at day 28 -0.103, and at day 180 -0.398). Similarly, only poor discriminant analysis models (Q^2 <0.1 in both cases) were generated when comparing those who recovered (Hb ≥9.0g/dl) to those who did not at both day 28 and day 180 time points. Models were constructed analysing whether admission plasma metabolome was related to subsequent mortality, however these were not valid (negative Q^2) therefore they are not presented.

Table 4.13 Summary of OPLS-DA models comparing TRACT interventions' effects on plasma metabolome at day 28 (n=179)

	OPLS-DA Model			
	TRACT A 30ml vs 20ml transfusion	TRACT B 30/20ml transfusion vs none	Cotrimoxazole vs None	Iron-Folate vs MVMM Supplementation
n:n	76:64	19:20	96:83	95:84
Components	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth	1 Pred + 1 Orth
R^2	0.830	0.958	0.699	0.758
Q^2	-0.116	0.041	-0.141	-0.172
CV-ANOVA p-value	1.000	0.803	1.000	1.000

Abbreviations: OPLS-DA, orthogonal partial least squares discriminant analysis; MVMM, multivitamin multi-mineral; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance

Table 4.14 Summary of OPLS and OPLS-DA models examining relationship between haemoglobin and plasma metabolome across all time points (n=543)

	Details of Model					
	OPLS or OPLS-DA	n	Components	R^2	Q^2	CV-ANOVA p-value
Admission Haemoglobin	OPLS	189	1 Pred + 1 Orth	0.631	0.118	<0.001
Day 28 Haemoglobin	OPLS	179	1 Pred + 1 Orth	0.806	-0.103	1.000
Not recovered : Recovered	OPLS-DA	88:91	1 Pred + 1 Orth	0.715	0.034	0.147
Day 180 Haemoglobin	OPLS	175	1 Pred + 1 Orth	0.650	-0.398	1.000
Not recovered : Recovered	OPLS-DA	99:76	1 Pred + 1 Orth	0.721	0.057	0.063

Abbreviations: OPLS-DA, orthogonal partial least squares discriminant analysis; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance; Recovery defined as haemoglobin ≥ 9.0g/dl

* indicates models where the addition of the orthogonal component decreased model predictive ability (Q^2)

4.2.2.5 DIETARY EFFECTS ON PLASMA METABOLOME

Summary details of multivariate statistical models exploring the effect of diet on plasma metabolic profiles are presented in table 4.15. In summary the dietary patterns and the foods with which they were most strongly associated were: DP1 "Carbohydrate-rich staples, legumes and beverages", DP2 "Meat and carbohydrate-rich staples", DP3 "Sweetened foods/drinks, fruit, eggs", DP4 "Milk-based, with low fruit and vegetable intake".

Regression and discriminant OPLS models assessing association with dietary diversity and the dietary patterns identified in Chapter 3 were determined to be invalid due to negative Q^2 scores and non-significant CV-ANOVA p-values. The same observation is true when food group regression and discriminant models based on tertiles 1 and 3 were constructed. Both OPLS and OPLS-DA models based on food group 1 (grains, roots, tubers and starchy banana) and food group 2 (legumes and nuts) generated significant CV-ANOVA p-values. However, on examining the models further they were determined to be invalid, since the addition of the orthogonal component decreased the predictive ability, suggesting overfitting, and the Q^2 values were generally low (i.e. below 0.3) in all cases. OPLS-DA models comparing the highest and lowest tertiles in food group 8 (sugar and highly sweetened foods) and food group 9 (other), also had significant CV-ANOVA p-values but low Q^2 values, and the orthogonal component lowered Q^2 values. As no models were generated which had high Q^2 values and significant CV-ANOVA p-values, it was deemed inappropriate to explore model loadings for discriminant metabolites.

Table 4.15 Summary of OPLS and OPLS-DA models examining the effect of diet on plasma metabolome across all time points (n=420)

	Details of Model					
Dietary factor	OPLS or OPLS-DA	n	Components	R^2	Q^2	CV-ANOVA p-value
Dietary diversity score	OPLS	420	1 Pred + 1 Orth	0.589	-0.101	1.000
Diverse (DDS<4) : not diverse (DDS≥4)	OPLS-DA	226:194	1 Pred + 1 Orth	0.618	-0.104	1.000
Dietary Pattern 1	OPLS	420	1 Pred + 1 Orth	0.533	-0.228	1.000
T1 : T3	OPLS-DA	142:140	1 Pred + 1 Orth	0.619	-0.200	1.000
Dietary Pattern 2	OPLS	420	1 Pred + 1 Orth	0.490	-0.239	1.000
T1 : T3	OPLS-DA	144:140	1 Pred + 1 Orth	0.600	-0.158	1.000
Dietary Pattern 3	OPLS	420	1 Pred + 1 Orth	0.498	-0.207	1.000
T1 : T3	OPLS-DA	142:149	1 Pred + 1 Orth	0.578	-0.273	1.000
Dietary Pattern 4	OPLS	420	1 Pred + 1 Orth	0.578	-0.220	1.000
T1 : T3	OPLS-DA	142:142	1 Pred + 1 Orth	0.665	-0.162	1.000
Grains, roots and tubers (FG1)*	OPLS	420	1 Pred + 1 Orth	0.311	0.076	<0.001
T1 : T3	OPLS-DA	131:160	1 Pred + 1 Orth	0.430	0.114	<0.001
Legumes and nuts (FG2)*	OPLS	420	1 Pred + 1 Orth	0.309	0.095	<0.001
T1 : T3	OPLS-DA	192:163	1 Pred + 1 Orth	0.367	0.115	<0.001
Dairy products (FG3)	OPLS	420	1 Pred + 1 Orth	0.663	-0.211	1.000
No intake : Any intake	OPLS-DA	356:64	1 Pred + 1 Orth	0.470	-0.257	1.000
Flesh foods (FG4)	OPLS	420	1 Pred + 1 Orth	0.313	-0.136	1.000
No intake : T3	OPLS-DA	221:199	1 Pred + 1 Orth	0.341	-0.125	1.000
Eggs (FG5)	OPLS	420	1 Pred + 1 Orth	0.479	-0.108	1.000
No intake : Any intake	OPLS-DA	394:26	1 Pred + 1 Orth	0.408	-0.133	1.000
Vitamin A-rich fruit and vegetables (FG6)	OPLS	420	1 Pred + 1 Orth	0.380	-0.209	1.000
T1 : T3	OPLS-DA	247:173	1 Pred + 1 Orth	0.319	-0.102	1.000
Other fruit and vegetables (FG7)	OPLS	420	1 Pred + 1 Orth	0.428	-0.071	1.000
No intake : Any intake	OPLS-DA	289:131	1 Pred + 1 Orth	0.534	-0.131	1.000
Sugar and highly sweetened food (FG8)	OPLS	420	1 Pred + 1 Orth	0.327	-0.030	1.000
T1 : T3	OPLS-DA	147:273	1 Pred + 1 Orth	0.483	0.051	0.053
Other (FG9)	OPLS	420	1 Pred + 1 Orth	0.313	-0.005	1.000
T1 : T3*	OPLS-DA	122:298	1 Pred + 1 Orth	0.410	0.072	0.003

Abbreviations: OPLS-DA, orthogonal partial least squares discriminant analysis; Pred, predictive component; Orth, orthogonal component; CV-ANOVA, cross-validated analysis of variance; DDS, dietary diversity score; DP, dietary pattern; T#, tertile; FG, food group
* indicates models where the addition of the orthogonal component decreased model predictive ability (Q^2)

4.2.3 FAECAL 16S RRNA PROFILING

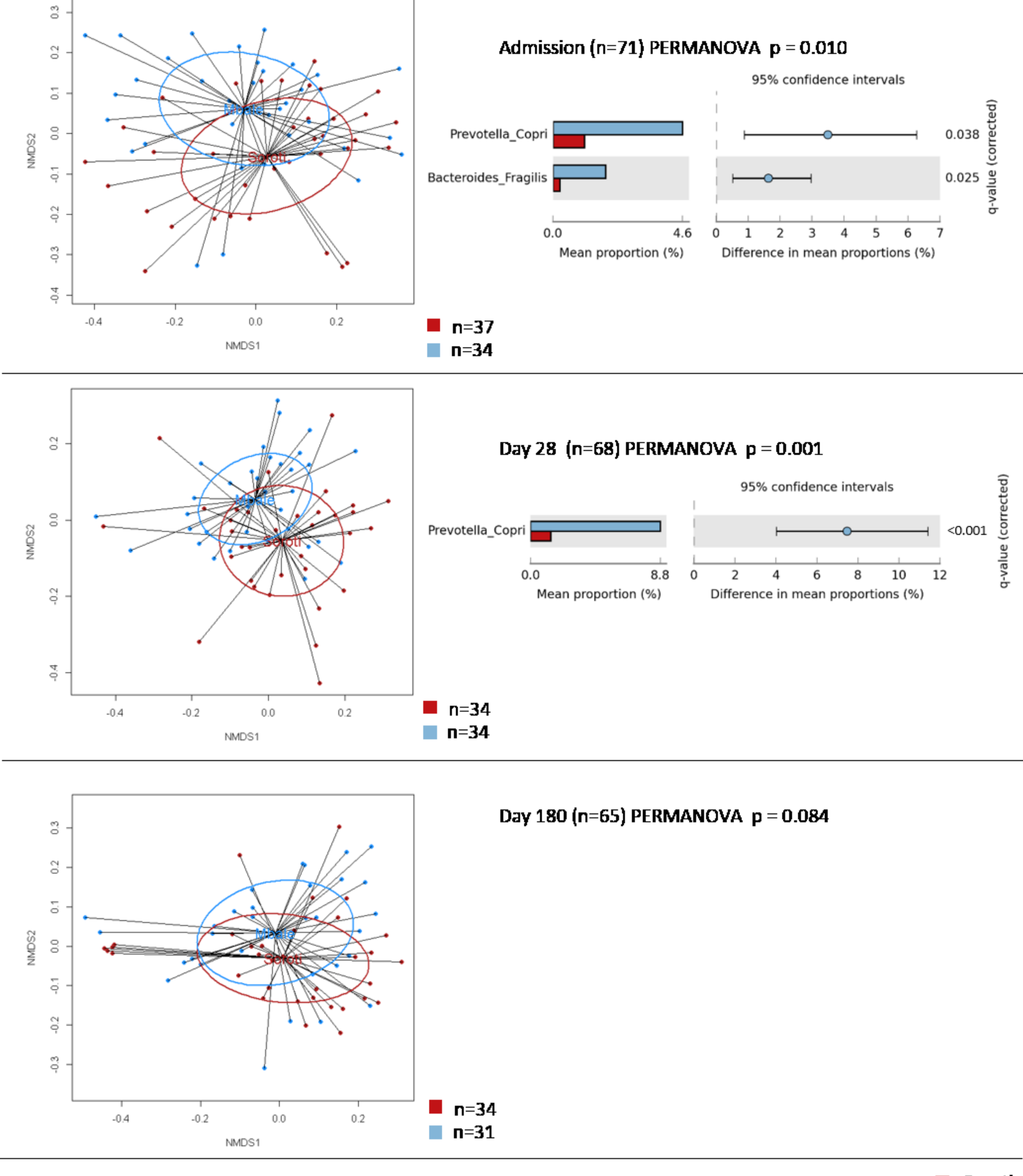
A total of 567 faecal samples were collected from n=327 children in the TRACT trial. Samples from all three study time points were collected for n=66 children (198 samples), and in addition to children who died but provided samples for all time points until their death (14 samples from n=11 children) these were selected for faecal DNA extraction and subsequent metataxonomic profiling. Therefore faecal DNA for downstream MiSeq 16s rRNA sequencing was extracted for a total of 212 samples from n=77 children. The forward primers (28F-YM, 28F-Borrellia, 28FChloroflex, 28F-Bifdo) were in a mix with a ratio of 4:1:1:1, and the reverse primer used was 388R.

Following data processing using the Mothur pipeline (detailed in section 2.5.2), the average number of reads per sample was 32,787 (SD ±8271), and 2 samples were found to have a low number of reads (<1000), and were therefore excluded from statistical analyses. After processing in the Mothur pipeline, 210 samples (from n=77 children) subsampled to 16,509 reads were available for statistical analysis. Subsampling to 16,509 reads resulted in Good's coverage of >99.0% for all samples, suggesting that the subsampled data provided excellent coverage.

4.2.3.1 SITE-RELATED DIFFERENCES

Figure 4.3 displays non-metric dimensional scaling (NMDS) plots, associated permutational analysis of variance (PERMANOVA) p-values, and extended error bar plots showing species-level differences in faecal microbial profiles between children from Mbale and Soroti at each time point. PERMANOVA tests were significant (p<0.05) at admission (false discovery rate [FDR] adjusted p = 0.01) and day 28 (FDR adjusted p = 0.001) time points, suggesting the observed divergences in NMDS are statistically significant. At admission two species in the *Bacteroidia* class were present at higher proportions in children from Mbale compared to Soroti: the proportion of *Prevotella copri* was greater by 3.50% (95% CI 0.89-6.28, relative frequency of 4.6% vs 1.1% respectively) and *Bacteroides fragilis* by 1.63% (95% CI 0.53-2.97, relative frequency of 1.8% vs 0.2% respectively), Benjamini-Hochberg (BH) corrected p=0.038 and p=0.025 respectively. By day 28 the proportion of *Prevotella copri* in samples from children in Mbale was greater than those from Soroti by 7.48% (95% CI 3.75-11.48), BH corrected p=0.010. NMDS and PERMANOVA suggest no difference between sites at day 180.

Figure 4.3 Non-metric dimensional scaling (NMDS) plots, associated permutational analysis of variance (PERMANOVA) and extended error bar plots assessing site-related differences in faecal microbial profiles



Differences in the richness and diversity of bacterial species found between sites at each time point are summarised in Table 4.16. No differences were found at admission or day 28, however by day 180 samples from Mbale were richer, i.e. more species were observed, and more diverse, i.e. the individuals are distributed more evenly among these species, than those from Soroti. At day 180 the mean S_{obs} was 258.0 (SD 99.6) in Mbale compared to 206.3 (SD 98.1) in Soroti, independent t-test p=0.039. Median Shannon index of diversity at day 180 in Mbale was 3.66 (IQR 0.72) compared to 3.16 (IQR 1.03) in Soroti, p=0.036.

Table 4.16 Site differences in faecal bacterial richness (S_{obs}) and α-diversity (Shannon index) (n=71)

	Admission			Day 28			Day 180		
Measure	Soroti (n=37)	Mbale (n=34)	p	Soroti (n=34)	Mbale (n=34)	p	Soroti (n=34)	Mbale (n=31)	p
S_{obs}* mean (SD)	177.6 (79.5)	192.0 (83.3)	0.458	205.1 (76.4)	204.2 (82.6)	0.966	206.3 (98.1)	258.0 (99.6)	0.039
Shannon index** median [IQR]	2.65 [1.44]	2.84 [1.09]	0.311	3.16 [0.82]	3.21 [0.78]	0.650	3.16 [1.03]	3.66 [0.72]	0.036

Abbreviations: S_{obs}, number of observed species; SD, standard deviation; IQR, interquartile range
*independent-samples t-test
**Mann Whitney U test

4.2.3.2 *BASELINE CHARACTERISTICS' EFFECT ON ADMISSION GUT MICROBIAL PROFILES*

NMDS and associated PERMANOVA analyses of baseline characteristics' effects on admission faecal microbial profiles are displayed in Figure 4.4. It was not possible to meaningfully assess any effect of wasting on admission faecal microbial profiles due to low numbers with this characteristic (n=2/71). Age group (over 5 years old vs. under 5 years old), gender, underweight status (weight-for-age z-score <-2.0), stunting status (height-for-age z-score <-2.0), fever (temperature >37.5°C), leucocytosis (WBC >12 x 10^9/L), malaria status and sickle cell status (confirmed by genotype SS) had no observable effect on the admission metataxonomic profiles of children, PERMANOVA p>0.05 in all cases. Due to lack of observable differences by NMDS and PERMANOVA, White's non-parametric t-test was not undertaken, therefore no extended error bar plots are presented.

Table 4.17 summarises differences in richness (S_{obs}) and diversity (Shannon index) of admission faecal samples based on baseline characteristics. No differences in richness or diversity were observed between children who were under age 5 vs. over age 5, underweight vs. not underweight, stunted vs. not stunted, with fever vs. without fever, leucocytotic vs. normal white blood cell count, positive for malaria vs. negative, and sickle cell SS homozygous vs. AA homozygous, p>0.05 in all cases. Female children had greater richness (S_{obs} 207.5 vs. 167.6 in males, p=0.039) and diversity (Shannon index 3.08 vs. 2.62 in males, p=0.042).

Figure 4.4 Non-metric dimensional scaling (NMDS) plots, associated permutational analysis of variance (PERMANOVA) assessing effect of baseline characteristics on faecal microbial profiles (n=71)

Under 5 years n=40 : n=31 over 5 years
PERMANOVA p = 0.554

Under 5
Over 5

Fever n=28 : n=43 no fever
PERMANOVA p = 0.878

Fever
No Fever

Female n=30 : n=41 male
PERMANOVA p = 0.561

Female
Male

Leucocytosis n=41: n=30 normal WBC
PERMANOVA p = 0.412

Leucocytosis
Normal WBC

Underweight n=11 : n=60 not underweight
PERMANOVA p = 0.459

Underweight
Not underweight

Malaria n=48 : n=23 no malaria
PERMANOVA p = 0.682

Malaria
No malaria

Stunted n=10 : n=61 not stunted
PERMANOVA p = 0.900

Stunted
Not Stunted

Sickle cell disease n=49: n=22 no sickle cell disease
PERMANOVA p = 0.117

Sickle Cell Dx
No Sickle Cell Dx

Table 4.17 Site differences in faecal bacterial richness (S_{obs}) and α-diversity (Shannon index) (n=71)

	Age Group			Gender			WAZ			HAZ		
	<5 years	>5 years		Female	Male		WAZ<-2	WAZ>-2		HAZ<-2	HAZ>-2	
Measure	n=40	n=31	p	n=30	n=41	p	n=11	n=60	p	n=10	n=61	p
S_{obs}* mean (SD)	169.3 (79.7)	204.0 (79.8)	0.073	207.5 (78.4)	167.6 (79.7)	0.039	168.2 (83.3)	186.0 (82.4)	0.489	161.4 (82.2)	188.3 (80.9)	0.335
Shannon index** median [IQR]	3.07 [1.33]	2.65 [1.18]	0.190	3.08 [1.23]	2.62 [1.31]	0.042	2.58 [1.43]	2.72 [1.32]	0.246	2.47 [1.91]	2.79 [1.21]	0.753

	Temperature			White Blood Cell Count			Malaria			Sickle Cell Status		
	>37.5°C	35-37.5°C		>12x10^9/L	4-12x10^9/L		Positive	Negative		SS Genotype	AA Genotype	
	n=28	n=43	p	n=41	n=30	p	n=48	n=23	p	n=22	n=49	p
S_{obs}* mean (SD)	180.9 (82.5)	186.8 (81.0)	0.766	172.2 (73.1)	205.9 (87.3)	0.084	190.4 (82.5)	172.1 (78.3)	0.378	197.3 (74.5)	179.7 (83.7)	0.402
Shannon index** median [IQR]	2.83 [1.31]	2.71 [1.31]	0.860	2.46 [1.65]	2.83 [1.05]	0.052	2.69 [1.37]	2.81 [1.06]	0.990	2.65 [1.26]	3.10 [1.11]	0.196

Abbreviations: S_{obs}, number of observed species; SD, standard deviation; IQR, interquartile range

*independent-samples t-test

**Mann Whitney U test

4.2.3.3 Gut microbiota changes over time

Figure 4.5 displays NMDS, PERMANOVA and associated White's t test extended error bar plots (at species level) assessing differences in faecal microbial profiles between the study time points. No differences were detected between the latter time points, day 28 and day 180 (PERMANOVA FDR adjusted p=0.219). Admission samples were significantly different from both day 28 and day 180 samples (PERMANOVA FDR adjusted p=0.002 in both cases). The major changes in species-level proportions from admission to day 28 were similar to those from admission to day 180. An unclassified species in the family *Enterobacteriaceae* decreased in mean relative frequency from 23.5% to 8.8% at day 28 (mean difference 14.7%, FDR adjusted p = 0.004), and from 23.5% to 13.6% at day 180 (mean difference 9.9%, FDR adjusted p=0.018). *Enterococcus faecium* relative frequency decreased from 11.5% at admission to 0.6% at day 28 (mean difference 11.0%. FDR adjusted p=0.004), and to 3.8% at day 180 (FDR adjusted p=0.007). *Akkermansia muciniphila* also decreased from admission (3.1%) to 0.6% at day 28 (FDR adjusted p=0.004) and to 0.5% at day 180 (FDR adjusted p=0.004). Species which displayed large increases from admission to day 28 and day 180 included unclassified species in the genera *Clostridium sensu stricto*, *Streptococcus*, and *Clostridium* cluster IX. The unclassified *Clostridium sensu stricto* species increased from 1.8% (admission) to 8.9% at day 28 (FDR adjusted p=0.005), and 9.0% at day 180 (FDR adjusted p=0.005). The unclassified *Streptococcus* species increased from 4.6% at admission to 11.6% at day 28 (FDR adjusted p=0.004) but this did not persist to day 180. The unclassified cluster IX *Clostridium* increased from 2.8% at admission to 8.5% at day 28 (FDR adjusted p=0.005) and to 8.1% by day 180 (FDR adjusted p=0.005). Species which increased by smaller proportions from admission to day 28 included *Faecalibacterium prausnitzii* (1.2% to 2.4%, FDR adjusted p=0.023), an unclassified species in the order *Clostridiales* (0.5% to 1.7%, FDR adjusted p=0.004) which was also elevated at day 180 (2.0%, FDR adjusted p=0.004), and an unclassified species in the family *Lachnospiraceae* (1.2% to 2.3%, FDR adjusted p=0.010) which was also elevated at day 180 (2.4%, FDR adjusted p=0.004). Other species that were present in higher proportions day 180 samples compared to admission samples included an unclassified species (unknown phylum) which increased from 1.3% to 3.7%, FDR adjusted p=0.015, an unclassified species in the family *Ruminococcaceae* (increased from 3.2% to 5.6%, FDR adjusted p=0.031), and an unclassified species in the order *Bacteroidales* (increased from 0.1% to 1.1%, FDR adjusted p=0.007). *Bifidobacterium longum* decreased from 1.6% at admission to 0.3% at day 180 (FDR corrected p=0.007).

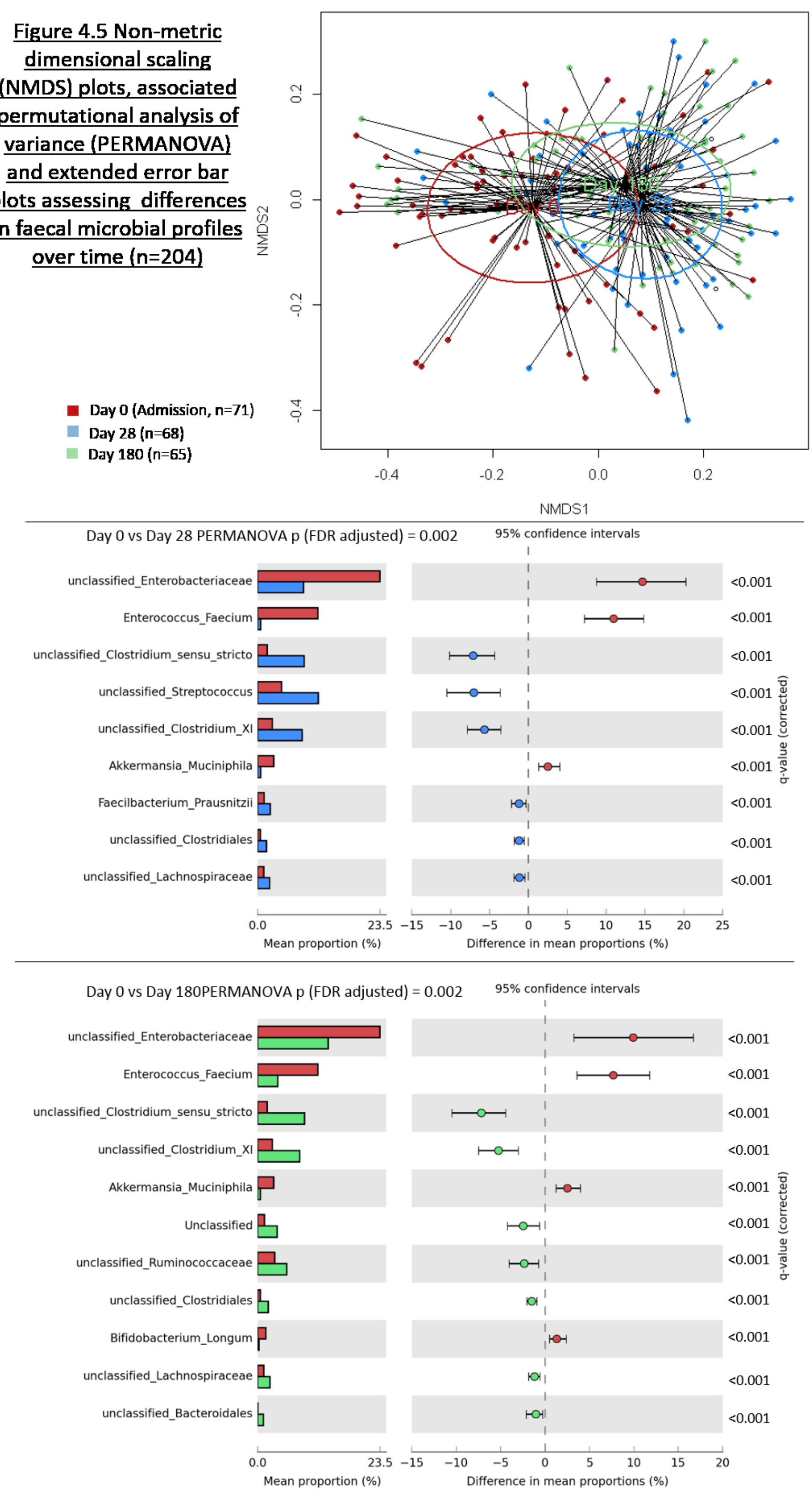


Figure 4.5 Non-metric dimensional scaling (NMDS) plots, associated permutational analysis of variance (PERMANOVA) and extended error bar plots assessing differences in faecal microbial profiles over time (n=204)

Day 28 vs Day 180PERMANOVA p (FDR adjusted) = 0.219 **Abbreviations: FDR, false discovery rate**

Differences that occurred in richness and α-diversity measures over time are detailed in Table 4.18. S_{obs} increased from 184.5 at admission to 204.7 at day 28, and to 231.0 at day 180 (one-way ANOVA p=0.009), but only the change from admission to day 180 was significant in pairwise comparisons. Diversity was higher in both later time points (mean Shannon index = 3.18 at day 28 and 3.40 at day 180) when compared to admission (mean Shannon index = 2.72) but not to each other, Kruskal-Wallis test p<0.001.

Table 4.18 Differences in faecal bacterial richness (S_{obs}) and diversity (Shannon index) over time

Measure	Admission (n=71)	Day 28 (n=68)	Day 180 (n=65)	p
S_{obs}*, mean (SD)	184.5 (81.1)^{D180}	204.7 (79.0)	231.0 (101.4)^{Adm}	0.009
Shannon index**, median [IQR]	2.72 [1.23]$^{D28, D180}$	3.18 [0.77]^{Adm}	3.40 [1.07]^{Adm}	<0.001

Abbreviations: S_{obs}, number of observed species; SD, standard deviation; IQR, interquartile range
*One-way analysis of variance (ANOVA)
**Kruskal-Wallis test
$^{Adm, D28, D180}$ denotes pairwise significant difference from admission, day 28, or day 180 sample respectively

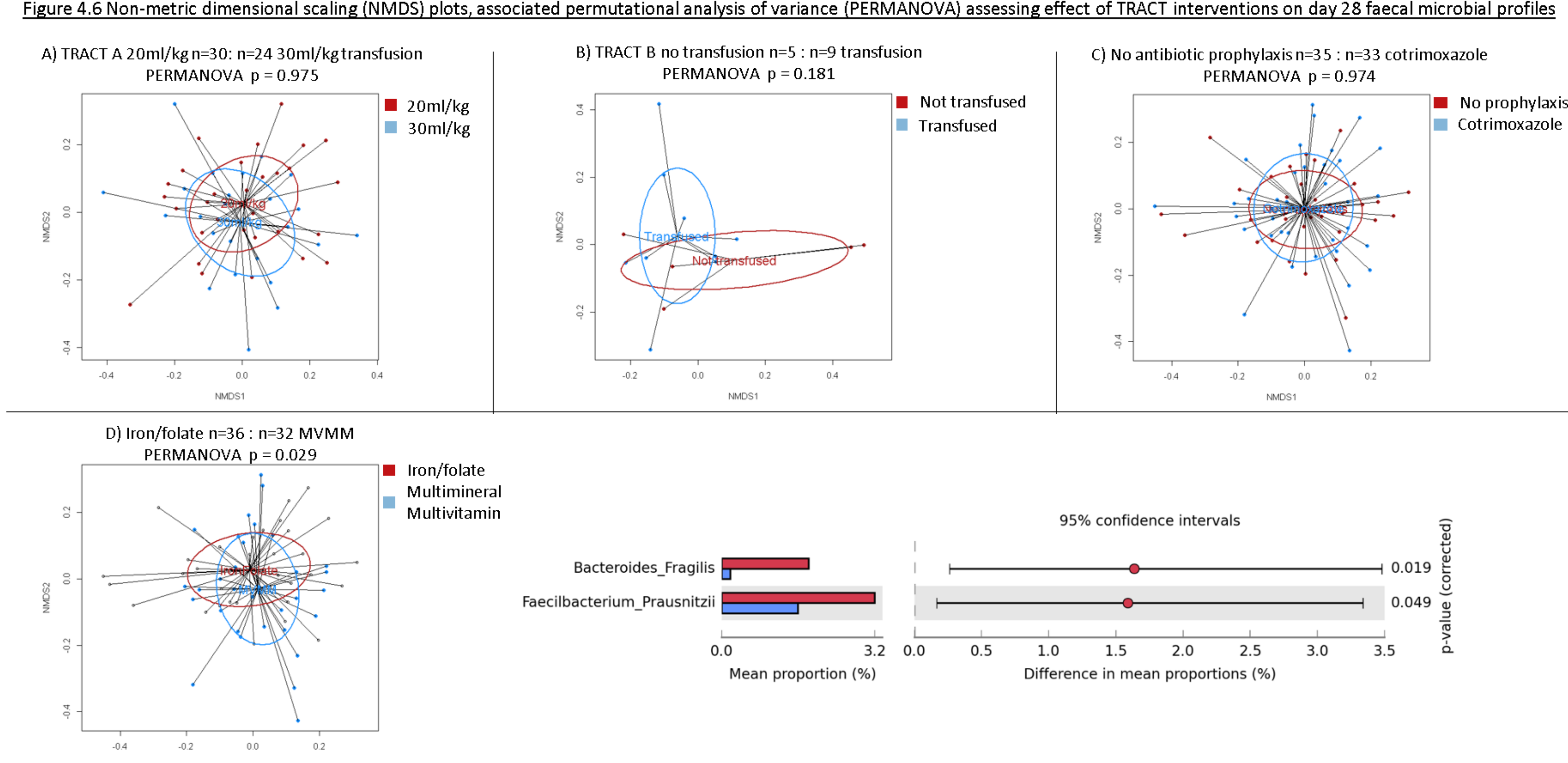
4.2.3.4 TRACT INTERVENTIONS AND HAEMOGLOBIN RECOVERY

Comparisons between children who died and those did not were conducted but indicated no differences between the gut microbial profiles, therefore these are omitted for brevity. The effect of TRACT study interventions, i.e. 20ml/kg vs. 30ml/kg whole blood equivalent transfusion (TRACT A arm), any transfusion vs. no transfusion (TRACT B arm), 12 weeks post discharge cotrimoxazole prophylaxis vs. no antibiotic prophylaxis, and 12 weeks post discharge iron and folate vs. 12 weeks post discharge multivitamin multi-mineral (MVMM) supplementation, are presented in this section. Figure 4.6 displays NMDS plots, PERMANOVA statistics and associated White's non-parametric t test extended error bar plots. Neither the TRACT A nor TRACT B arm transfusion comparisons resulted in any differences in gut microbial profiles at day 28 (PERMANOVA p>0.05), however it should be noted that the numbers in TRACT B were low (no transfusion n=5 vs. n=9 who were transfused). Contrary to expectations, antibiotic prophylaxis with Cotrimoxazole did not result in any detectable difference in 16S profiles by day 28. Section 4.2.3.6 below presents further results related to antibiotic treatment and its effect on gut microbial profiles, but briefly due to large numbers of children receiving antibiotics of different classes in different combinations, a specific effect related solely to cotrimoxazole treatment would be difficult to observe. The nutritional intervention caused separation in microbial profiles (PERMANOVA p=0.029), and this was found to be caused by higher proportions of two bacterial species in the iron and folate arm. Day 28 relative frequency of *Bacteroides fragilis* were 0.2% in the MVMM arm compared to 1.8% in the iron and folate arm (White's nonparametric t test p=0.019). At day 28 *Faecalibacterium prausnitzii* had mean relative frequency of 1.6% in the MVMM arm compared to 3.2% in the iron and folate arm (White's nonparametric t p=0.049).

Table 4.19 summarises comparisons of faecal bacterial richness and diversity between the TRACT interventions at day 28. No significant differences were detected, contrary to expectations. An incidental finding was that Shannon index at day 180 was significantly lower in the cotrimoxazole arm (median 3.12 [IQR 1.10]) compared to 3.55 [IQR 0.57], Mann Whitney U p=0.013.

Associations between mortality and gut microbial profiles at admission were also conducted, however no significant differences between the metataxonomic profiles of children who died (n=7/71) vs. those who survived (n=64/71), were observed.

Figure 4.6 Non-metric dimensional scaling (NMDS) plots, associated permutational analysis of variance (PERMANOVA) assessing effect of TRACT interventions on day 28 faecal microbial profiles



TRACT interventions: TRACT A: 20ml/kg vs 30ml/kg transfusion; TRACT B: 20/30ml/kg transfusion vs no transfusion; Antibiotic prophylaxis (cotrimoxazole for 3 months) vs none; Iron + folate vs multivitamin-multimineral supplementation

Table 4.19 Differences in day 28 faecal bacterial richness (S_{obs}) and α-diversity (Shannon index) comparing TRACT interventions (n=68)

	TRACT A transfusion			TRACT B transfusion			Antibiotic prophylaxis			Micronutrients		
Measure	20ml/kg n=30	30ml/kg n=24	p	Not transfused n=5	Transfused n=9	p	No prophylaxis n=32	Cotrimoxazole n=36	p	Iron & folate n=36	Multivitamin multi-mineral n=32	p
S_{obs}* mean (SD)	200.2 (74.1)	209.6 (78.6)	0.654	188.8 (128.1)	214.9 (77.0)	0.693	205.2 (71.8)	204.1 (85.8)	0.955	192.7 (83.1)	218.1 (73.1)	0.189
Shannon index** median [IQR]	3.15 [0.68]	3.23 [1.12]	0.903	3.22 [0.53]	3.17 [2.08]	0.699	3.13 [0.94]	3.18 [0.70]	0.951	3.09 [0.87]	3.29 [0.83]	0.210

Abbreviations: S_{obs}, number of observed species; SD, standard deviation; IQR, interquartile range

*independent-samples t-test

**Mann Whitney U test

Table 4.20 Differences in day 28 faecal bacterial richness (S_{obs}) and α-diversity (Shannon index) between haemoglobin recovery† vs. non-recovery‡

	Day 28 (n=68)			Day 180 (n=65)		
Measure	Hb≥9.0g/dl n=39	Hb<9.0g/dl n=29	p	Hb≥9.0g/dl n=37	Hb<9.0g/dl n=28	p
S_{obs}* mean (SD)	210.5 (85.6)	196.8 (71.2)	0.478	247.7 (98.6)	209.1 (110.3)	0.155
Shannon index** median [IQR]	3.25 [0.76]	3.00 [0.90]	0.398	3.52 [0.85]	3.17 [1.11]	0.108

Abbreviations: S_{obs}, number of observed species; SD, standard deviation; IQR, interquartile range

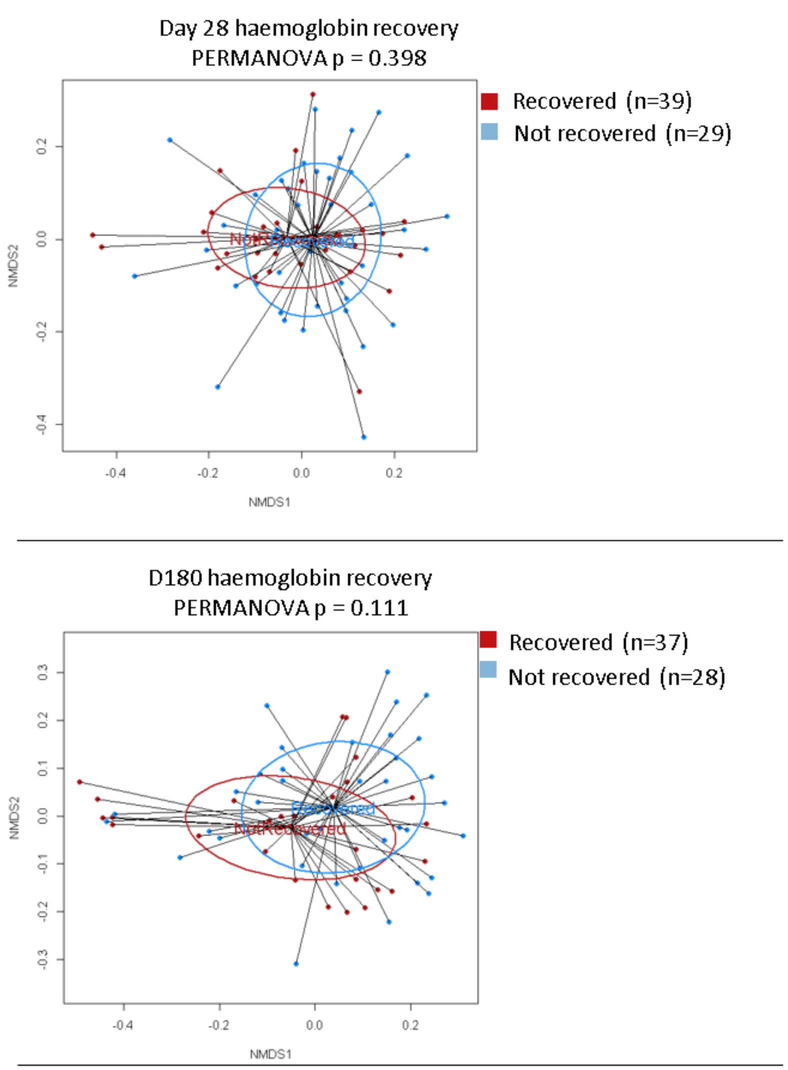
*independent-samples t-test

**Mann Whitney U test

† recovery: Hb≥9.0g/dl ‡ non-recovery: Hb<9.0g/dl

NMDS and PERMANOVA analyses did not reveal any associations in gut microbial profiles between gut microbial profiles and haemoglobin recovery (Hb≥9.0g/dL) at day 28 or day 180 (Figure 4.7), nor were there any differences in richness or diversity data between those whose haemoglobin had recovered and those who did not (Table 4.20).

Figure 4.7 Non-metric dimensional scaling (NMDS) plots and associated permutational analysis of variance (PERMANOVA) comparing faecal microbial profiles of children who recovered vs. those who did not



Recovery defined as Hb ≥9.0g/dl , non-recovery as Hb<9.0g/dl at review

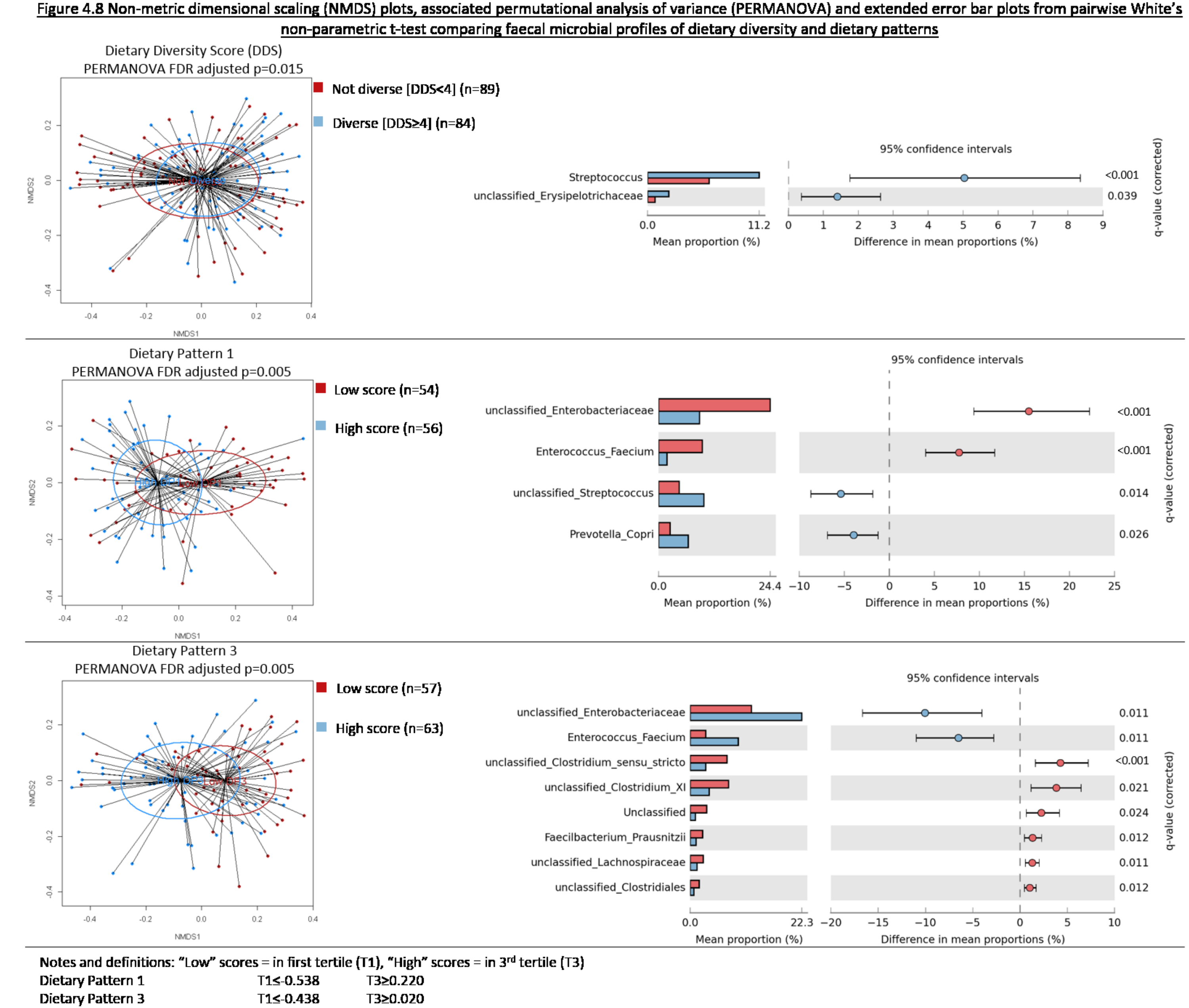
4.2.3.5 *Dietary effects on the gut microbiota*

Results from NMDS and PERMANOVA assessing association between gut microbial profiles and dietary diversity and dietary patterns are presented in Figure 4.8. In summary the dietary patterns and the foods with which they were most strongly associated were: DP1 "Carbohydrate-rich staples, legumes

and beverages", DP2 "Meat and carbohydrate-rich staples", DP3 "Sweetened foods/drinks, fruit, eggs", DP4 "Milk-based, with low fruit and vegetable intake".

Additional non-significant results for dietary patterns, nutrients, and food groups are displayed in Appendix XI, along with the specific values of tertile cut offs for dietary patterns, nutrients and food groups. An unclassified species in the genus *Streptococcus* had mean relative frequency of 11.2% in the group with dietary diversity score (DDS) ≥4 compared to 6.2% in those with lower dietary diversity (DDS<4), White's non-parametric t-test p=0.010 (FDR-adjusted). An unclassified species in the family *Erysipelotrichaceae* was also higher in those with a more diverse diet (2.1% vs 0.7%, FDR adjusted p=0.039). Both dietary pattern 1 and dietary pattern 3 were associate with differences in the prevalence of several species. Those whose scores fell in the highest tertile for DP1 had higher relative frequency of unclassified *Streptococcus* (9.9% vs. 4.5%, FDR adjusted p=0.014) and *P. copri* (6.5% vs. 2.5%, FDR adjusted p=0.026), while those with the lowest scores had higher proportions of unclassified *Enterobacteriaceae* (24.4% vs. 8.9%, FDR adjusted p=0.005) and *E. faecium* (9.6% vs. 1.8%, FDR adjusted p=0.005). Conversely, higher scores in dietary pattern 3 were associated with unclassified *Enterobacteriaceae* (22.3% vs. 12.1%, FDR adjusted p=0.011) and *E. faecium* (9.5% vs. 3.1%, FDR adjusted p=0.011). Lower scoring diets in DP3 were associated with higher proportions of *Clostridium sensu stricto* (7.4% vs. 3.1%), an unclassified *Clostridium XI* (7.6% vs. 3.8%), *F. prausnitzii* 2.5% vs.1.1%), unclassified *Lachnospiraceae* (2.6% vs. 1.3%) and unclassified *Clostridiales* (1.8% vs 0.7%), FDR adjusted p<0.05 in all cases.

Figure 4.8 Non-metric dimensional scaling (NMDS) plots, associated permutational analysis of variance (PERMANOVA) and extended error bar plots from pairwise White's non-parametric t-test comparing faecal microbial profiles of dietary diversity and dietary patterns



Notes and definitions: "Low" scores = in first tertile (T1), "High" scores = in 3rd tertile (T3)

Dietary Pattern 1	**T1≤-0.538**	**T3≥0.220**
Dietary Pattern 3	**T1≤-0.438**	**T3≥0.020**

Associations between faecal microbial profiles and selected nutrients are presented in Figure 4.9. PERMANOVA p-values were adjusted for multiple comparisons by the total number of nutrients tested (carbohydrate, protein, fat, sugar and fibre). Results for carbohydrate and fat are in Appendix XI. Only fibre was directly associated with differences in faecal microbial profiles. Sugar intake and protein intake had PERMANOVA FDR-adjusted p-values near to significance (p=0.052 and p=0.068 respectively). Higher intakes of fibre were associated with higher proportions of *Ruminococcaceae* (9.7% vs. 5.9%) and *Clostridiales* (1.7% vs. 0.7%), but lower *Enterobacteriaceae* (12.6% vs. 25.9%), FDR adjusted p<0.05.

As PERMANOVA p-values prior to correction were considered valid, White's non-parametric t-tests were conducted to assess if any differences in specific bacteria were evident. The highest tertile intake of sugar i.e. over 35g was associated with lower proportions of cluster IX *Clostridium* (4.2%) compared to those with low intake i.e. <17.8g (7.7%), mean difference 3.6% (FDR corrected p=0.033). Conversely high intakes of sugar were associated with higher proportions of *Eubacterium biforme* than those with lower intakes (3.5% vs. 1.4%), mean difference 2.1% FDR corrected p=0.033. Higher intakes of protein (>28.1g) were associated with a higher relative frequency of *Streptococcus* (9.0% vs 4.2%), mean difference 4.8% (95% CI = 1.3%-8.0%), but lower proportions of *Enterobacteriaceae* (11.8% vs 22.2%), mean difference 10.5% (95% CI = 2.7%-18.6%).

Figure 4.9 Non-metric dimensional scaling (NMDS) plots and associated permutational analysis of variance (PERMANOVA) comparing faecal microbial profiles based on selected nutrient intakes

Sugar Intake
PERMANOVA FDR adjusted p=0.052

Low sugar intake (n=59)
High sugar intake (n=52)

Protein Intake
PERMANOVA FDR adjusted p=0.068

Low protein intake (n=52)
High protein intake (n=52)

Fibre Intake
PERMANOVA FDR adjusted p=0.013

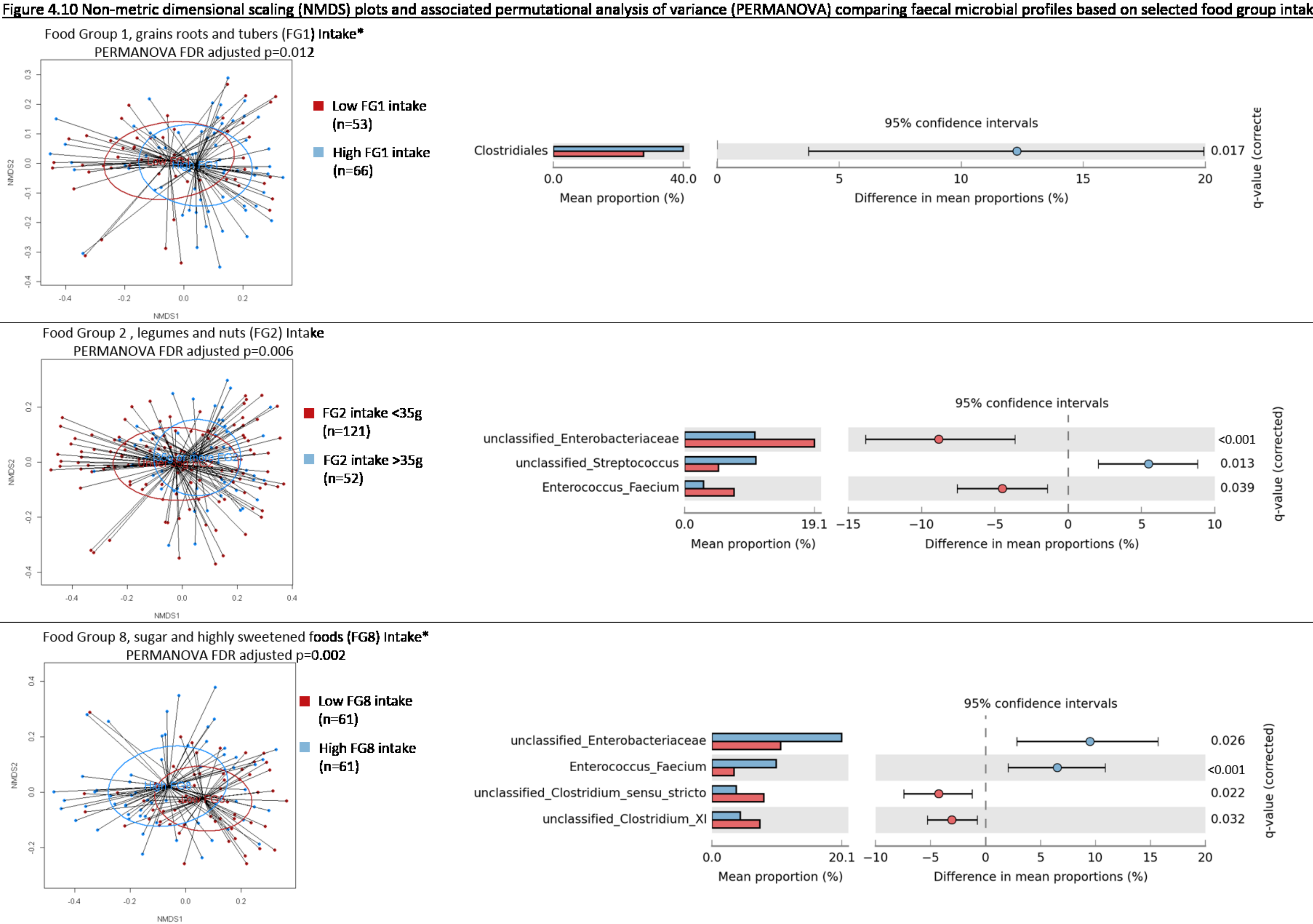
Low fibre intake (n=43)
High fibre intake (n=71)

Notes and definitions: "Low" scores = in first tertile (T1), "High" scores = in 3rd tertile (T3)

Sugar	T1≤17.8g	T3≥35.0g
Protein	T1≤14.4g	T3≥28.1g
Fibre	T1≤1.2g	T3≥5.4g

Associations between microbial profiles and food groups are presented in Figure 4.10. Figure 4.11 shows extended error bar plots for food groups with significant differences identified by NMDS and PERMANOVA. Only food group 1 (grains, roots, tubers and starchy banana, PERMANOVA p=0.012), food group 2 (legumes and nuts, PERMANOVA p=0.006), and food group 8 (sugar and highly sweetened foods, PERMANOVA p=0.002) were found to have statistically significant associations with gut microbial profiles. In FG1, those with the highest intakes were associated with higher proportions of *Clostridiales* (40.0% vs 27.7%), but at the genus level only *Sarcina* was associated high FG1 intake (3.8% vs. 0.5%). Those who ate less than 35g (10th percentile of intake) of FG2 foods in the 24hrs preceding faecal sampling were found to have lower proportions of the genus *Streptococcus* (5.0% vs 10.5%) than those with higher intakes. Low intakes of legumes and nuts (FG2) were also associated with higher proportions of the family *Enterobacteriaceae* (19.1% vs. 10.3%), and the species *E. faecium* (7.3% vs. 2.8%). FG8 closely followed the patterns seen in DP3, i.e. high intake of sugar and sweetened foods was associated with elevated *Enterobacteriaceae* (20.1% vs 10.6%, FDR corrected p=0.018) and *E. faecium* (10.0% vs. 3.4%, FDR corrected p=0.029), but lower proportions of *Clostridium sensu stricto* (3.8% vs. 8.0%, FDR corrected p=0.046) and cluster IX *Clostridium* (4.4% vs 7.4%, FDR corrected p=0.043).

Figure 4.10 Non-metric dimensional scaling (NMDS) plots and associated permutational analysis of variance (PERMANOVA) comparing faecal microbial profiles based on selected food group intakes



Notes and definitions: "No intake" = 0g, "Any intake" >0g; *"Low" scores = in first tertile (T1), "High" scores = in 3rd tertile (T3)
Food Group 1: T1≤309.6g T3≥595.1g Food Group 8: T1≤20.5g T3≥58.0g

Table 4.21 summarises statistical analyses assessing differences in richness and diversity associated with dietary factors including dietary diversity, dietary patterns and selected macronutrients. Dietary diversity was not associated with differences in measures of richness or diversity, $p>0.05$. Those who scored in the top tertile of dietary pattern 1 had significantly greater richness (S_{obs}=222.1 vs. T1=185.6, $p=0.034$) and diversity (Shannon index=3.23 vs. T1=2.70, $p=0.005$) than those scoring in the lowest tertile. The opposite pattern was observed for dietary pattern 3 where those in the lowest tertile had greater richness (T1 S_{obs}=227.9 vs. T3=188.8, $p=0.014$) and diversity (T1 Shannon index=3.37 vs. T3=Shannon index 2.87, $p=0.002$). Neither DP2 nor DP4 was associated with any difference in richness or diversity measures. Of the macronutrients analysed only fibre was associated with differences in diversity (T1 Shannon index 2.72 vs. T3 Shannon index 3.30, $p=0.018$). Richness was also greater in samples from children who reported higher fibre intakes (S_{obs} T3=220.0 vs. T1=186.0) but this did not reach the level of statistical significance ($p=0.051$). Sugar intake was also observed to be negatively associated with bacterial diversity (Shannon index T1=3.36 vs. T3=3.00), but this also did not reach statistical significance ($p=0.053$).

Table 4.22 displays statistical analysis comparing richness and diversity measure differences between differing intakes of food groups 1-9. Only food group 8 (sugar and highly sweetened foods) was associated with any differences in richness or diversity. Those with the highest intakes of FG8 (T3≥58.0g) had lower richness (S_{obs} = 192.0) and diversity (Shannon index = 2.87) compared to those with the lowest (T1<20.5g), S_{obs}=229.6 Shannon index=3.37, $p=0.021$ and $p=0.005$ respectively.

The above analyses comparing gut microbiota profiles across diets of differing diversity, food group, and nutrient content were also conducted within time points. These analyses resulted in fewer significant results, however significant results broadly agreed with the above, therefore the within time-point dietary analyses are not presented here for brevity.

Table 4.21 Differences in faecal bacterial richness (S_{obs}) and α-diversity (Shannon index) associated with dietary diversity, diet patterns, and nutrients

Measure	Dietary Diversity DDS<4 n=89	Dietary Diversity DDS≥4 n=84	p	Dietary Pattern 1 T1 n=54	Dietary Pattern 1 T3 n=56	p	Dietary Pattern 2 T1 n=46	Dietary Pattern 2 T3 n=68	p	Dietary Pattern 3 T1 n=57	Dietary Pattern 3 T3 n=63	p
S_{obs}* mean (SD)	198.7 (88.2)	215.3 (89.9)	0.220	185.6 (87.6)	222.1 (90.9)	0.034	202.4 (95.1)	209.0 (82.4)	0.699	227.9 (85.6)	188.8 (86.6)	0.014
Shannon index** median [IQR]	3.04 [1.36]	3.25 [0.92]	0.100	2.70 [1.62]	3.23 [0.96]	0.005	2.92 [1.02]	3.16 [1.15]	0.673	3.37 [1.06]	2.87 [1.29]	0.002

	Dietary Pattern 4 T1 n=69	Dietary Pattern 4 T3 n=53	p	Carbohydrate Intake T1 n=53	Carbohydrate Intake T3 n=57	p	Protein Intake T1 n=52	Protein Intake T3 n=52	p	Fat Intake T1 n=58	Fat Intake T3 n=49	p
S_{obs}* mean (SD)	200.8 (87.5)	214.1 (95.1)	0.429	201.6 (88.3)	214.8 (90.9)	0.439	199.8 (94.8)	212.6 (93.2)	0.489	204.4 (95.7)	200.1 (95.7)	0.828
Shannon index** median [IQR]	3.09 [0.99]	3.22 [1.13]	0.298	3.04 [1.42]	3.27 [1.19]	0.202	3.02 [1.48]	3.25 [1.15]	0.247	3.15 [1.15]	3.10 [1.27]	0.975

	Sugar Intake T1 n=59	Sugar Intake T3 n=52	p	Fibre Intake T1 n=43	Fibre Intake T3 n=71	p
S_{obs}* mean (SD)	222.3 (90.6)	195.6 (88.0)	0.120	186.0 (92.5)	220.0 (87.2)	0.051
Shannon index** median [IQR]	3.36 [0.94]	3.00 [1.00]	0.053	2.72 [1.74]	3.30 [0.79]	0.018

Abbreviations: S_{obs}, number of observed species; SD, standard deviation; IQR, interquartile range
*independent-samples t-test **Mann Whitney U test

Table 4.22 Site differences in faecal bacterial richness (S_{obs}) and α-diversity (Shannon index) (n=71)

	Food Group 1			Food Group 2			Food Group 3		
Measure	T1 n=53	T3 n=66	p	<35g n=121	≥35g n=52	p	No FG3 n=150	Any FG3 n=23	p
S_{obs}* mean (SD)	203.4 (89.2)	212.2 (87.6)	0.589	201.36 (90.5)	219.3 (85.7)	0.219	206 (86.2)	205.7 (109.0)	0.962
Shannon index** median [IQR]	2.80 [1.54]	3.28 [0.95]	0.141	3.10 [1.31]	3.13 [0.83]	0.322	3.13 [0.99]	3.09 [1.33]	0.848

	Food Group 4			Food Group 5			Food Group 6		
	<24g n=105	≥24g n=68	p	No FG5 n=169	Any FG5 n=4	p	<30g n=117	≥30g n=56	p
S_{obs}* mean (SD)	204.1 (94.6)	210.9 (80.6)	0.612	207.3 (89.5)	184.8 (82.9)	0.627	201.2 (85.9)	218.4 (95.3)	0.255
Shannon index** median [IQR]	3.10 [1.04]	3.26 [1.08]	0.538	3.10 [1.04]	2.72 [1.74]	0.353	3.07 [1.08]	3.27 [1.07]	0.208

	Food Group 7			Food Group 8			Food Group 9		
	No FG7 n=103	Any FG7 n=70	p	T1 n=61	T3 n=61	p	T1 n=49	T3 n=56	p
S_{obs}* mean (SD)	208.3 (86.6)	204.4 (93.5)	0.779	229.6 (88.3)	192.0 (89.9)	0.021	207.5 (94.1)	224.8 (88.6)	0.334
Shannon index** median [IQR]	3.10 [1.13]	3.11 [0.92]	0.699	3.37 [0.90]	2.87 [1.39]	0.005	3.10 [1.35]	3.34 [1.03]	0.167

Abbreviations: S_{obs}, number of observed species; SD, standard deviation; IQR, interquartile range

*independent-samples t-test

**Mann Whitney U test

4.2.3.6 ANTIBIOTIC EFFECTS ON THE GUT MICROBIOTA

Table 4.23 summarises the antibiotic treatments received in the subgroup of children in TRACT who also provided faecal samples that were selected for 16S rRNA profiling. A large proportion of children (72/74) received antibiotic treatment during their admission, inclusive of those randomised to receive cotrimoxazole prophylaxis. No differences in gut microbial profiles was observed between those receiving any antibiotic (including cotrimoxazole, n=51) and those receiving none (n=17) at day 28. At day 180, those receiving antibiotics (17/65) were found to have lower *Clostridiaceae* at the family-level than those not receiving antibiotics (7.2% vs 13.8% respectively, White's non-parametric t-test FDR corrected p=0.038) as well as lower *Blautia* at the genus level (2.3% vs 4.1%, p=0.015). Otherwise no differences were observed between those receiving antibiotics vs. those who were not. Because many children received different classes if antibiotics and different combinations across many classes of antibiotics, it was not possible to determine if cotrimoxazole prophylaxis had a lasting effect on gut microbiota populations.

Table 4.23 Summary of antibiotic treatment in TRACT children

	Time point		
Antibiotic treatment	Admission n=74	Day 28 n=68	Day 180 n=65
Any antibiotic (exc. cotrimoxazole)	68/74	24/68	17/65
Any antibiotic (inc. cotrimoxazole)	72/74	51/68	-
More than 1 antibiotic (exc. cotrimoxazole)	25/74	2/68	4/65
Amoxicillin	5/74	18/68	13/65
Ampicillin	7/74	0/68	0/65
Ampicloxacillin	3/74	2/68	2/65
Cefalexin	0/74	1/68	0/65
Ceftriaxone	57/74	3/68	3/65
Chloramphenicol	2/74	0/68	0/65
Ciprofloxacin	1/74	0/68	0/65
Cloxacillin	3/74	0/68	0/65
Cotrimoxazole (TRACT)	39/74	36/68	-
Gentamycin	18/74	2/68	1/65
Penicillin	1/74	0/68	0/65
Rifampicin	0/74	0/68	1/65

4.3 Discussion

Summary of main findings:

- Both urine and plasma metabolic profiles differed between sites and over time
- Urinary metabolomic changes included increased glucose and ketones at admission, and increased amino acids at later time points
- Plasma metabolomic changes included increased triacylglycerol, branched-chain amino acids and other amino acids at admission, and elevated lipid transport proteins at later time points.
- Metabolic profiles did not significantly differ between children with different reported dietary intakes, nor did they differ between TRACT interventions
- Gut microbiota richness and diversity increased over time, with simultaneous reductions in the relative proportions of some groups (*Enterobacteriaceae* & *E. faecium*) and increases in others (*Clostridia* and *Streptococcus*)
- Dietary diversity, certain dietary patterns, certain food groups and fibre intake were all associated with alterations in the gut microbiota
- Iron and folate supplementation was associated with increased *B. fragilis* and *F. prausnitzii*, but other TRACT interventions including antibiotic prophylaxis had no longer term effect on gut microbial populations

4.3.1 Site-associated Differences in Metabolic and Metataxonomic Profiles

4.3.2 Energy metabolism

Children being treated for severe anaemia in the TRACT study displayed significant alterations in their metabolic profiles associated with location (urine and plasma), study time point (urine and plasma), and some clinical measurements such as haemoglobin recovery (urine) and sickle cell status (plasma). Conversely TRACT study interventions including transfusion dose, micronutrient supplementation, and prophylactic antibiotics, and dietary intake did not appear to have significant effects on metabolic profiles.

In the current study the urine of children on admission contained relatively elevated concentrations of glucose and 3-hydroxybutyrate, indicative of metabolic stress associated with critical illness and dietary restriction. Glucose metabolism is known to be altered in severe illness, through increased secretion of cytokines and counter-regulatory stress hormones, increased gluconeogenesis and glycogenolysis, and peripheral insulin resistance, resulting in hyperglycaemia (Robinson and van

Soeren, 2004). Malaria, which was present in 71.7% of children, is also specifically associated with insulin resistance (Acquah et al., 2014), acute renal impairment (Sowunmi, 1996; Silva et al., 2017) and elevations in urinary ketones (Ephraim et al., 2015; Tobon-Castano et al., 2017). The observed metabolic alterations at admission are likely to be the result of these combined factors of elevated blood glucose, renal impairment, and dietary insufficiency. Raised plasma 3-hydroxybutyrate has also been associated with sepsis and inflammation in children in the acute care setting in developed countries (Mickiewicz et al., 2013), however only urinary 3-hydroxybutyrate was found to be elevated in this study. Citrate, a major intermediary in the tricarboxylic acid cycle was elevated by day 180 in children from Mbale, suggesting a return to this as a main energy metabolism pathway. Trigonelline (1-methyl nicotinate) was found to be elevated in children from Soroti at day 180, likely reflecting increased intake of niacin and increased carbohydrate energy metabolism (Kanehisa and Goto, 2000).

Plasma metabolic profiles partially support the hypothesis that the energy substrate of children had switched from carbohydrate to ketones derived from fat and/or protein catabolism. In both Mbale and Soroti, plasma concentrations of triacylglyceride were found to be elevated at admission, which is a frequently noted finding in studies examining the metabolic effects of acute undernutrition in children (Mayneris-Perxachs and Swann, 2019). From anthropometric data it is also clear that although similar numbers of children were underweight at admission, significantly more were wasted in Soroti (31.8%) compared to Mbale (13.7 %) suggesting acute undernutrition is of more concern at this location. Children in both study sites also displayed increasing concentrations of a further unknown lipid species (δ3.23-3.25broad) over time. Blood lipids are also observed to be dysregulated in critical illness: hypocholesterolaemia, particularly low levels of LDL cholesterol, are seen in adults and children with acute illness and sepsis (Dunham et al., 2003; Vermont et al., 2005). Dunham et al. (2003) also note that increasing LDL cholesterol over time is associated with recovery from severe illness, but low levels persist if infection or other insult occurs. VLDL/LDL cholesterol concentration was also observed to be higher in children from Mbale at all time points, when compared to Soroti, which may be partly accounted for by differences in nutritional status (wasting) and diet.

One relevant study examined the effect of iron deficiency anaemia on the serum metabolome of infants in Peru, however this study focusses on sex associated metabolic differences (McClorry et al., 2018). The predominant changes found in this study suggest that all iron deficiency anaemic children had reductions in serum concentrations of TCA cycle intermediates, namely succinate and fumarate. This was not found in the current study, although citrate was observed to be relatively increased in concentration by day 180. This study differed significantly from the current study in two important ways: 1) anaemia was defined as Hb <11.0g/dl (mild), and 2) none of the children had active malaria. Since the current study was concerned with severe anaemia (<5.0g Hb/dl) and over 70% of children

had active malaria infection, the studies are not directly comparable and the aetiology of anaemia is significantly different.

4.3.3 Amino acid derangement

Admission plasma phenylalanine concentration was consistently elevated at admission in both sites, while in Soroti tyrosine was also elevated. Elevated plasma phenylalanine levels have been previously reported in children and adults with malaria (Enwonwu et al., 1999; Lopansri et al., 2006; Rubach et al., 2015). One pathophysiological pathway of severe malaria, a decreased bioavailability of nitric oxide (NO), is related to phenylalanine metabolism. A cofactor of phenylalanine hydroxylase and tyrosine hydroxylase, tetrahydrobiopterin (BH_4), is required for NO production and reduced NO availability is associated with increased disease severity and impaired function in the microvasculature (Yeo et al., 2015). Rubach et al. (2015) have clearly demonstrated that there is reduced BH_4 availability in severe falciparum malaria, though the cause of the reduced availability remains unclear. Unlike other studies in malaria, the current study did not observe alterations in arginine metabolism (Olszewski et al., 2009). Elevation in plasma phenylalanine has been associated with increased disease severity (Yeo et al., 2015) and so the reduction in phenylalanine concentration by day 28 is associated with the successful treatment of malaria and reduction in the prevalence of 71% at admission to 9% by day 28. Phenylalanine, tyrosine and tryptophan are important precursors for synthesis of dopamine, norepinephrine and serotonin (Enwonwu et al., 1999), and it is thought that impaired production of these neurotransmitters, in combination with limited NO and the direct effects of hyperphenylalaninaemia on the brain can result in the progression to severe cerebral malaria (Ghosh et al., 2016).

Plasma N-acetylneuraminic acid concentrations were also observed to be elevated at admission in both study sites. N-acetylneuraminic acid is expressed on all human cell surfaces, and is utilised as a binding site by *Plasmodium falciparum* on erythrocytes (Varki and Gagneux, 2012). It is possible that this observation is related to red cell lysis, and thus the presentation of severe anaemia associated with malaria. Only one other unpublished study has reported an increase in this metabolite in severe malaria (Leopold, 2018), and its relevance to malaria was unclear.

Particular to children in Soroti, the concentration of branched chain amino acids (BCAAs: isoleucine, leucine, valine) were observed to be elevated at admission compared to day 28 (Table 4.12). These essential amino acids are frequently reported to be elevated in conditions of overnutrition, including childhood obesity and type 2 diabetes and inflammatory states generally (Butte et al., 2015). Conversely lower concentrations of serum BCAAs are associated with stunting in otherwise healthy

children (Semba et al., 2016; Mayneris-Perxachs and Swann, 2019), however the children in the current study were critically ill on admission. It is likely that in the current study, and taking into account evidence of acute dietary energy insufficiency (discussed in 4.3.1), the elevated levels of BCAAs particular to children from Soroti on admission are related to protein catabolism and their downstream use as a source of ketone bodies for energy metabolism. BCAAs have also been shown to play important roles in the signalling and regulation of adipose tissue catabolism and insulin resistance (Lerin et al., 2016; Nie et al., 2018). Although less likely it is possible that previously reported insulin resistance in severe anaemia (Acquah et al., 2014) and acute illness in children (Robinson and van Soeren, 2004) is mediated through elevation of circulating BCAAs, although this would require further investigation.

Children with sickle cell anaemia were observed to have elevated phosphorylcholine, however this was a relatively weak OPLS-DA model (Q^2 0.192). Since choline containing compounds including phosphorylcholine are an important component of the red blood cell membrane (Sathi et al., 2014), the limited lifespan and increased clearance of erythrocytes in this condition may explain this finding. In the same model phenylalanine concentrations were higher in those who did not have sickle cell anaemia. Sickle cell disease protects the host from progression to more severe forms of *Plasmodium* infection such as cerebral malaria, thought to be due to upregulation of haem oxygenase-1 (Ferreira et al., 2011). The finding that phenylalanine is not elevated in sickle cell disease compared to non-sickle cell counterparts with malaria may indicate a further mechanism, however due to the low model quality and lack of corroboration in Mbale, this should be interpreted with caution.

4.3.4 Gut Microbial Changes

The admission time point was the most distinctive with regards to the gut microbial profiles, with day 28 and day 180 differing little. Faecal microbial communities of admission samples were enriched by *Enterobacteriaceae* and *E. faecium* in line with the finding that critical illness is associated with a gut microbial population that favours pathogenic species (Wolff et al., 2018; Xu et al., 2019). Loss of diversity in the gut microbiome is also broadly associated with illness and poorer outcomes (Wolff et al., 2018). Reductions in diversity of gut microbial species are also associated with impaired host immune response (Holmes et al., 2012; Jacobs et al., 2017), potentially further increasing the risk of life threatening opportunistic infection in severely anaemic children.

Normal differences that would be expected between children of different age groups such as greater bacterial diversity with age, lower proportions of *Bifidobacterium longum* with age, and increases of *Firmicutes* and *Bacteroidetes* with age (Ximenez and Torres, 2017; Stewart et al., 2018) were not

observed here when younger and older children were compared. This may be due to the age grouping chosen (<5 and >5yrs) as many of changes associated with maturation of the gut microbiome are known to take place in the first 2-3 years of life (Stewart et al., 2018). Both richness and diversity increased over time, which is expected as age increases (Yatsunenko et al., 2012), however since there were little differences observed between children at day 28 and day 180, the age-related changes were less significant than those related to critical illness and medical treatment at admission. Although both richness and diversity increased from day 28 to day 180, this was not significant, however since it was a relatively short duration (5 months), it is possible that over time the difference would become significant as in other studies.

Other previously reported differences related to wasting or stunting that were not observed in the current study include greater proportions of *Gammaproteobacteria*, and reduced *Ruminococcaceae* and *Faecalibacterium prausnitzii* in undernourished children (Robertson et al., 2019). It is probable that the high usage of antibiotics, combined with limited dietary diversity, in the context of critical illness had greater effects on the gut microbiota, obliterating the usual expected differences related to age/nutritional status. One aspect that was not investigated in this study due to the emergency nature of the medical condition, is the priming effect of poor dietary diversity, sub-optimal nutritional status, and food hygiene practices on the gut microbiota. It is known that in LMIC settings even relatively healthy children have gut microbial populations which may include greater proportions of potentially pathogenic species including *Escherichia coli* and *Staphylococcus aureus* (Robertson et al., 2019). When a child from LMIC settings goes on to develop a critical condition such as severe anaemia, their pre-existing 'pathogen-favouring' gut microbes could combine with antibiotic use and further reduced dietary intake, to produce a uniquely pathogenic gut environment. The possibility of pre-existing gut microbe affecting response to antibiotics has been previously expressed in the literature, however the author is unaware of studies which have attempted to conclusively test this hypothesis (Dethlefsen et al., 2008; Yoon and Yoon, 2018). A prospective cohort study would be necessary to address the question of pre-existing risks associated with gut microbial populations definitively, however this would be prohibitively expensive.

4.3.5 Effect of TRACT interventions on metabolic profiles and gut microbiota

No differences in metabolic or metataxonomic profiles was evident between children who died and those who survived, possibly due to the statistical limitations of the low number of deaths (n=26/339). Of the TRACT study interventions, only the micronutrient randomisation was associated with any difference in gut microbial profiles, and no TRACT interventions were associated with metabolic

changes. Other studies have found that the administration of antibiotics has a profound effect on the composition, richness and diversity of the gut microbiota, and although cotrimoxazole was administered for 90 days in the current study, at day 28 and day 180 there was no effect on metabolic profile or gut microbiota. Iron and folate supplementation when compared with multivitamin multimineral supplementation encouraged the proliferation of *B. fragilis*, a known and frequently isolated opportunistic pathogen (Wexler, 2007). It appears that this is either a dose-specific response to iron, since the MVMM contained 10mg of iron vs 25mg in the iron-folate arm, or it is possible that the presence of other micronutrients has an additional effect, however this is less likely. Iron supplementation has been associated with other changes in the gut microbiota of children in sub-Saharan countries including expansion of *Enterobacteriaceae*, and contraction of *Lactobacilli* and *Bifidobacteria* (Zimmermann et al., 2010; Jaeggi et al., 2015). Although the current study did not find changes in the latter two genera, the finding that *B. fragilis* is also increased in high-dose iron supplementation is novel. *F. prausnitzii* is a member of *Ruminococcaceae* (*Firmicutes*), which is a commensal species, was also shown to be increased with oral iron, a finding which agrees with other studies (Dostal et al., 2013). Deschemin et al. (2016) suggest an interesting downstream effect of this finding: *F. prausnitzii* can induce elevations of ferritin in the colon, thereby increasing the iron storage and sequestration capability of the gut playing a possible role in maintaining immunity.

The relationship of *P. falciparum* malaria with the gut microbiota of children in Mali and Malawi has been investigated by Yooseph et al. (2015). They found that children at the lowest risk of contracting malaria had relatively higher proportions of *Enterobacteriaceae Escherichia/Shigella*, whereas in the current at admission when a majority of children had active malaria the highest proportions of *Enterobacteriaceae* (unclassified) were observed. Since the exact genera in the current study were not identified, it is possible that the genera concerned are different, or that active infection is associated with a different profile than those at high risk. The finding in the current study is more in line with critical illness being associated with a gut microbial population that lacks diversity and richness and favours pathogenic species which include many in the family *Enterobacteriaceae* and also *E. faecium* (Wolff et al., 2018; Xu et al., 2019).

4.3.6 Dietary Effects on Metabolic and Metataxonomic Profiles

While recent dietary intake is known to be a major contributor to the metabolic profile of urine (Garcia-Perez et al., 2017; Guasch-Ferre et al., 2018), diet did not appear to be an important determinant in the current study. Neither regression (OPLS-R) nor discriminant (OPLS-DA) multivariate models which considered dietary factors was deemed valid. It is hypothesised that the lack of dietary

effect was due to the sampling method: a spot sample when the child's fasting status was not controlled. This limited the systematic differences that would be expected and detectable by the methods of the current study, and is discussed in detail in the limitations section below (4.3.8). Many studies have reported that dietary patterns have been associated with reliable changes in plasma metabolites (Altmaier et al., 2011; Floegel et al., 2013; Bondia-Pons et al., 2015). The dietary patterns observed in the current study were not associated with any changes in the plasma metabolome of the children studied, despite significant differences in the foods/food groups eaten (Chapter 3). This finding can be explained using the rationale that the metabolism of these critically ill children is largely defined by their medical condition. As previously discussed, malaria-associated changes in the plasma metabolome (hyperphenylalaninaemia), alongside changes in other amino acids and lipid transport molecules are explainable by medical status. Since diet and clinical status improved in tandem over time, it is possible that the observed metabolic changes over time are a product of changes in both of these factors.

Some metabolic changes in both urine and plasma over time can be conceptually tied to dietary factors. For example elevation of urinary trigonelline, a metabolite of niacin, in later time points may reflect the increased carbohydrate energy metabolism which uses niacin as a cofactor (Wishart et al., 2018). Trigonelline can also be found in coffee (Wishart et al., 2018), however this was infrequently consumed in this study group. Hippuric acid, which also appears in urine at later time points, is a mammalian-microbial co-metabolite so it can reflect the combined effects of increased dietary substrates (polyphenols), increased gut bacterial metabolism and normalised gut microbial populations to produce benzoic acid and finally renal/hepatic conversion of benzoic acid to hippuric acid (Lees et al., 2013). As the production of hippuric acid requires adequate diet, and effective microbial and host metabolism, it is a non-specific indicator that all three components are in place and in the current study is related to generally improved clinical condition.

Metataxonomic profiles displayed stronger associations with diet than metabolic profiles. Greater dietary diversity resulted in higher proportions of the genus *Streptococcus* and *Erysipelotrichaceae*. *Erysipelotrichaceae* contain a diverse number of genera, with some being associated with inflammation while others support the gut immune system (Kaakoush, 2015). They have also been strongly associated with fat intake (Kaakoush, 2015), but this was not the case in the current study. The importance of the presence *Streptococci* is similarly contradictory, with some species being associated with a high risk of translocation and septicaemia, while others appear to be normal commensal bacteria in early life transmitted from the mother's skin (Adlerberth and Wold, 2009; Cassidy-Bushrow et al., 2016). The two dietary patterns that were associated with alterations in the gut microbiota (DP1 and DP3), appear to more closely align to what may be considered a more healthy

and less healthy gut microbiota, respectively. Children with a high intake of DP1 (carbohydrate-rich staples, legumes and beverages), which was also more associated with later time points, had higher proportions of *Prevotella copri*, and *Streptococci*, while those with lower intakes of this pattern had greater proportions of *Enterobacteriaceae* and *E. faecium*. There is a clear overlap with the time-associated changes, but since diet also varied with time these factors cannot easily be disentangled. *Prevotella copri* has the clearest relationship to this dietary pattern, being capable of metabolising dietary fibre (De Filippis et al., 2019) which both the staple foods (maize, millet) and legumes contain in high quantities. The expansion of *Enterobacteriaceae* and *E. faecium* is likely related more to the host's health, as both have been suggested to be markers of gut microbial "symbiosis" and poor general health (Shin et al., 2015). *Enterobacteriaceae* in particular is associated with undernutrition (Pham et al., 2019), although conversely it has been associated with conditions of overnutrition (Shin et al., 2015). An "oxygen hypothesis" has been proposed to explain the finding of elevated *Enterobacteriaceae* in illness. In health, gut oxygen is depleted by the gut epithelium, a capacity which is lost in the presence of inflammation (Rizzatti et al., 2017). The subsequent increase in gut oxygen leads to decreases in anaerobic genera, and expansion of aero-tolerant genera.

Fibre was the only individual nutrient to have a statistically significant association with the gut microbiota, which is unsurprising given it is the major energy substrate for many commensals. Higher intake of fibre (>5.4g/day) was associated with a lower proportion of *Enterobacteriaceae*, higher proportion of *Ruminococcaceae*, and higher measures of richness and diversity. *Ruminococcaceae* have been shown to be associated with higher fibre diets and lower risk of weight gain in the context of obesity (Flint, 2012; Menni et al., 2017), while greater richness and diversity are generally associated with better overall health. Food group analysis also suggests that *Clostridiales* are elevated with higher intakes of starchy foods (FG1), while legumes (FG2) are associated with lower proportions of *Enterobacteriaceae* and *E. faecium*. Only food group 8 (sugar and highly sweetened foods) appeared to have a "negative" effect on the gut microbiota, with higher intakes (>58.0g/day) being associated with lower richness and diversity and expansions of *Enterobacteriaceae* and *E. faecium*. This finding in particular highlights the difficulty in separating the effect of diet from other aspects of host physiology. Parents provided high amounts of sugar and sweet drinks to unwell children with poor appetites on admission (Chapter 3), so in addition to acute illness, intake of the protective dietary fibre was at its lowest. A direct causal effect cannot be inferred, however taken together this provides information about the combined effects of illness and inadequate diet.

4.3.7 Strengths

All three experiments (^{1}H NMR of urine and plasma and 16S rRNA profiling) were successful and showed a high degree of instrumental stability. In both of the ^{1}H NMR spectroscopy experiments, the quality control samples demonstrated high similarity and no evidence of systematic drift (Appendix X). Subsampling to 16,509 reads in the faecal microbial profiling experiment resulted in excellent coverage (>99% in all cases), suggesting that the subsampled data was a good representation of the total reads for each sample.

The gut microbiota of children with severe anaemia has only been characterised in one previous study (McClorry et al., 2018), which focussed on iron deficiency anaemia and gender differences in infants. This study selected mildly anaemia children who did not have malaria, therefore the current study represents a significant addition to the area of research.

Most published research on both metabolomics and gut microbiota in free living populations has largely ignored the dietary component. The current research provides the strongest example to date where high quality and highly detailed dietary data has been concurrently collected which reflects the acute effect of diet on both biofluid metabolomes and gut microbiota.

4.3.8 Limitations

This sub-study was part of a larger study already developed and underway at the time of commencing the sub-study. Sample collection procedures were therefore unable to be altered to a significant extent without incurring additional cost, time, and ethical review. Hence some procedures were not optimal for metabolomic or metataxonomic samples.

Although sample handling protocols were in place for plasma, urine and faeces, no monitoring of these systems took place, therefore it is not possible to confirm that all samples were handled per protocol. Indeed although plasma samples were to be collected in lithium heparin collection tubes, EDTA (ethylenediaminetetraacetic acid) was detected in 13 plasma samples. Samples may become contaminated, or contain microbes if the subject has systemic infection, or in the case of urine samples, renal or urinary tract infection. The metabolic activity of these microbes may alter the metabolome, especially at the higher ambient temperatures in Eastern Uganda, frequently >33°C. Microbial activity within faecal samples also continues after defaecation, altering the populations of microbes available for downstream analysis proportional to the time spent at ambient temperature. Anaerobic species will also be affected by atmospheric oxygen, making the microbiota profile of the stored sample further removed from the true physiological profile (Gratton et al., 2016). In the current

study, pragmatic controls were put in place (limiting time spent on ice on the ward to 30 minutes, and use of sterile collection containers), but especially on review visits this resulted in significant numbers of faecal samples not being collected if the child did not defecate during their time at the hospital. Admission faecal samples also had to be collected in a pragmatic fashion, given the emergency medical setting. It would have been unethical to withhold necessary medical treatment, including antibiotics, until a child had passed stool, therefore admission faecal samples are most likely from sometime within the first 48hrs of admission when a child had been stabilised. The literature indicates that antibiotics have profound effects on the gut microbiota within 4 days (Dethlefsen et al., 2008; Uhr et al., 2019), so necessary medical treatment may have already had a profound effect on the gut microbial profiles by the time of sampling in the current study. While this may be the case, the findings of this study (elevated *Enterobacteriaceae*/*E. faecium* and reduced diversity and richness) would be expected in a population of critically ill children. Future studies should take care to record the time/date of sampling and provision of treatments so this can be reported and considered in the interpretation of results. A further limitation is comparison of the richness estimate used in this study (S_{obs}) to other studies. S_{obs}, or the total number of observed species is uncorrected for relative abundance. Other studies which have reported on the gut microbial profiles of children in low or middle income countries have either failed to report richness or chosen another measure of richness such as Chao1 or ACE (Abundance-based Coverage Estimator) (Gotelli and Chao, 2013).

As changes in lipid metabolism and/or transport were a main finding of the plasma metabolomic profiling experiment, it is prudent to comment on the limitations of ^{1}H NMR spectroscopy in this respect. ^{1}H NMR spectroscopy is ideally suited to the analysis and identification of small (<1500kDa) compounds, other methods such as LC-MS are better suited to the classification and quantification of lipids (Yang and Han, 2016). Future studies of the plasma metabolome of children with acute illnesses should consider bearing this in mind, and running a dedicated method to analyse lipid classes and concentrations alongside untargeted techniques.

Urine samples collected in this study were spot samples that were not controlled for fasting state, also related to the emergency medical nature of the condition being investigated. Spot urine samples provide information only on the metabolism which took place up to the most recent emptying of the bladder. The effect of diet is clearer when using 24-hour pooled samples (Garcia-Perez et al., 2017; Maitre et al., 2017), which was not practical for the current study. As such the negative findings of any effect of diet or TRACT secondary interventions on urine metabolome could be explained by this limitation. The absorption, distribution, metabolism and excretion of both dietary components, micronutrient supplements, and drugs inherently vary and also vary between individuals, therefore any effect on a spot urine sample would be minimal and may be below the limit of detection for this

technology. Care should be taken in future studies to optimise urine sampling technique by 1) recording the time/date of sample collection and noting the most recent meal time/date, and 2) considering the collection of 24hr pooled samples if possible within the study design. Ethically it would be questionable to withhold food or intentionally fast a critically ill child who may also present with acute malnutrition, therefore controlling or specifying that fasted samples were required would likely be unethical and counterproductive.

As NMR is poorly tailored to explore changes in lipids, a more targeted and fully quantitated approach such as LC-MS would further clarify the relationship between acute illness in children, and lipid transport/metabolism derangements. Sample collection protocols must be monitored and additional information such as time and date of collection and fasting status will make interpretation of metabolic profiling and gut microbial profiling results more robust. There is currently no gold-standard method for the integration of metabolic, dietary and gut microbiota datasets. Each data type has its own characteristics, for example metabolomic and dietary data display high levels of intra-correlation, while 16S rRNA data are proportional rather than absolutely quantitative. Because of these issues, data integration is not straightforward, and although some methods have been proposed to address this, e.g. canonical correlation analysis (Hardoon et al., 2004), they are subject to error. Correlation analyses between these datasets are fraught due to the characteristics of proportional and highly-intra-correlated data, and liable to generate spurious results. Whole-genome shotgun sequencing of faecal DNA is now possible (Heather and Chain, 2016), which has the advantage of assessing the fungi, amoebae, viruses and phages also inhabiting the gut alongside bacteria, however it is expensive and the best methods for data processing and analysis is a subject of debate (Ranjan et al., 2016). Use of this method would also enable better identification at the genus and species level, which limited interpretability in the current study. Assessment of the faecal metabolome may also provide further insight into the environment and metabolic activity of the gut microbiome.

4.3.9 CONCLUSIONS AND FUTURE WORK

This study assessing metabolic and gut microbial profiles in severe childhood anaemia shows that the specific aetiology (e.g. malaria), the effects of acute illness and reduced dietary intake, and antibiotic treatment have the largest effects on these host metabolism and gut microbiota. Since clinical condition, diet, metabolism and gut microbiota all experienced changes over the course of the study, it is difficult to definitively determine causal or directional relationships. It may be inappropriate to do so since diet, metabolism and the gut microbiota are all connected, and perturbation of one has

effects on the others. The interactions between these factors should continue to be explored with the development of novel multivariate, multi-omics approaches.

Critical illness and diet are important determinants of the gut microbiota in severe anaemia. Reduced richness and diversity in gut microbiota are associated with poorer outcomes, but to date the dietary component has been underappreciated. This study questions whether the association between illness and altered gut microbiota is in fact the combined product of dietary changes associated with illness (reduced diversity, reliance on sugar as a source of energy) in addition to pathophysiological effects of illness and associated treatments. Dietary fibre was associated with the preservation of gut microbial richness and diversity, and of 'helpful' bacteria such as *Ruminococcaceae*, and reduction of pathogenic species e.g. *Enterobacteriaceae* and *E. faecium*. As such, the provision of prebiotics to prevent pathogen overgrowth in severely anaemic children warrants investigation.

Future studies in critical illness in children in African settings should confirm whether inclusion of a prebiotic compound promotes 'healthy' gut microbial profiles, and whether it is associated with improved outcomes.

5. Chapter 5 – General Discussion

5.1 Summary of findings

- A 24hr dietary recall tool augmented with physical portion size estimation tools provides similar estimates of energy, protein and iron intakes to independent weighed records
- Diets of children in eastern Uganda lack diversity and are micronutrient poor
- There are significant local variations in children's diets within eastern Uganda
- Four dietary patterns were evident with two patterns being strongly associated with plasma haemoglobin concentration
- Dietary pattern 1 (carbohydrate-rich staples, legumes and beverages) was positively associated with plasma haemoglobin concentration, as well as with weight and height
- Dietary pattern 3 (sweetened foods/drinks, fruit and eggs) was negatively associated with plasma haemoglobin
- Intake of legumes was associated with greater odds of recovery by day 180, and with reduced odds of being underweight
- Intake of energy and protein and most nutrients improves over time as children recover from acute illness making dietary effects difficult to isolate
- Urine and plasma metabolic profiles show clear differences between the study sites, possibly due to differences in age and general dietary intakes
- Metabolic profiles showed stark changes over time, most deemed to be associated with critical illness and related acute dietary insufficiency
- Reported dietary intakes did not show strong relationships with metabolic profiles, nor did TRACT study interventions
- Gut microbial species richness and diversity increased over time in tandem with clinical recovery, dietary improvement and resolution of metabolic derangements
- The proportions of *Enterobacteriaceae* and *E. faecium*, potential pathogenic species, which dominated admission faecal samples reduced over time while commensals such as *Clostridia* and *Streptococcus* increased
- Dietary diversity, dietary patterns, food groups, and fibre were all associated with differences in bacterial diversity, richness and certain genera
- Higher dose iron supplementation resulted in increased proportions of *B. fragilis*, a potential pathogen, but also *F. prausnitzii*, a symbiont

5.2 DIET AND SEVERE ANAEMIA

The 24hr dietary recall tool developed for use in the TRACT study overestimated energy, protein and iron intakes by minimal amounts (2.8%, 9.4%, and 8.3%), similar to other tools (Reilly et al., 2001). Other measures of agreement confirm a high correlation with the gold standard weighed food record including high intraclass correlation coefficient, high agreement of quartile cross-classification and associated Cohen's κ. Wide ranges of agreement were evident, however this was partially due to some extreme outliers with atypical food intakes, and it was determined that in a large sample size the mean error would be low.

As with other studies examining severe childhood anaemia, the sub-study of TRACT identified several likely mechanisms for the development of severe anaemia in this group including malaria, sickle cell disease, and inadequate nutrient intakes. A major finding of this study is that dietary diversity is a valuable measure that is associated with improved haemoglobin concentrations: each additional food group is associated with a 0.71g/dl improvement in haemoglobin concentration. Legumes were also observed to be associated with improved Hb recovery by day 180 (OR 1.13, p=0.047), as well as negatively associated with underweight (OR 0.92, p=0.017). As previously discussed legumes are a concentrated source of iron, protein and dietary fibre, and can also be grown in unfavourable conditions, so would be a suitable target for highlighting in local public health nutrition programmes.

Although previous studies have found that iron status is not an important factor in the development of severe anaemia (van Hensbroek et al., 2010), the current study found that even when children were recovered from acute illness by day 180 and likely to be consuming a "normal" diet, only 2.5-25.9% of children met requirements for iron. The intake of those who were not meeting requirements was approximately 36-54% of their estimated requirement, suggesting that iron deficiency is the background upon which malaria or other acute insult occurs. Indeed the majority of dietary iron came from non-haem sources in this study, in conjunction with foods which contain phytate, therefore the iron available for absorption may be even lower. To offset this, however, the intake of vitamin C was above requirements in in most children (75.5-85.2%) which promotes non-haem iron absorption Regardless, a significant association between dietary iron and haemoglobin recovery by day 180 was observed, which could indicate that although supplementary iron appears fraught with risks (Sazawal et al., 2006), dietary sources of iron may be a suitable alternative and cost-effective recommendation for children recovering from severe anaemia. The consumption of legumes as mentioned previously in addition to offal (organs) are locally acceptable and highly concentrated sources of iron and other nutrients known to be deficient in this population (vitamin A), and this approach could be safely

recommended as dietary advice for children who present with severe anaemia. The effectiveness of dietary strategies to improve iron status in this population warrants further research.

One concerning incidental finding is that a common method of screening for malnutrition, mid upper arm circumference <11.5cm, did not identify children who were underweight (19.8-22.4%) or wasted (13.7-31.8%) in this study. Nor did it reflect the acute dietary insufficiency of children, who showed evidence of ketosis. Inclusion of a brief screening question regarding recent dietary intake, e.g. assessing the number of food groups/dietary diversity taken in the past 24hrs, may identify children who could benefit from further assessment of micronutrient status or referral to the hospital nutrition unit for short term nutrition support.

5.3 TRACT Interventions

The TRACT study provided a unique opportunity to describe the roles of diet, metabolism and the gut microbiota in recovery from severe childhood anaemia, a condition with a high mortality rate of 19% within 6 months (Phiri et al., 2008). TRACT was a randomised controlled trial assessing the effectiveness of acute and long term treatments in severe anaemia, namely immediate transfusion strategy, post-discharge micronutrient supplementation strategy (90 days), and post-discharge antibiotic prophylaxis (90 days). Although the results of the main TRACT study were not published at the time of preparation of this thesis, the main findings were made available to the author and are restated for reference here from section 3.3.2. No difference was observed in the day 28 mortality between children with profound (Hb<4.0g/dl) or complicated severe anaemia (Hb<6.0g/dl) who received 30ml/kg (n=1598) vs. 20ml/kg transfusion (n=1598), Hazard Ratio 0.76 (95% CI 0.54-1.08, p=0.120). Nor was there any difference in day 28 mortality between children with uncomplicated severe anaemia who were randomised to receive immediate transfusion (n=778) vs. those who only received transfusion if triggered by complication (n=787), HR 0.54 (95% CI 0.22-1.36, p=0.190). Children who were randomised to receive iron and folate only (n=1901) had a similar risk of mortality by day 28 as those randomised to multivitamin multimineral supplementation (n=1911), HR 0.97 (95% CI 0.79-1.21, p=0.810). Lastly children randomised to receive cotrimoxazole (n=1922) vs. no antibiotic prophylaxis (n=2061) had a similar risk of day 28 mortality, HR 1.07 (95% CI 0.86-1.32, p=0.560). No interaction between the micronutrient strategy and the antibiotic prophylaxis strategy was observed.

In the dietary sub-study of TRACT, additional information was collected which supports the finding of the main study. For instance metabolic profiling revealed some significant metabolic changes over time reflecting malaria (hyperphenylalaninaemia), critical illness (raised plasma lipids, glycosuria), and acute dietary insufficiency (ketosis). However no TRACT interventions were associated with

differences in metabolic profiles. In addition they had little impact on gut microbial profiles, except for the higher dose of oral iron supplement (25-60mg/day in the IF arm vs. 10mg in the MVMM arm). Although the transfusion strategy was expected to have little impact on metabolic profiles, it was surprising that neither cotrimoxazole nor micronutrient supplementation had any effects on urine or plasma profiles.

5.4 Time point associated changes in diet, metabolic and gut microbial profiles

Perhaps the clearest results from dietary assessment, metabolomic and gut microbiota experiments were those comparing the time points. When children were most critically ill at admission they also had inadequate energy intake, a high reliance on sugar as an energy source (food group 8/dietary pattern 3), glycosuria, dyslipidaemia, ketosis and elevated BCAAs which may lead to insulin resistance (Robinson and van Soeren, 2004; Nie et al., 2018), and gut microbial profiles dominated by potential pathogens (*Enterobacteriaceae* and *E. faecium*) and those which can rely on non-dietary energy sources like mucin-degrading *Akkermansia*. Although clear relationships between the metabolomic and 16S experiments were not drawn, a general profile of a critically ill child with impaired nutritional intake, metabolic derangement and altered gut microbiota became evident. The directionality and correlation of these factors should be further investigated, but the current study clearly shows that these changes occur in tandem, so all three facets must be considered together.

It appears that a spontaneous approach of parents in these areas to nutritionally support children who are acutely unwell, is to provide dense sources of dietary energy in the form of sugar, frequently in as sugary beverages. The finding of glycosuria in children on admission suggests that this is not being adequately metabolised and/or that insulin resistance and renal insufficiency associated with critical illness (possibly via increased circulating BCAAs) inhibits its use as an energy substrate. The practice of providing sugary foods and drinks may therefore be maladaptive in children with severe anaemia.

5.5 Interplay of diet, metabolism and the gut microbiota

Metabolites of dietary origin were observed to change over time, such as trigonelline from niacin, however dietary data did not directly explain any of the metabolic changes observed in this study. This is most likely related to the dominant effects of sampling procedures used. Both plasma and urine have been shown to contain metabolites that can be related to acute dietary intake and nutritional status (Holmes et al., 2008; Garcia-Perez et al., 2017; Mayneris-Perxachs and Swann, 2019). In the current study, the precise time of collection or fasting status of the plasma and urine samples was not

available, therefore any association with recent dietary intake is difficult to assess. Urine samples were also taken as a single spot sample, and since many dietary metabolites are excreted over variable timescales depending on individual factors, it would be simply a fluke if the spot urine sample contained sufficient concentration to be detectable. Future studies may consider using a more robust collection technique, such as a pooled 24hr sample, however these approaches are not without drawbacks. Pooled samples are at greater risk of microbiological contamination and require refrigeration or the addition of preservative compounds that can interfere with the metabolic profile. Since refrigeration and refrigerated transport is not universally available in LMIC settings, the issue of optimising urine sample collection in LMICs for association with dietary data remains.

Several dietary factors were observed to have distinct effects on the gut microbiota. Dietary diversity, in addition to being associated with improvements in Hb concentration, was associated with enrichment of *Erysipelotrichaceae* and *Streptococcus*. Dietary pattern 1 (carbohydrate-rich staples, legumes and beverages) was associated with lower risk of underweight and also recovery of haemoglobin, in addition to greater gut microbial diversity and richness and lower proportions of *Enterobacteriaceae* and *E. faecium*. Dietary pattern 3 (sweetened foods/drinks, fruit, eggs was negatively associated with haemoglobin recovery, and positively associated with elevations potentially pathogenic *Enterobacteriaceae*. At a single nutrient level, it appears that fibre is likely to be a significant driver of these relationships. Both staple foods and legumes found in dietary pattern one are rich sources of dietary fibre, while the major foods found in dietary pattern 3 are low in fibre. Intake of sugar was found to have the inverse associations to fibre, i.e. it was associated with lower richness and diversity and greater potential intestinal pathogen load. It is clear from changes in diet over time that intakes of sugar and fibre were anti-correlated, so the ultimate effect may be combinatorial. This is a clear highlight of one issue of dietary data previously discussed, that there is significant intra correlation in dietary data.

The provision of supplemental dietary fibre to support the gut microbiota (prebiotic) has been assessed in the context of antibiotics, and this has been proven to have the potential to limit the deleterious effects of antibiotic therapy on gut microbial populations in vitro (Johnson et al., 2015). Since legumes are a locally available and affordable source of dietary fibre, a future study could assess whether their provision, or a prebiotic product derived from these locally available sources improves gut microbial profiles and outcome in critically ill children.

5.6 Limitations and future recommendations

The completion of the current study has highlighted some areas that warrant further research, and ways to optimise research in the area. Since the dietary assessment approach was directly concerned with assessing acute intake, future studies may wish to augment this. Additional study visits or additional dietary assessment methods such as food frequency relating to the intervening periods between visits could help to establish more reliably the relationship between habitual diet and recovery from severe anaemia. Recovery from severe anaemia by day 180 post admission in this study was affected by food-based dietary iron intake, but in the parent TRACT trial supplemental iron dosage did not affect outcome. Additional studies assessing the effect of food-based iron improvement strategies are warranted, since previous research has been focussed on supplemental forms which have well documented drawbacks. Supplemental iron and dietary fibre both appeared to affect the gut microbiota in the current study, however interactions and type of fibre were uncontrolled. Additional analysis of local food composition is required to improve the accuracy of dietary assessment, and this could include the characterisation of different dietary fibres in the main staple foods. High antibiotic use was observed in this study, and since dietary fibre was associated with improved gut microbial characteristics (fewer *Proteobacteria* and greater diversity/richness), the concomitant use of prebiotics warrants investigation in this group at high risk of enteric pathogen translocation. The function of the gut barrier was not assessed in this study, which would provide additional information regarding the elevated risk of infection. Sampling of urine and plasma for metabolic profiling requires high adherence to protocols to ensure differences observed are physiological, therefore future studies should pay particular attention to monitoring adherence so this limitation can be overcome.

5.7 Conclusions

From this study a profile of a typical severely anaemic child may be summarised as being acutely malnourished with grossly insufficient dietary intake, metabolically stressed as evidenced by ketosis and modified fatty acid transport systems, and with potentially pathogenic gut bacterial species enriched by the effects of acute illness, low diversity diet and/or antibiotic use. Dietary iron, but not supplemental iron was associated with Hb improvement, while dietary fibre was associated with a less pathogenic gut microbiota with greater diversity. The metabolic profiles of children with severe anaemia confirm the multifactorial nature of the condition with clear signatures of malaria and dietary insufficiency, and potential indicators of sickle cell anaemia.

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APPENDICES

APPENDIX I – TRACT STUDY PROTOCOL

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Trials

STUDY PROTOCOL Open Access

Transfusion and Treatment of severe anaemia in African children (TRACT): a study protocol for a randomised controlled trial

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Abstract

Background: In sub-Saharan Africa, where infectious diseases and nutritional deficiencies are common, severe anaemia is a common cause of paediatric hospital admission, yet the evidence to support current treatment recommendations is limited. To avert overuse of blood products, the World Health Organisation advocates a conservative transfusion policy and recommends iron, folate and anti-helminthics at discharge. Outcomes are unsatisfactory with high rates of in-hospital mortality (9–10 %), 6-month mortality and relapse (6 %). A definitive trial to establish best transfusion and treatment strategies to prevent both early and delayed mortality and relapse is warranted.

Methods/Design: TRACT is a multicentre randomised controlled trial of 3954 children aged 2 months to 12 years admitted to hospital with severe anaemia (haemoglobin < 6 g/dl). Children will be enrolled over 2 years in 4 centres in Uganda and Malawi and followed for 6 months. The trial will simultaneously evaluate (in a factorial trial with a 3 x 2 x 2 design) 3 ways to reduce short-term and longer-term mortality and morbidity following admission to hospital with severe anaemia in African children.
The trial will compare: (i) R1: liberal transfusion (30 ml/kg whole blood) versus conservative transfusion (20 ml/kg) versus no transfusion (control). The control is only for children with uncomplicated severe anaemia (haemoglobin 4–6 g/dl); (ii) R2: post-discharge multi-vitamin multi-mineral supplementation (including folate and iron) versus routine care (folate and iron) for 3 months; (iii) R3: post-discharge cotrimoxazole prophylaxis for 3 months versus no prophylaxis. All randomisations are open. Enrolment to the trial started September 2014 and is currently ongoing. Primary outcome is cumulative mortality to 4 weeks for the transfusion strategy comparisons, and to 6 months for the nutritional support/antibiotic prophylaxis comparisons. Secondary outcomes include mortality, morbidity (haematological correction, nutritional and infectious), safety and cost-effectiveness.

Discussion: If confirmed by the trial, a cheap and widely available 'bundle' of effective interventions, directed at immediate and downstream consequences of severe anaemia, could lead to substantial reductions in mortality in a substantial number of African children hospitalised with severe anaemia every year, if widely implemented.

Trial registration: Current Controlled Trials ISRCTN84086586, Approved 11 February 2013

Keywords: Children, Africa, Anaemia, Malaria, Sepsis, Transfusion, Micronutrients, Emergency medicine, Haemoglobinopathies, Antibiotic prophylaxis

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Background

In sub-Saharan Africa severe anaemia (SA) in children is a leading cause of hospital admission, a major cause of direct mortality [1] and a key factor in the approximately 600,000 malaria deaths/year [2]. Guidelines developed by the World Health Organisation (WHO) encourage the rational use of blood transfusion to preserve this scarce resource and to reduce the risk of transfusion-transmitted infections [3]. However, the evidence base supporting these guidelines is weak, adherence is poor and national transfusion recommendations vary between sub-Saharan Africa countries [4]. Outcomes following SA are unsatisfactory with high rates of in-hospital (9–10 %) [5] and 6-month (12 %) mortality, and relapse or re-hospitalisation (6 %) [6], indicating that the current recommendations and their implementation are not working in practice. Further, the aetiology of SA is frequently multi-factorial, including potentially treatable co-morbidities such as bacteraemia and multiple vitamin deficiencies – key determinants of outcome [7] that are not addressed in current treatment guidelines. Although the 2 most recent systematic reviews (both published in 2000) indicated the need for formal evaluation of the restrictive transfusion policy supported by the World Health Organisation (WHO) in a controlled trial [5, 8], little progress has been made in the intervening decade. The poor outcomes and recurrent morbidity of children with SA warrant a definitive trial to establish best transfusion and treatment strategies to prevent both early and delayed mortality and relapse.

Current WHO recommendations

Transfusion

To avert overuse of blood products the WHO advocates a conservative transfusion policy, reserving blood for children with a haemoglobin (Hb) < 4 g/dl (or < 6 g/dl if accompanied by complications). Although not systematically evaluated, this conservative transfusion policy has been incorporated in WHO paediatric hospital practice guidelines. However, the specific recommendations in these guidelines contain inconsistencies and ambiguities [9] resulting in variation in practice across African countries, most particularly in the subgroup with 'uncomplicated' SA (Hb 4–6 g/dl without severe symptoms) where transfusion avoidance is recommended [4, 8]. A Cochrane review including the only 2 African randomised controlled trials (RCTs) [10, 11] conducted to date (involving 114 and 116 children randomised to blood transfusion or oral haematinics) concluded that there was insufficient information on whether routinely giving blood to clinically stable children with SA either reduces death or results in a higher haematocrit measured at 1 month, and indicated the need for a definitive trial [8].

Overall mortality in children with Hb < 4 g/dl or SA with life-threatening complications is 15 % [5]. Clinical studies in Kenya [12, 13] have shown that profound anaemia (Hb < 4 g/dl) is independently associated with death (odds ratio; OR = 2 · 5), as is SA (defined in this study as a Hb < 5 g/dl) complicated by reduced consciousness (OR = 7 · 4) or respiratory distress (OR = 4 · 1). Many deaths occur within 48 hours of admission, with 25–50 % [14, 15] occurring within 6 hours. In the FEAST trial, which enrolled children with shock, a higher case fatality was found in those with anaemia compared to those without anaemia, irrespective of intervention group [16]. In children with uncomplicated SA – Hb 4–6 g/dl without prostration or respiratory distress – overall case fatality is 4–6 %, being lower in parasitaemic children (2–3 %) [17] than in those with negative malarial slides (8–10 %) [12]. The ratio of complicated to uncomplicated SA is commonly 1:1 [18].

Current transfusion guidelines are conservative not only in terms of criteria applied for administering a transfusion at all, but also in terms of the volume of blood transfused. Currently, 20 ml/kg of whole blood (or 10 ml/kg packed cells) are recommended for all levels of anaemia below Hb < 6 g/dl [19]. Using standard formulae to calculate volume required [20] this under-treats children with profound anaemia by approximately 30 % and this volume may not, therefore, be sufficient to correct anaemia [4].

Other treatment recommendations for severe anaemia

WHO treatment guidelines deal specifically with acute treatment of malaria and with folate and iron deficiency, together widely held as the most important causes of anaemia. There are no specific recommendations for subsequent infection prophylaxis (including against malaria) [3]. In the only comprehensive case-control study (SeVana) of children hospitalised with SA in Africa [7], key aetiological factors for SA were bacteraemia (OR = 5.3; 95 % confidence interval; CI 2.6–10.9), malaria (2.3; 1.6–3.3), hookworm (4.8; 2.0–11.8), HIV infection (2.0; 1.0–3.8), vitamin A deficiency (2.8; 1.3–5.8) and vitamin B_{12} deficiency (2.2; 1.4–3.6). A subsequent publication, reporting the long-term outcome of children in this study, HIV infection was found to be the major risk factor both for 18-month post-discharge mortality (hazard ratio (HR) 10.5, 95 % CI 4.0–27.2) and for recurrence of SA (HR 5.6, 95 % CI 1.6–20.1). Children admitted with bacteraemia were also at an increased risk of post-discharge all cause mortality (HR 2.2, 95 % CI 0.8–5.6).

With respect to current treatment recommendations neither iron nor folate deficiencies were factors for SA being less prevalent among cases than controls (without SA) in the SeVana study. Thus, although folate supplementation is recommended, folate deficiency was not found in the

Malawian SeVana study [6], in agreement with previous reports [21] and observations that folate supplementation in anaemic children with malaria failed to raise Hb concentrations [22]. Moreover, vitamin B_{12} and vitamin A supplementation are not recommended in guidelines for the management of SA. Iron supplementation is effective for reduction of iron deficiency and anaemia in iron-deficient children. However, a community-based randomised controlled trial in Zanzibar designed to evaluate the impact of zinc and iron plus folic acid supplementation on morbidity and mortality in young children showed that supplementation may also be associated with adverse effects, specifically increased risk of hospitalisation (primarily due to malaria and infectious disease), and mortality in malaria-endemic areas [23]. The WHO has revised its recommendations to advise that iron and folic acid should only be targeted towards those who are anaemic and at risk of iron deficiency. Establishing iron status in children hospitalised with SA, and more generally in paediatric populations living in malarial areas, is technically challenging [24, 25] and is rarely available in resource-limited hospitals, making the implementation of WHO guidelines challenging in the very areas that are most affected. The development of micronutrient powders (eg Nutromix™ or Sprinkles™; Hexagon Nutrition Pvt Ltd, Nashik, Maharastra, India), as a novel approach for delivering iron and other micronutrients, offers a chance to correct relevant nutrient deficiencies [26] and provide iron in lower doses; with good adherence in population-based studies [27].

With regard to infection prophylaxis the substantial mortality benefits (allied with extremely low rates of toxicity) associated with cotrimoxazole prophylaxis in HIV-infected children [28] have generally been attributed to reductions in bacterial infections [29, 30]. Of note, these benefits have been observed even in areas of high background resistance [31]. The fact that mortality benefits cannot be attributed solely to pneumonia [29, 30] raises the intriguing possibility that cotrimoxazole may act on a number of different pathways – the most important with regards to SA relapse being enteropathy and intestinal permeability, although any benefits of cotrimoxazole on microbial translocation and/or systemic immune activation, or on reducing recurrent infections during recovery from SA, could also impact longer-term morbidity. Cotrimoxazole has been shown to be effective in preventing malaria in HIV-uninfected children aged > 5 years [32], and in HIV-exposed uninfected (HIV-uninfected children born to HIV-infected mothers) and HIV-infected children [33], despite high levels of background parasite resistance to sulphamethoxazole.

In summary, the best available evidence suggests that key factors for poor long-term outcome following SA hospitalisation are nutritional factors and recurrent bacterial infection, the strongest potentially modifiable underlying causes of morbidity and mortality which we propose to address in this trial.

Methods/Design

Study objectives

The primary objective of the trial is to identify effective, safe and acceptable interventions to reduce short-term and longer-term mortality and morbidity following admission to hospital with SA in sub-Saharan Africa. There are 2 hypotheses being tested:

1) A liberal rather than a conservative blood transfusion policy will decrease mortality (cumulative to 4 weeks) in children admitted to hospital with SA (Hb < 6 g/dl).
2) Supplementary multi-vitamin multi-mineral (MVMM) treatments or additional cotrimoxazole prophylaxis or both for 3 months post discharge will reduce rates of readmission, SA relapse, re-transfusion or death (cumulative to 6 months) compared to current recommendations (iron and folate) and anti-helminthics in all (anti-helminthics if aged > 1 year).

Secondary objectives

i. To identify the most cost-effective interventions to reduce early mortality, and assess their budget impact
ii. To determine efficacy of long-term support strategies (MVMM and cotrimoxazole prophylaxis) on other markers of nutritional status and causes of death
iii. To determine the effect of transfusion strategies and long-term support strategies on markers of inflammation and immunological activation and function
iv. To identify the mechanism of action of the most effective interventions through focussed investigations of human genetic polymorphisms, molecular diagnostics, immunological activation, markers of gut barrier dysfunction, and haematological and nutritional response

Study design and population

TRACT is a multicentre randomised controlled trial of 3954 children aged 2 months to 12 years admitted to hospital with a Hb < 6 g/dl. Children will be enrolled over 2 years from 2 countries and followed for 6 months. The trial will simultaneously evaluate 3 ways to reduce short-term and longer-term mortality (primary endpoint) and morbidity following admission to hospital with SA in sub-Saharan Africa using a 3 x 2 x 2 factorial design. All randomisations will be open. Inclusion/exclusion criteria are detailed in Table 1.

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Aged 2 months to 12 years	Malignancy or other terminal illness
Severe anaemia (Hb < 6 g/dl) on the day of admission to hospital	Acute trauma or burns as main reason for admission
Care-giver willing/able to provide consent	Surgery as main reason for admission
	Chronic renal or liver failure
	Signs of bi-ventricular heart failure
	Known congenital or valvular heart disease (non-surgically corrected)
	Children who are exclusively breast fed (thus unable to take nutritional support)

Trial interventions

Each intervention addresses one of the potential approaches to reducing mortality and morbidity in children with SA (Fig. 1: Trial flow schema)

R1: Immediate liberal transfusion (30 ml/kg) versus immediate conservative transfusion (20 ml/kg) versus no transfusion (the last strategy only for children with uncomplicated SA with Hb 4–6 g/dl).
R2: Post-discharge MVMM supplementation for 3 months (which includes folate and iron) and anti-helminthics if aged > 1 years versus routine care (folate and iron at standard treatment doses (varies with age) for 3 months) and anti-helminthics if aged > 1 year.
R3: Post-discharge cotrimoxazole prophylaxis for 3 months versus no prophylaxis.

R1 addresses both conservative aspects of current guidelines: 'whether to give blood' in uncomplicated SA (4-6 g/dl without complications), and 'how much blood to give' in all children with SA. The transfusion and post-discharge interventions will be open-label for reasons of practicality and compliance.

Potential for interactions between the trial interventions

Because the transfusion, nutritional and antibiotic prophylaxis interventions approach different mechanisms for reducing short-term and long-term mortality/morbidity following SA, we consider that important interactions between randomised groups are unlikely. Further, any interactions that do exist are likely to be quantitative (slightly smaller/larger effects) rather than qualitative (effect on one background, no effect on another).

Ethics approvals

The trial protocol was reviewed and obtained approval from the Imperial College Research Ethics Committee (ICREC_13_1_11). In Uganda the trial was approved by the Research Ethics Committee in Uganda, Makerere University School of Medicine Research and Ethics Committee, Kampala (#REC ref 2013–050) and in Malawi by the College of Medicine Research and Ethics Committee, (P.03/13/1365). In both countries regulatory authorities' approval was also received.

Consent

Prospective written, informed consent will be obtained from parents or guardians of children who are considered to be sufficiently stable. Parents or guardians will be given an information sheet in their usual language containing details of the TRACT trial. 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. The right of the participant to refuse to participate without giving reasons must be respected. However, we anticipate a number of children presenting as emergencies where delay in study enrolment, and thus treatment, through a consent procedure would be unacceptable. A two-stage consent process in this circumstance and the details are covered under section 'Consent process for severe life-threatening anaemia' below.

Treatments given in the trial

Standard management

All trial patients will receive standard of care (SOC) including antibiotics (given intravenously or orally) and/or anti-malarial drugs following national guidelines, based on WHO syndromic patient management [3]. We will collect data on all administered drugs. Antipyretics, anticonvulsants and treatment for hypoglycaemia will be administered according to nationally agreed protocols. If required, maintenance fluids will be run at 3–4 ml/kg/hour irrespective of age until the child can drink and retain oral fluids. At discharge from hospital all aged children > 1 year will be receive empiric treatment for helminths (500 mg mebendazole or 400 mg albendazole in Malawi) in accordance to current recommendations (SOC) regardless of randomised allocation.

Trial treatments

Randomisation procedures

Randomisation in each part of the factorial will be stratified by centre and the other randomisations in the factorial. Randomisation lists, using variable block sizes, will be generated and kept at the Medical Research Council Clinical Trials Unit (MRC CTU), London. The randomisation envelopes and clinical packs will be prepared before the trial, with 1 set for complicated SA (R1a) and 1 for uncomplicated SA (R1b). Eligible children will be screened and recruited at the time of hospital admission. At enrolment sealed consecutively numbered opaque envelopes (opened in strict numeric order) will assign a TRACT trial number and indicate a clinical pack number. The clinical pack will be within the first 10 packs

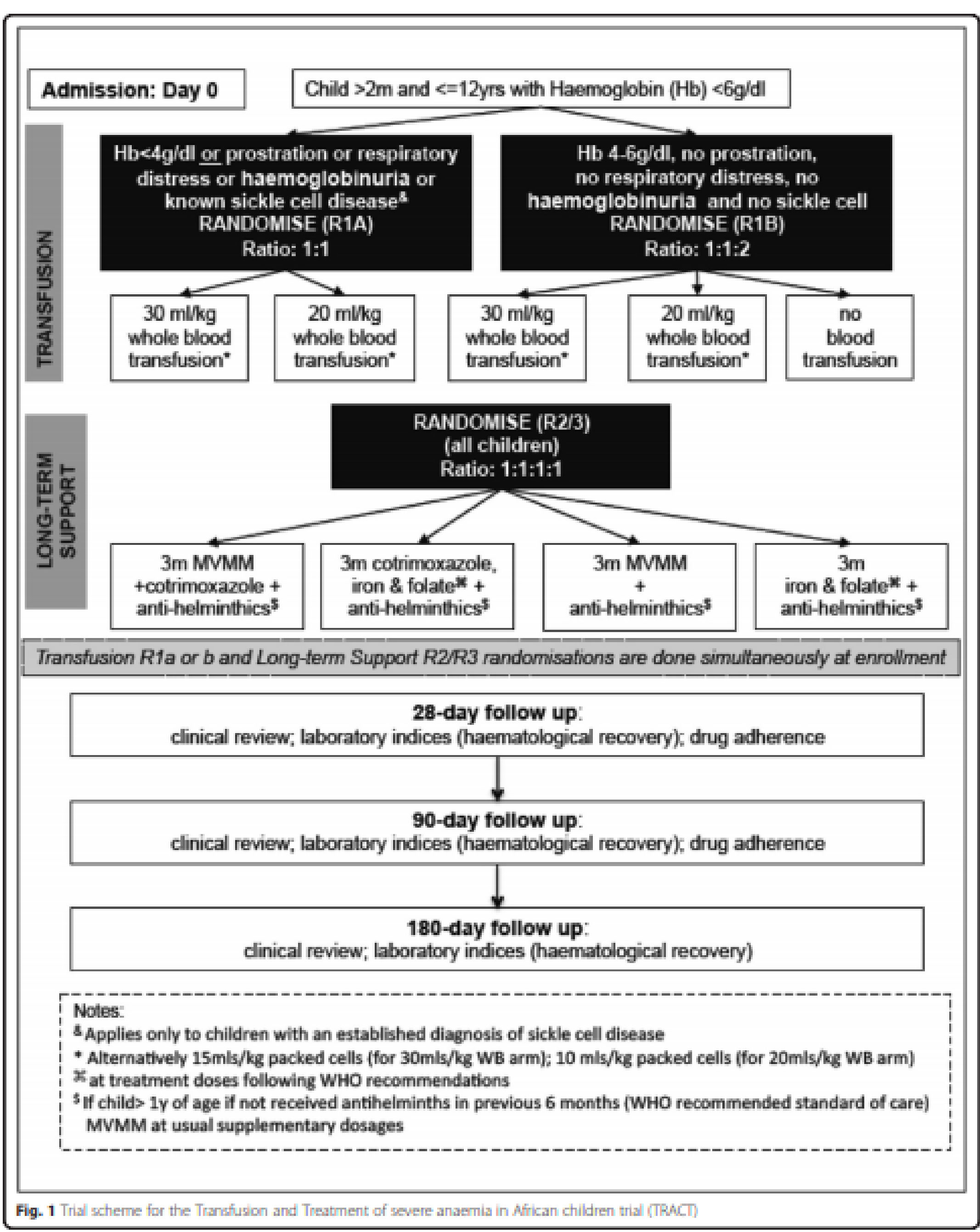


Fig. 1 Trial scheme for the Transfusion and Treatment of severe anaemia in African children trial (TRACT)

in the study filing cabinet but will not necessarily be the first remaining clinical pack number (an additional level of allocation concealment). Once opened, the clinical pack contains case report forms (CRFs) and a card which confirms the TRACT trial number, and simultaneously assigns transfusion interventions R1a/b (according to SA strata), micronutrient support (R2) and antimicrobial prophylaxis (R3) randomly. A similar system has worked well in the emergency care trial, FEAST [16].

The randomisation procedures and adherence to allocated treatments are reviewed at each independent monitoring visit. The process for this is detailed within the monitoring Standard Operating Procedure (SOP). The monitors are requested to check at each participating site whether: a) the randomisation envelopes are being correctly stored and used; b) whether enrolled children are allocated to the correct arm and c) that the allocated trial treatments (transfusion, MVMM/iron or folate and cotrimoxazole) were received by the child. It is also monitored electronically through central database monitoring, and also that both the Data Monitoring Committee (DMC) and the Trial Steering Committee (TSC) will monitor overall adherence to each of the randomised allocations.

R1 transfusion strategies

At enrolment children will be assessed at admission and will be divided into 2 groups for randomisation (R1a and R1b: see Table 2) based on: i) haemoglobin level, and ii) assessment of clinical severity children or complications (reduced conscious level; respiratory distress, acute history of haemoglobinuria or an established diagnosis of sickle cell disease).

R1a complicated severe anaemia

Children fulfilling these severity criteria will be randomly allocated on a 1:1 basis to receive one of the following:

- Whole blood transfusion 20 ml/kg, alternatively 10 ml/kg packed cells; or
- Whole blood transfusion 30 ml/kg, alternatively 15 ml/kg packed cells

R1b uncomplicated severe anaemia

Children with an Hb 4–6 g/dl without any severity features will be randomly allocated on a 1:1:2 basis to receive one of the following:

- Whole blood transfusion 20 ml/kg alternatively 10 ml/kg packed cells, or
- Whole blood transfusion 30 ml/kg alternatively 15 ml/kg packed cells, or
- No transfusion (control, SOC)

Table 2 Transfusion sub-groups

R1a Complicated severe anaemia: Hb < 4 g/dl *or* a Hb < 6 g/dl *plus* 1 or more signs of severity or complications
R1b Uncomplicated severe anaemia: Hb ≥ 4 and < 6 g/dl without any of the severity features or complications

Transfusion treatment schedule

A clinician or medical officer will prescribe the blood, using a calculator, to determine the volume of whole blood required (20 or 30 ml/kg). If only packed cells are available then the clinician must re-calculate the equivalent volumes of packed cells (10 or 15 ml/kg). Transfusions will be administered in gauged blood burettes; an initial aliquot (2 ml) will run into a sterile apex tube using an aseptic technique (and ensuring that the tip of the infusion set does not touch anything, to prevent contamination) and 1 drop taken from this to record the Hb and haematocrit of the donor blood. Whole blood will be run over 3–4 hours and packed cells can be administered over 2–3 hours.

For all children in the trial an additional, or initial (for SOC control group in R1b only), transfusion(s) will be permitted after 8 hours (at the point of the first reassessment of Hb) for children who still have either: (i) profound anaemia Hb < 4 g/dl, irrespective of other signs of severity; (ii) SA 4–6 g/dl and one or both de novo signs of severity (respiratory distress or impaired consciousness); (iii) uncorrected SA 4–6 g/dl in children with an acute history of haemoglobinuria or known sickle cell disease. Early sampling of Hb (<8 hours from baseline), and additional transfusion, will be permitted in children randomised to any group in the R1b strata (uncomplicated SA) developing de novo signs of severity.

If a child randomised to no-transfusion control (R1b only) meets the above criteria, they will receive 20 ml/kg whole blood or 10 ml/kg packed cells, as recommended by the WHO [3]. Children randomised to initially receive blood (R1a and R1b) who subsequently meet the above criteria will follow their randomisation arm: that is they will receive either an additional transfusion of 20 ml/kg or 30 ml/kg of whole blood (or 10 ml/kg or 15 ml/kg packed cells respectively). Any child who has already received 2 transfusions and subsequently fulfils the criteria above will receive a maximum of 20 ml/kg (or 10 ml/kg packed cells) irrespective of randomisation. Frusemide or other diuretics will be prescribed at the discretion of the attending physician and not used routinely in the trial.

R2 micronutrient support

Simultaneously to R1 randomisations, all children entering the trial will also be randomly allocated on a 1:1 basis to receive either multi-vitamin multi-mineral mix (MVMM: Nutromix™ which contains iron, folate and other MVMM) or iron and folate alone (at WHO-recommended doses) for 3 months post discharge. Nutromix™ has been

specifically designed for children of 6–24 months of age with SA [34–36]. The formulation, meets the recommended nutrient intake (RNI), particularly for vulnerable groups during emergencies [37]. RNI is defined as the daily dietary intake of a nutrient sufficient to meet the nutrient requirements of nearly all apparently healthy individuals in a specific population group, usually by age and sex. The dosage is 1 sachet to be taken daily by the child [26] and will be prescribed at the time of discharge from hospital (or 5 days from randomisation in those not discharged by this timepoint). In those children receiving iron (syrup or tablets) and folate tablets, for children aged < 2 years the recommended dosages are 25 mg iron: 100–400 μg folate; and for children > 2 years and < 12 years, 60 mg iron: 400 μg folate. Iron, folate and MVMM will all be given for 3 months, according to WHO guidelines for the management of SA.

The use of nutritional supplementation, including MVMM randomisation, will be pragmatic in that all children for whom these supplements should be received, according to WHO or national guidelines (e.g. those initially admitted with severe malnutrition), will receive them. Children aged < 6 months who are not weaned (fully breast-fed) will be excluded from the trial. For children with severe malnutrition, iron-containing supplements are not recommended during the first 7 days of acute rehabilitation (WHO guidelines) [3] but can be used effectively after this. For children with severe malnutrition discharged on ready-to-use therapeutic food (RUTF) which contains MVMM, children will essentially ignore their allocated MVMM randomisation, but will receive their standard post-discharge supplementation within the RUTF which would be recorded on study CRFs. The number of children with severe malnutrition as their admission diagnosis is expected to be small (< 5 %).

R3 antimicrobial prophylaxis

Children will be randomly allocated on a 1:1 basis to receive either receive: (i) cotrimoxazole prophylaxis for 3 months post discharge or (ii) no antibiotic prophylaxis post discharge (control, SOC). Cotrimoxazole dispersible tablets (240 mg: trimethoprim 40 mg/sulphamethoxazole 200 mg) will be used and dosing will follow WHO recommendations for prophylaxis in HIV-infected children: age 2 to 6 months: 120 mg; age 6 months to 5 years: 240 mg; children > 5 years: 480 mg [38]. The dispersible tablets may be taken with water or mixed with feeds. Cotrimoxazole will be prescribed from discharge (or 5 days from randomisation in those not discharged by this timepoint). The cotrimoxazole prophylaxis randomisation will be pragmatic in that all children for whom cotrimoxazole prophylaxis should be prescribed according to WHO or national guidelines (e.g. HIV-infected children) will receive it regardless of randomisation, and no child in whom it is contraindicated (e.g. known GP6D deficiency according to local testing) will receive it. Such children will essentially ignore their allocated cotrimoxazole randomisation; any cotrimoxazole received per guidelines would be recorded on study CRFs. The number of children with these conditions is expected to be small (< 5 %). HIV-infected children will receive antiretrovirals and will continue in the trial with HIV management and follow-up tailored in collaboration with local HIV clinics.

Co-enrolment guidelines

Patients will not ordinarily be permitted to participate in any other clinical intervention trial or research protocol while on the TRACT trial. Participation in other studies that do not involve an intervention may be acceptable, following agreement from the TRACT trial management group (TMG). The TRACT TMG will consider co-enrolment of TRACT participants onto other trials where the interventions do not conflict with the TRACT objectives on a case-by-case basis.

Measurement of endpoints

Assessment during hospital

The clinicians will complete a detailed clinical review on the CRF and perform a physical examination at enrolment. A symptom checklist and targeted physical examination will be performed at each subsequent clinical assessment. Children will be intensively monitored on the day of admission by the clinical team, and during any transfusion and then reviewed daily by the study team until discharge, with Hb testing performed at least 8 hourly in the first 24 hours, and daily thereafter. At each review conscious level, vital signs (heart rate, oxygen saturation, respiratory rate, axillary temperature, blood pressure) will be recorded, and examinations will specifically review the child for solicited adverse events. The doctor will be responsible for documenting and reporting serious adverse events (SAEs). Admission and final diagnoses will be recorded in the CRF.

Follow-up

A symptom checklist and targeted physical examination will be performed at each clinic visit post discharge. Medical history since last visit including hospital readmissions, transfusions, specific solicited adverse events, and grade 3 or 4 adverse events related to nutritional and antibiotic interventions will be documented by a doctor, including severity and likely relationship of any adverse events to trial interventions. At Day 28 adherence to and acceptability of MVMM and/or cotrimoxazole will be queried by carer self-report, and carers will be provided with a supply of drugs sufficient to last for the next 2

APPENDIX II – STANDARD OPERATING PROCEDURE (SOP) FOR DIETARY RECALLS (TRACT SUB-STUDY)

Standard Operating Procedure for Dietary Recalls

SOP Number:	Effective Date: 30/03/2015
Version Number & Date: Version 1.0 25/03/2015	Review Date: 06/07/2015
Superseded Version Number & Date: Draft version 06/02/2015	

Author: Kevin Walsh Position: PhD Student/Dietary sub-study coordinator E: k.walsh13@imperial.ac.uk	Signature: [redacted] Date: 25/03/2015
Reviewed by: Mudoola Macreen Position: Data Manager	Signature: [redacted] Date: 02/04/2015
Approved by: Dr Peter Olupot-Olupot Position: Site PI	Signature: [redacted] Date: 02/04/2015
Approved by: Prof Kathryn Maitland Position: Chief Investigator	Signature: [redacted] Date: 02/Apr/2015

Disclaimer

When using this document please ensure that the version you are using is the most up to date by contacting the author to confirm the current version.

Out of date documents must not be relied upon.

Acronyms

24DR	24 hour Dietary Recall
CRF	Case Report Form
CTU	Clinical Trials Unit
SD	Source Document
SOP	Standard Operating Procedure
TRACT	Transfusion and Treatment of severe anaemia in African Children: a randomised controlled Trial

1.0: Background

TRACT (TRansfusion and TReatment of severe Anaemia in African Children: a randomised controlled Trial) is a randomised controlled trial involving 3954 children aged 2 months to 12 years with severe anaemia (SA) (defined as a haemoglobin <6g/dl). Children will be enrolled at admission to hospital over 2 years from 2 countries (Malawi and Uganda) and followed for 6 months to make sure longer-term outcomes are captured. The trial has been designed to address the poor outcomes following severe anaemia in children in sub-Saharan Africa, indicating that the current recommendations and/or management strategies are not working in practice.

Nutritional intake is fundamentally important to the health of the child and there is an intimate relationship between nutritional intake, nutritional status and infection. Poor nutritional intake is also linked to anaemia, poor gut barrier function and impaired immunity. Nutritional intake can also be used as a surrogate marker of wellbeing. The measurement of nutritional intake is a balance between the complexity of user involvement and engagement of the target group. This sub-study of the TRACT trial is designed to use an interactive 24-hour dietary recall (24DR) to assess the nutritional intake of children at each assessment point. This will be undertaken before discharge at the initial presentation, at Day 28, and at Day 180. 24DR refers to food eaten the day prior to the assessment, for a full 24 hour period. Micro and macro-nutrient intakes will be assessed with Dietplan™ software. Results of dietary analysis will be related to markers of infection, appetite regulatory gut hormones, gut barrier function, and treatment outcome.

2.0: Purpose of SOP

The purpose of this SOP is to provide guidance for conducting, recording, and analysing the results of 24DRs. Procedures for conducting 24DRs, estimating portion sizes, calculating food weights using standard food items and volume, and recording data using the source document (SD) and case report forms (CRF) are clearly outlined in this SOP.

3.0: Responsible Personnel

In accordance with Good Clinical Practice (GCP), the TRACT sponsor has ensured that only qualified individuals are responsible for clinical and dietary data collection in the TRACT trial. Dietary recall analysis will be conducted by appropriately trained research staff in the Mbale and Soroti study sites.

4.0: Basic Terms and Concepts

Case report form: A printed, optical, or electronic document designed to record all the protocol-required information to be reported to the sponsor on each trial subject. In this case it refers to the TRACT Dietary Recall Sub-Study CRF v1.2 (Appendix I), where data from the source document is entered after being quantified using the Weight from Food Item and/or Weight from Volume databases.

Data: Refers to all the types of data collected stored and processed by our trials unit, i.e. both the scientific data garnered during the TRACT trial itself and the administrative data used in managing the trial.

Portion size estimation: Using standard terms of reference to establish the quantity (in grams) of foods or drinks reported.

Source document: These are original documents, data or records; in this case it refers to the TRACT Dietary Recall Sub-Study Source Document v1.2 (Appendix II) that is completed during the dietary recall process, where the food and drinks taken are recorded.

Triple-pass approach: A method to conduct 24DR to maximize the accuracy of information, in order to enable precise quantitation of micro and macronutrients.

5.0: Procedures

5.1 Overview, recruitment, and additional samples

All patients who enter the TRACT trial should be approached to be included in the sub-study unless they are partially breastfed (fully breastfed children are already excluded from TRACT). Only patients/guardians who consent to BOTH the main TRACT study AND the sub-study are eligible for the sub-study. They will have dietary recalls, and additional clinical samples stored at admission, Day 28 and Day 180 (NB not at day 90).

It should also be noted that recruitment to the sub-study will have a daily cap to ensure the additional burden on clinical and laboratory staff is not excessive. These caps may be reviewed in time as staff become more familiar with the procedures:

- Mbale site: maximum of 2 sub-study patients per day up to 14:00hrs (to allow 3hrs for PBMC isolation)
- Soroti site: maximum of 2 sub-study patients per day, no restriction on time recruited (PBMC will not be completed in Soroti)

Staff should refer to the main and sub-study flow charts for information on what samples/procedures are required at each time point. Once a patient is recruited, they will be randomized in the usual way according to the TRACT protocol. For patients who are to take part in the sub-study, an additional "Sub-study sample pack", and dietary recall SD and CRF should be collected. This will be available near to the usual packs. It is the responsibility of clinical staff to inform laboratory staff when additional packs are required, who will make up these packs as required.

Time point specific information:

<table>
<tr><td>Admission</td><td>Routine sub-study samples & dietary recall
Give faecal sample collection container + 1 x A4 paper
Instruction to collect 4 scoops from any stool passed on morning of Day 28 review
Re-state this during reminder call</td></tr>
<tr><td>Day 28 Review</td><td>Routine sub-study samples & dietary recall
If faecal sample not collected prior to review, to be collected during*
Give faecal sample collection container + 1 x A4 paper
Instruction to collect 4 scoops from any stool passed on morning of Day 180 review
Re-state this during reminder call</td></tr>
<tr><td>Day 180</td><td>Routine sub-study samples & dietary recall
If faecal sample not collected prior to review, to be collected during*</td></tr>
<tr><td colspan="2">*It is acknowledged that stool sample collection may prove difficult. Options to increase chance of successful collection:
Give sample collection container to take home
Reminder call to include instruction about faecal sample
Give small meal (e.g. porridge) to child on arrival
The study site coordinator should arrange this process, and it will be reviewed on an ongoing basis.</td></tr>
</table>

5.2 24 Hour Dietary Recall Assessment - Research Team

Equipment: TRACT Dietary Recall Sub-Study Source Document v1.2, portion size estimation props (teaspoon, tablespoon, cup, measuring jug, uncooked rice, play dough, water). Play dough recipe: 2 parts wheat flour: 1 part salt: approx. 1 part water → mix well and add extra flour/water until desired consistency.

TRACT ID, visit number, DOB, gender, weight (kg), height (cm) should be transcribed from the main TRACT form. Additional information must be obtained from the child or parent/guardian including

date the diet recall refers to (i.e. day prior to assessment), the day of the week it refers to, what time the child woke up, and who is providing the information (child/mother/father/aunt/uncle/guardian etc.).

The TRACT dietary recall source document is used to collect information about what the child ate the day prior to the assessment from the time the child woke, to that time the following day. It is conducted as an interview, and will take approximately 20-45 minutes.

Children who are old enough (approximately 5yrs and older) can be asked questions directly with support from parents. If the child is too young to understand, then their parent or guardian can provide the information on their behalf.

The dietary recall is introduced, and then conducted in three stages, known as passes with different functions.

Stage	Instructions	What to ask
Introduction		We would like to assess what your child ate yesterday starting with the first food or drink they had after they woke up, and finishing 24hours later. We will be using this information to see if the type of food children eat affects the bacteria "bugs" in their gut, and their appetite.
First pass Purpose of first pass is to get a detailed list of foods/drinks taken and how they were prepared. Portion sizes may be volunteered but are not required at this stage.	Begin with first food or drink Clarify the exact item taken and any additions Move onto the next food/drink	"You told me they woke at [time], when did they first eat or drink after this?" "What time was this taken?" "How was this food/drink prepared?" (fried/boiled/raw etc.) "What were the ingredients?" "Was anything added to it? Butter/sugar/oil?" "What did you/your child eat next?

	Closing the first pass	"You/your child went to sleep at [time]. Did they wake overnight, and if so did they eat anything?"
Second pass Purpose of second pass is to confirm what you have been told, and check for any misunderstanding. Check that no omissions have been made particularly with reference to added items (sugar, butter etc.).	Explain the 2nd pass Read the list from first to last item, allowing them to make corrections	"I will repeat the list of what you/your child ate yesterday. Please let me know if you would like to add or take away anything." "The first food/drink was [food/drink item] at [time] - was anything added to this?" "The next food/drink was [food/drink item] at [time] - was anything added to this?"
Third pass Purpose of third pass is to quantify the intake of food/drink	Explain that quantification is needed Request quantities for each food/drink listed Use equipment to assist with portion size estimates Standard sized foods may be used as reference e.g. small/medium/large bread bun Closing the third pass	"I would like you to tell me approximately how much of each food and drink you/your child took" "You/your child had [food/drink] to eat or drink first yesterday. How much do you think they ate/drank?" "Was the cup he/she ate/drank from this size? How full was it? Was there any left over?" "Was it a big spoon like this? Or a small spoon like this? How many spoonfuls? Were they heaped/level?" "Can you use this rice to show me how full the bowl/cup/plate was?" "Can you pull off some play-dough which is about the same size?" "How much was the bun in USH? Did he/she eat it all? How much was left over?" "Is there anything else you would like to add?"

Once dietary recall assessment is completed, the remaining empty sections of the SD should be crossed out, to show that there is not missing data.

Example of completed section of source document:

TRACT Dietary Recall Sub-study - Source Document v1.3 Valid from: 26th Feb 2015

TRACT ID: AXXXXC **Hospital no:** 123456	**Date of assessment:** 21 / 01 / 2015 **Date food eaten:** 20 / 01 / 2015
TRACT Visit: ☐ Admission X Day 28 ☐ Day 180 ☐ Extra Visit: ________ Day **Date of birth:** 01 / 01 / 2010 **Gender:** X Male ☐ Female **Weight (kg):** 25.0 **Height (cm):** 120.0 *24 hr dietary recall refers to food eaten the day prior to the assessment visit	**Day food eaten:** ☐ Wednesday ☐ Saturday ☐ Monday X Thursday ☐ Sunday ☐ Tuesday ☐ Friday **Time child woke up (24hr clock):** 07 : 30 **Who is providing info:** Child's mother

Time (24hr clock)	Food/Drink	Preparation	Portion Size
07.45 07.45	Milk Donut	Boiled with sugar and tea leaves Baked – no sugar	300ml cup 2 x tbsp. sugar 1 x medium donut
09.00	Egg	Boiled	1 x egg

5.3 Weight Calculation - Research Team

Equipment: Weight Calculation Database (Appendix III), TRACT Dietary Recall Sub-Study CRF v1.2

After the 24 hour dietary recall assessment is completed, portion sizes must be converted into weight in grams using the Volume to Weight or Food Item to Weight Calculator as appropriate.

Volume to Weight: Any portions estimated in millilitres can be calculated using the Volume to Weight tab in the Weight calculation database by finding the row for the item that was taken (e.g. milk), typing the volume into the blue column (e.g. 150 if the child took 150mls). This figure should be copied from the orange column into the Case Report Form.

Food Item to Weight: Any portions estimated in whole items or fractions of items (e.g. 1 x medium doughnut) can be calculated using the Food Item to Weight tab in the weight calculation database by finding the row for the food item size (e.g. doughnut - medium) and entering 1 in the blue column. The orange column will calculate the final weight, which should be copied onto the Case Report Form.

Once all weights have been calculated and recorded on the Case Report Form, the assessor should not save values in the blue columns. All other columns are locked and can only be amended by the dietary sub-study coordinator. If weights for any items cannot be calculated, or details are missing, the assessor should attempt to collect the missing information where possible. If this is not possible, the reason should be recorded. If the weight cannot be calculated due to an item not being present in the database, please contact the dietary study coordinator, who will arrange for the new food to be added to the database.

Example of weight calculations using Volume to Weight and Food Item to Weight tabs in the Weight Calculator Database:

Food Group	Item	Weight of 100ml	Portion Size (ml)	Weight (g)	
Drinks/Liquids	Milk/Milk tea/African tea	100	300	300	

Food Group	Item	Size	Weight of a whole item	Portion Size (number)	Weight (g)
Misc	sugar	teaspoon - level (small spoon)	6		0
		teaspoon - heaped (small spoon)	10		0
		tablespoon - level (large spoon)	11	1	11
		tablespoon - heaped (large spoon)	19		0
Snacks	Doughnut (Kaswa)	small	25		0
		medium	47	1	47
		large	176		0
Meat and Eggs	egg (boiled, no shell)		45	1	45
	egg (fried)		54		0
	egg yolk only		14		0

5.4 Case Report Form

Once dietary data has been converted into weight in (g), it is ready to be transferred onto the case report form. Each food/drink/added item should be listed as a separate line e.g. if sugar has been added to tea, it will be listed as a separate item with its own weight calculation. The preparation method should be added, but can be omitted if not applicable e.g. sugar is not "prepared". The original portion size estimation in millilitres or standard food sizes should be entered in the relevant column (only one of these columns should be filled), followed by the weight in (g) in the final column.

If the portion size of a certain food item cannot be estimated by the patient/guardian (e.g. amount of sugar added to bought fruit juice), code 9999 should be entered in the weight column. Where the assessor has not asked about the portion size and is unable to go back to the patient/guardian to clarify, code 8888 should be entered. The CRF may be handwritten or typed using the electronic form. An example of a section of a completed CRF is shown below:

TRACT Dietary Recall Sub-study – Case Report Form v1.3 Valid From: 26th Feb 2015

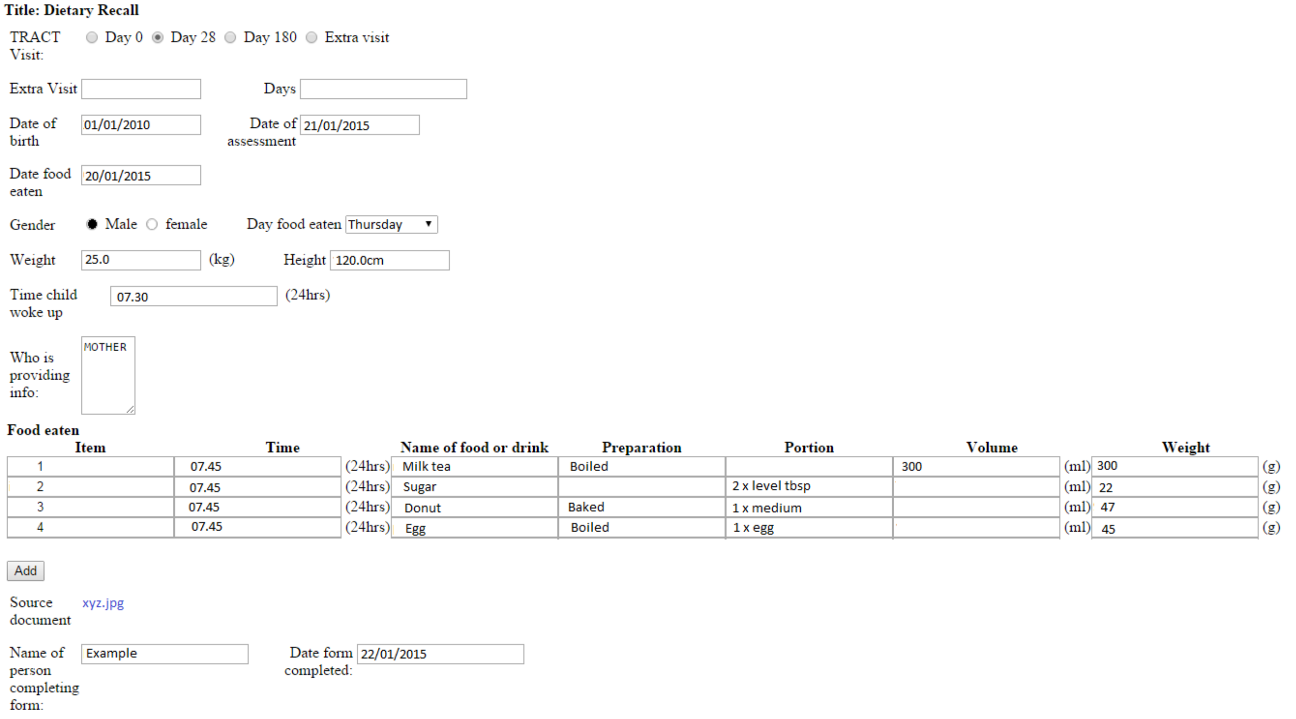
TRACT ID: AXXXXC			Hospital no: 123456			
	Time	Name of food or drink	Preparation	Portion *If estimated by Weight From Item*	Volume (ml) *If estimated using Weight from Volume*	Weight (g)*
Item 1	*07.45*	*Milk tea*	*Boiled*		*300*	*300*
Item 2	*07.45*	*Sugar*		*2 x level tbsp.*		*22*
Item 3	*07.45*	*Donut*	*Baked*	*1 x medium*		*47*
Item 4	*09.00*	*Egg*	*Boiled*	*1 x egg*		*45*

5.5 Data Entry

Data entry timeline:

Before discharge/during review visit	Complete diet recall and source document
Within 24hrs of completion of source document	Complete weight calculation and CRF
Within 48hrs of completion of CRF	Data from source document and CRF should be entered on database Source document should be scanned and uploaded to database
Within 1 week of database entry	Data should be double checked by sub-study coordinator and entered into Dietplan

An example of a completed section of the online form is shown below:



Title: Dietary Recall

TRACT Visit: ○ Day 0 ◉ Day 28 ○ Day 180 ○ Extra visit

Extra Visit: [ ] Days: [ ]

Date of birth: 01/01/2010 Date of assessment: 21/01/2015

Date food eaten: 20/01/2015

Gender: ● Male ○ female Day food eaten: Thursday

Weight: 25.0 (kg) Height: 120.0cm

Time child woke up: 07.30 (24hrs)

Who is providing info: MOTHER

Food eaten

Item	Time		Name of food or drink	Preparation	Portion	Volume		Weight	
1	07.45	(24hrs)	Milk tea	Boiled		300	(ml)	300	(g)
2	07.45	(24hrs)	Sugar		2 x level tbsp		(ml)	22	(g)
3	07.45	(24hrs)	Donut	Baked	1 x medium		(ml)	47	(g)
4	07.45	(24hrs)	Egg	Boiled	1 x egg		(ml)	45	(g)

Add

Source document: xyz.jpg

Name of person completing form: Example Date form completed: 22/01/2015

6.0 Data Storage

Once data has been entered onto the OpenClinica database, it will be saved in a separate database from main TRACT trial. As discussed with the trial statistician and database management, the study coordinator will have access to this database on an ongoing basis. This does not constitute unblinding, since no data relating to TRACT treatment arms will be included. It will also facilitate analysis of dietary data in a timely manner.

7.0 Quality Controls

Data will be double-entered by data entry clerks who will:

- Check that data on the CRF corresponds to that on the source document, with no omissions or errors in transcription
- Check for any missing information on the CRF including food/drink entered with no portion size or calculated weight

If either of the above are noted, this will be raised with the person who completed the dietary recall, to provide missing data. Within 1 week of uploading the source and data entry from the CRF completed, the study coordinator will endeavour to code this data into Dietplan software. This will constitute a further detailed quality control including:

- Checking that data on CRF corresponds to the source document
- Checking for missing data
- Check for errors in portion size calculation using the database (these will be corrected where obvious, or returned to the original interviewer for clarification/correction)
- Review data to assess if intakes of various foods/nutrients are realistic and physiologically plausible (these will be corrected where obvious, or returned to the original interviewer for clarification/correction)

APPENDIX III – DIETARY RECALL SOURCE DOCUMENT (TRACT SUB-STUDY)

TRACT Dietary Recall Sub-study - Source Document v1.3   **Valid from: 26th Feb 2015**

<table>
<tr><td>TRACT ID:
Hospital no:</td><td rowspan="2">Date of assessment: DD / MM / YYYY
Date food eaten: DD / MM / YYYY
Day food eaten: ☐ Wednesday ☐ Saturday
☐ Monday ☐ Thursday ☐ Sunday
☐ Tuesday ☐ Friday
Time child woke up (24hr clock): HH : MM
Who is providing info:</td></tr>
<tr><td>TRACT Visit: ☐ Admission ☐ Day 28 ☐ Day 180
☐ Extra Visit: ________ Day
Date of birth: DD / MM / YYYY
Gender: ☐ Male ☐ Female
Weight (kg): Height (cm):
*24 hr dietary recall refers to food eaten the day prior to the assessment visit</td></tr>
</table>

Time (24hr clock)	Food/Drink	Preparation	Portion Size

Once dietary recall is completed, please cross out any unused spaces

Was the child's food intake on this day unusual and/or were there problems obtaining or eating food on this day? ☐ **Yes** ☐ **No**

If Yes, why?

Assessor's Name:

APPENDIX IV – DIET RECALL CASE REPORT FORM (TRACT SUB-STUDY)

TRACT Dietary Recall Sub-study – Case Report Form v1.3 Valid From: 26th Feb 2015

TRACT ID:			Hospital no:			
	Time	**Name of food or drink**	**Preparation**	**Portion** *If estimated by Weight From Item*	**Volume (ml)** *If estimated using Weight from Volume*	**Weight (g)***
Item 1						
Item 2						
Item 3						
Item 4						
Item 5						
Item 6						
Item 7						
Item 8						
Item 9						
Item 10						
Item 11						
Item 12						
Item 13						
Item 14						
Item 15						
Item 16						
Item 17						
Item 18						
Item 19						
Item 20						
Item 21						
Item 22						
Item 23						
Item 24						
Item 25						
Item 26						
Item 27						
Item 28						
Name of person completing this form:						
Date form completed:						
***If food portion size is not known/remembered by the patient enter '8888 in 'Weight (g)' column** ***If food portion size was not requested by the assessor enter '9999' in the 'Weight (g)' column**						

Appendix V – Volume/portion size to weight conversion tables (TRACT sub-study)

Volume to weight:

Food Group	Item	Weight of 100ml	Portion Size (ml)	Weight (g)
Staples	posho	97		0
	rice (cooked)	79		0
	irish potato (boiled)	168		0
	sweet potato (boiled)	138		0
	millet/maize porridge	115		0
	bread/bun	37		0
	atapa (millet bread)	136		0
	maize flour	70		0
	millet flour	80		0
	cassava flour	50		0
	matoke	131		0
	yam (cubed and boiled)	57		0
Meat and Eggs	beef/cow meat (cooked)	100		0
	chicken (cooked)	99		0
	silver fish/mukene/omena (dried)	40		0
	Nile perch/tilapia (cooked)	98		0
	eggs, boiled	105		0
Vegetables	greens (any, cooked)	120		0
	greens (any, raw)	40		0
	beans in soup	88		0
	onions	76		0
	tomatoes	91		0
	carrot	91		0
	green pepper	71		0
	mushrooms (sliced, raw)	30		0
	mushrooms (sliced, fried)	46		0
	eggplant	79		0
Fruits	mango	110		0
	banana, ripe	101		0
	orange	96		0
	katogo fruit (mixed pineapple/mango/melon etc)	88		0
	jackfruit	118		0
	avocado	97		0
	paw paw	75		0
Drinks/Liquids	Water/Black tea/Dry tea	100		0
	Milk/Milk tea/African tea	100		0
	Lucozade	100		0
	Ribena	100		0
	Fruit juice (any)	110		0
	Soda (any)	110		0
	Soup from meat/beans	100		0
Snacks	Biscuit	40		0
	Mandazi	48		0
	Doughnut	48		0
	Popcorn	53		0
	Cake	80		0
	Sugar cane	98		0
	Cassava chips	100		0
	Pancake/Kabalagala	80		0

	soybeans (roasted/deep fried)	78		0
	groundnut (raw/roasted/fried)	62		0
Other	Groundnut/gnut paste	112		0
	Simsim paste	110		0
	Sugar	90		0
	Vegetable oil/cooking oil	90		0
	F75 supplement milk	106		0
	F100 supplement milk	106		0
	yoghurt	104		0

Portion size to weight

Food Group	Item	Size	Weight of a whole item	Portion Size (number)	Weight (g)
Staples	bread	small slice	16		0
		medium slice	24		0
		large slice	32		0
	irish potatoes (boiled)	small	58		0
		medium	106		0
		large	159		0
	chapatti	small	73		0
		medium	122		0
		large	188		0
Meat, fish and eggs	beef	small piece (approx 3cm each side)	26		0
		medium piece (approx 3.6cm each side)	46		0
		large piece (approx 4.5cm each side)	88		0
	chicken	small piece (approx 3cm each side)	26		0
		medium piece (approx 3.6cm each side)	45		0
		large piece (approx 4.5cm each side)	87		0
	fish (Nile perch/tilapia)	small piece (approx 3cm each side)	25		0
		medium piece (approx 3.6cm each side)	45		0
		large piece (approx 4.5cm each side)	86		0
	egg (boiled, no shell)		45		0
	egg (fried)		54		0
	egg yolk only		14		0
Vegetables	onion	small	31		0
		medium	71		0
		large	122		0
	tomatoes	small	52		0
		medium	76		0
		large	103		0
	carrot	small	41		0
		medium	64		0
		large	134		0
	green pepper	small	17		0
		medium	38		0
		large	68		0
	eggplant	small	7		0
		medium	13		0
		large	24		0
	boiled/roasted maize (on cob)	small	117		0
		medium	207		0
		large	399		0
Fruits	mango (without stone)	small	37		0
		medium	66		0
		large	239		0
	banana/matoke	small	50		0
		medium	91		0
		large	301		0
	orange	small	61		0
		medium	102		0
		large	134		0
	guava	small	28		0
		medium	56		0
		large	88		0
	avocado	small	100		0
		medium	145		0
		large	195		0
	paw paw	medium	280		0
Snacks	Biscuit	magic milk	1		0
		glucose	2		0
	samosa	small	29		0
		large	43		0
	Mandazi	small	25		0

		medium	47		0
		large (500 /=)	176		0
	Doughnut (Kaswa)	small	25		0
		medium	47		0
		large	176		0
	Sugar cane	20 cm piece	30		0
	Cassava chips	short (approx 11cm x 2cm)	26		0
		medium (approx 15cm x 1.5cm)	40		0
		long (approx 21cm x 1.5cm)	53		0
	Pancake/Kabalagala	small	29		0
		medium (100 /=)	43		0
		large	200		0
	Ban (bread)	small (100 /=)	25		0
		medium (200 /=)	50		0
		large (500 /=)	111		0
	cake	medium (500 /=)	112		0
	white ants	teaspoon - level (small spoon)	1		0
		teaspoon - heaped (small spoon)	3		0
		tablespoon - level (large spoon)	4		0
		tablespoon - heaped (large spoon)	6		0
Misc	vegetable oil	1 tablespoon	11		0
	sugar	teaspoon - level (small spoon)	6		0
		teaspoon - heaped (small spoon)	10		0
		tablespoon - level (large spoon)	11		0
		tablespoon - heaped (large spoon)	19		0

Appendix VI – Dietary recall coding issues and resolutions

Issue	General resolutions	Specific examples
Parent/guardian not able to recall	Reject	N/A
Ambiguous food description	Record as closest most common food item	"Beans" - assume stewed/with soup (USF TRACT 5) "Biscuits" - assume semi-sweet Ugandan biscuits (UKN 11 521) "Bread" - assume sliced white bread (UKN 11-468) "Cake" - use generic Ugandan cake (USF TRACT1) "Chapati" - assume made with fat (UKN 11-458) "Dried fish" - assume silverfish/mukene (USF TRACT 13) "Fish" - assume tilapia (UKN 16-154) "Fried pork" - assume fried pork loin steaks, lean and fat (UKN 18-266) "Greens" - use cowpea leaves (USF TRACT 11) "Irish potatoes" - assume boiled old potatoes (UKN 13-476) "Juice" - assume ready to drink juice (UKN 17-195) "Meat" - assume beef stewing steak (UKN 18-081) "Milk tea" - assume whole cow's milk (USF TRACT3) with no sugar unless specified "Peanuts" - assume roasted and salted (UKN 14-834) "Porridge" - assume maize porridge made on water (USF TRACT11) "Samosa" - assume vegetable (UKN 15 305) "Soda" - assume sweetened carbonated fruit juice drink (UKN 17-177) "Tea" - assume black with no sugar (UKN17-165) unless specified
Unspecified cooking method	Use standardised method	Chicken - assume fried Doughnut - assume fried Dried fish - assume boiled and pasted Fresh fish - assume fried Greens - assume boiled Meat/beef - assume stewed Mushrooms - assume fried Pork - assume fried Porridge (maize, millet) - assume boiled with water Samosa - assume fried
Portion size not estimated/recorded	>25% foods missing portion size: reject >1 staple food missing portion size: reject Otherwise use standardised values	Atap/posho/matooke lunch or dinner portion - 150g Banana - medium 91g Beans - 50g Beef/pork/chicken/fish - small piece 25g Biscuits - 4g Bogoya - small banana 50g Bread roll - medium 50g Bread slice - medium 24g Chapati - medium 122g Greens - 50g Groundnut paste - 10g Groundnut sauce - 20g Juice/water/milk - 1 cup 200ml Maize - 1 small 117g Peanuts - 10g Pineapple - 1 slice 50g Silverfish/mukene - 20g Sugar in porridge - 1tbsp 11g Sugar in tea - 1tbsp 11g

APPENDIX VII – RECIPES ADDED TO DIETPLAN FOR NUTRITIONAL ANALYSIS

Recipe Code	Description	Food or Ingredient	Quantity (%)
TRACT1	cassava chips, deep fried, Uganda	Cassava, boiled in unsalted water	75
		Vegetable oil, blended, average	25
TRACT2	posho, thick maize dough, Uganda	Water, distilled	66
		maize flour, boiled	34
TRACT3	millet porridge, made without milk, Uganda	Water, distilled	86
		Millet flour	14
TRACT4	Millet porridge, made with milk, Uganda	Water, distilled	80
		whole milk, Ugandan, cow's, raw	12
		Millet flour	7
TRACT5	beans in soup, stew, Uganda, boiled with tomatoes and onion	Pinto beans, dried, boiled in unsalted water	45
		Water, distilled	35
		Pinto beans, dried, raw	10
		Tomatoes, raw	9
		Onions, boiled in unsalted water	1
TRACT6	Boiled tomato soup, with onion, vegetable oil, Uganda	Tomatoes, raw	78
		Vegetable oil, blended, average	13
		Onions, boiled in unsalted water	9
TRACT7	meat soup only, no pieces of meat, Uganda	meat stock, Uganda, boiled	91
		Vegetable oil, blended, average	9
TRACT8	fish soup only, no pieces of fish, Uganda	fish stock, Uganda, boiled	94
		Vegetable oil, blended, average	6
TRACT9	pasted fish soup only, no pieces of fish, Uganda	fish stock, Uganda, boiled	80
		gnut powder, boiled, Uganda (groundnut powder, peanut powder)	18
		Tomatoes, raw	2
TRACT10	pasted fish soup with fish pieces, Uganda	Tilapia, Fresh, Boiled, Uganda	50
		pasted fish soup only, no pieces of fish, Uganda	50
TRACT11	maize porridge, Uganda, made without milk	Water, distilled	89
		maize flour, boiled	11
TRACT12	maize porridge, Uganda, made with milk	Water, distilled	75
		whole milk, Ugandan, cow's, raw	14
		maize flour, boiled	11
TRACT13	atap, millet bread, Uganda	Water, distilled	58
		cassava flour, raw, Uganda	34
		Millet flour	8

Recipe Code	Description	Food or Ingredient	Quantity (%)
TRACT14	Greens, Pasted, Uganda	Water, distilled	59
		Groundnut, Paste, Raw, Uganda	24
		Greens, average, boiled, Uganda	18
TRACT15	Groundnut sauce, Uganda	Water, distilled	69
		gnut powder, boiled, Uganda (groundnut powder, peanut powder)	20
		Groundnut, Paste, Raw, Uganda	5
		Tomatoes, raw	5
		Onions, boiled in unsalted water	1
TRACT16	Katogo matooke, tomato, onion (fried)	Matooke, boiled, Uganda	80
		Tomatoes, fried in blended oil	15
		Vegetable oil, blended, average	3
		Onions, fried in blended oil	2
TRACT17	Cassava bread, mingled, Uganda	Water, distilled	66
		cassava flour, raw, Uganda	34
TRACT18	Cabbage, fried w/ tomato and onion, Uganda	Cabbage, raw, average	80
		Tomatoes, raw	15
		Vegetable oil, blended, average	3
		Onions, raw	2
TRACT19	F-75 Supplemental Feed	Water, distilled	84
		Sugar, white	10
		Vegetable oil, blended, average	3
		Dried skimmed milk	3
TRACT20	F-100 Supplementary Feed	Water, distilled	81
		Dried skimmed milk	8
		Vegetable oil, blended, average	6
		Sugar, white	5
TRACT32	Tilapia, pasted, Uganda	Tilapia, Fresh, Boiled, Uganda	80
		Groundnut, Paste, Raw, Uganda	20
TRACT33	Silverfish, pasted, Uganda	Silverfish, dried, boiled, Uganda	80
		Groundnut, Paste, Raw, Uganda	20
TRACT34	Tilapia, fried, Uganda	Tilapia, Fresh, Boiled, Uganda	95
		Vegetable oil, blended, average	5
TRACT36	Meat, tomatoes, onions fried, Uganda	Beef, stewing steak, stewed, lean and fat	32
		Onions, raw	32
		Tomatoes, raw	32
		Vegetable oil, blended, average	4

Recipe Code	Description	Food or Ingredient	Quantity (%)
TRACT37	Fish soup, with fish pieces, boiled, Uganda	fish soup only, no pieces of fish, Uganda	75
		Tilapia, Fresh, Boiled, Uganda	25
TRACT38	Cassava, fried, with onions and tomatoes, Uganda	Cassava, boiled in unsalted water	76
		Tomatoes, fried in blended oil	11
		Onions, fried in blended oil	11
		Vegetable oil, blended, average	3
TRACT39	Mixed vegetable stew, Uganda	Water, distilled	19
		Tomatoes, canned, whole contents	11
		Tomato juice	7
		Haricot beans, dried, boiled in unsalted water	7
		Aduki beans, dried, boiled in unsalted water	7
		New potatoes, average, raw	7
		Parsnip, boiled in unsalted water	6
		Carrots, old, boiled in unsalted water	5
		Mushrooms, common, raw	5
		Courgette, raw	5
		Cauliflower, boiled in unsalted water	5
		Onions, fried in blended oil	4
		Peppers, capsicum, red, raw	4
		Lentils, green and brown, whole, dried, raw	3
		Cream, fresh, double	2
		Sacla Basil Pesto	1
		Garlic, raw	1
		Stock cubes, chicken	0

APPENDIX VIII – FOOD ITEMS ADDED TO DIETPLAN FOR NUTRITIONAL ANALYSIS

Dietplan Ref code	Food group	Uganda FCT Name	Uganda FCT Code	Description	Dietplan Food Name
TRACT1	AN	Cake, Basic Recipe, Baked	1351	Generic plain sponge cake from Ugandan ingredients	Cake, Ugandan
TRACT2	AN	Pancake, Banana/Cassava Flour, Fried	1350	Pancake, Banana/Cassava Flour, Fried	Pancakes, Ugandan (kabalagala), fried
TRACT3	BA	Milk, Cow, Whole, Fresh, Raw	9001		Whole milk, Ugandan, cow's, raw
TRACT4	AA	Maize flour , white variety, refined, raw *Boiled*	801041	Boiled maize flour for Ugandan posho recipe	Maize flour, boiled
TRACT5	G	*Groundnuts, powder, raw, *Boiled*	808007	Ground, boiled peanuts for use in groundnut paste	Gnut powder, boiled, Uganda (groundnut powder, peanut powder)
TRACT6	W	Soup, Stock, Beef, Home-prepared	41010	Beef stock	Meat stock, Uganda boiled
TRACT7	W	Soup, Stock, Fish, Home-prepared	43000	Fish stock	Fish stock, Uganda, boiled
TRACT8	SEC	Sugarcane, Raw	26001		Sugar cane, raw, whole cane, Uganda
TRACT9	AP	Mandazi, Inflated, Fried	1381	Deep fried African doughnut	Mandazi, Uganda doughnut
TRACT10	AA	Cassava flour, raw	2020		Cassava flour, raw, Uganda
TRACT11	DG	Cowpea leaves, fresh, boiled	18072	Boiled cowpea leaves for Eboo recipe	Cowpea leaves, fresh, boiled, Uganda
TRACT12	JA	*Fish, Tilapia (type 2), Fresh, Raw *Boiled*	814001	Boiled tilapia	Tilapia, Fresh, Boiled, Uganda
TRACT13	JA	*Fish, Silver Fish (Mukene), Dried, Raw *Boiled*	815021	Small silver fish, dried, then boiled and eaten whole	Silverfish, dried, boiled, Uganda
TRACT14	G	Groundnuts, Paste, Raw	8010	Paste of groundnuts (peanuts)	Groundnut paste, Raw, Uganda
TRACT15	Y	N/A	N/A	Nutritional supplement - company supplied composition info	Plumpy Nut
TRACT16	BNE	Yoghurt, Full Fat, Plain, Fresh, Raw	9201	Yoghurt-like fermented milk product	Bongo, fermented milk, Uganda
TRACT17	F	Banana, Matooke, Ripe, Fresh, Steamed	5013	Boiled mashed matooke banana	Matooke, boiled, Uganda
TRACT18	DG	Other green leaves, fresh, fried	18354	Average shallow fried greens (amaranth, cowpea, spinach etc)	Greens, average, fried, Uganda

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Validation of triple pass 24-hour dietary recall in Ugandan children by simultaneous weighed food assessment

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Abstract

Background—Undernutrition remains highly prevalent in African children, highlighting the need for accurately assessing dietary intake. In order to do so, the assessment method must be validated in the target population. A triple pass 24 hour dietary recall with volumetric portion size estimation has been described but not previously validated in African children. This study aimed to establish the relative validity of 24-hour dietary recalls of daily food consumption in healthy African children living in Mbale and Soroti, eastern Uganda compared to simultaneous weighed food records.

Methods—Quantitative assessment of daily food consumption by weighed food records followed by two independent assessments using triple pass 24-hour dietary recall on the following day. In conjunction with household measures and standard food sizes, volumes of liquid, dry rice, or play

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Ethics approval and consent to participate: This study was approved by the Mbale Research Ethics committee (Reference: 2013-050). Verbal consent was sought from parents or guardians for researchers to measure the child's weight, observe and weigh the child's food intake over one day, and for two separate 24 hour dietary recalls to be undertaken the following day. The child's assent and ongoing verbal parental/guardian consent was also sought prior to each activity.

Consent for publication of individual data: Not applicable

Availability of Data and Materials
The dataset supporting the conclusions of this article is included within the article and its additional files.

Competing interests: None.

Authors' contributions: HN, GF, KM, POO, and CE designed and planned the study. HN, TS, JN, CM, MN, MA, DA developed the method and carried out data collection. KJW analysed and interpreted the data. HN and KJW drafted the manuscript, and all authors commented on the manuscript.

dough were used to aid portion size estimation. Inter-assessor agreement, and agreement with weighed food records was conducted primarily by Bland-Altman analysis and secondly by intraclass correlation coefficients and quartile cross-classification.

Results—19 healthy children aged 6 months to 12 years were included in the study. Bland-Altman analysis showed 24-hour recall only marginally under-estimated energy (mean difference of 149kJ or 2.8%; limits of agreement -1618 to 1321kJ), protein (2.9g or 9.4%; -12.6 to 6.7g), and iron (0.43mg or 8.3%; -3.1 to 2.3mg). Quartile cross-classification was correct in 79% of cases for energy intake, and 89% for both protein and iron. The intraclass correlation coefficient between the separate dietary recalls for energy was 0.801 (95% CI, 0.429-0.933), indicating acceptable inter-observer agreement.

Conclusions—Dietary assessment using 24-hour dietary recall with volumetric portion size estimation resulted in similar and acceptable estimates of dietary intake compared with weighed food records and thus is considered a valid method for daily dietary intake assessment of children in communities with similar diets. The method will be utilised in a sub-study of a large randomised controlled trial addressing treatment in severe childhood anaemia.

Trial Registration—This study was approved by the Mbale Research Ethics committee (Reference: 2013-050). Transfusion and Treatment of severe Anaemia in African Children: a randomized controlled Trial (TRACT) registration: ISRCTN84086586.

Keywords

dietary assessment; validation; children; portion size estimation; Uganda; undernutrition

Background

Undernutrition, estimated to affect 100 million children in the developing world, is implicated in approximately 45% of childhood mortality globally[1 2] and its reduction has been one of the United Nations Millennium Development Goals since 2000s[3]. Aside from affecting mortality, poor nutrition in the first 1,000 days of life is also associated with impaired cognitive ability, and reduced school and work performance[4]. Nutritional intake is fundamentally important to the health of the child and there is an intimate relationship between nutritional intake, nutritional status and infection. In order to develop and assess nutritional strategies and policies aimed at reducing childhood undernutrition, evaluation and validation of reliable methods of quantifying an individual's macro- and micronutrient intakes are therefore of critical importance.

Several studies in African countries have used single methods for assessing diet including household consumption surveys[5], weighed food records[6], food frequency questionnaires[7 8], and 24-hour dietary recall (24hDR)[5–7 9] with variable success. Common methodologies, such as food frequency questionnaires and retrospective information on dietary history, are largely qualitative and considered as poor barometers of daily intake due to their imprecision[10–12]. Quantitative methods, measuring individual foods consumed (weighed food records, WFR) are the most precise methods for providing quantitative dietary data[13]. These are, however, time-consuming to conduct that results often in a small sample size, as they have been found to be burdensome and disruptive to the respondents. Interactive dietary

recall is a potential substitute for a weighed food record. This has been investigated in Ghanaian children14 and in Malawian children15 in studies using a single 24hDR the day following independent weighed food assessment. This method of dietary recall could only be considered partially validated in the study groups due to some biases and imprecision. The Ghanaian study reported that averaged 24hDR assessments tended to underestimate energy and nutrient intake compared with WFR, while the Malawian study reported the opposite. The over- and under-estimation of energy and nutrients may be reduced by the modifying the triple pass method for 24hDR, which has been shown to maximise recall accuracy for quantitation16–18 by including volumetric portion size estimation, but this has yet to be evaluated in African children.

The current pilot study sought to establish the relative validity of an interactive 24hDR method with volumetric portion size estimation, compared to concurrent WFR in children in rural Uganda. The tool is intended for future use to assess the impact of daily dietary intake on outcome for a controlled trial of children hospitalised with severe anaemia (Transfusion and Treatment of severe anaemia in African children: a randomised controlled Trial (TRACT), ISRCTN84086586)19.

Methods

Aim

The study's aims were first, to establish the relative validity of a 24hDR method compared to a weighed food record in estimating intakes of macro- and selected micro-nutrients in children in rural Uganda. Second, to ensure the recall method is feasible and culturally acceptable in this population.

Design

Dietary data from a weighed food record carried out by an independent researcher in the home of the subject was compared to estimated intakes from 24hDR assessments carried out by two other independent researchers the following day, to assess the relative validity of 24hDR. These researchers (clinicians and nurses) were not aware of the outcome of either the weighed food record or the other dietary recall. We opportunistically recruited 24 well children aged 6 months to 12 years attending Mbale and Soroti Regional Referral Hospitals over a two-week period in May 2014. We excluded infants who were entirely breastfed and children currently unwell. Prospective consent was sought from parents or guardians.

Pre-study training involved role-play simulations of data collection including recall and weighed food interviews with non-study child-parent pairs attending hospital to consolidate clinician and nurse training.

Portion size estimation

In developing the 24hDR method for this population, issues specific to East African diets emerged such as the estimation of portion sizes for semi-solid foods since much of the diet is a semi-solid consistency (such as a maize flour-based paste known as 'posho' or 'ugali') and eaten by hand, often from one communal family bowl20. Thus, it was problematic to

estimate by volume using standard household measures (bowlfuls, spoonfuls). We developed a number of novel approaches to estimate portion sizes (see Table 1). We considered an alternative method of estimating portions of semi-solid foods by utilising play dough and volume displacement, previously proposed16 but not yet validated. Estimated volumes or number of items eaten were then converted into grams. For this a database of local foodstuffs was generated with weight per 100mls or weight of a whole food item. Local reference sizes were used where appropriate (for example small/medium/large mango) or for certain foods including cassava chips or sugar cane three using representative lengths to which they were closest. Consensus approaches were agreed for other items, for example loaves of bread were classified by price, since these are consistently sized in this community.

Dietary data collection

Dietary data collection occurred in three stages: weighed food record (WFR) and two dietary recalls (DR) each carried out by a separate member of the research team following published protocols14, 15. Each researcher completed only one stage with each child and guardian in the home of the child and were blinded to details recorded by other observers. The details of each stage are summarised in Table 1.

For all measures the specific time frame was from the time the child awoke in the morning to the time they slept at night. Any food taken after this time was not included in either WFR or DR since it was not realistic to expect researchers to remain in participants' houses overnight.

The triple pass 24-hour recall, shown to maximise recall accuracy for quantitation18, used the following algorithm. The first pass encourages the respondent (guardian/parent) to freely report all food and drink intake for the prior day uninterrupted; in the second pass the interviewer probes for greater details on the exact time, type and quantity of food or drink taken; in the third and final pass the interviewer reviews all food reported in order, prompting for omissions and clarifying ambiguities. Completion of both DR used the same methodology and the same guardian and child to provide information about inter-assessor reliability and reproducibility. Interviews and assessments were carried out English or local languages to ensure accuracy.

Calculation of estimated requirements

Total daily energy and protein requirements were estimated using the methods recommended by the relevant World Health Organization (WHO), Food and Agriculture Organisation of the United Nations (FAO), United Nations University (UNU) or joint publications21 22. Iron requirements were based on the age and gender specific recommended daily allowances presented by Food and Nutrition Board of the US Institute of Medicine23.

Data entry and analysis

Data from WFR, DR1 and DR2 were entered into Dietplan 6 (Forestfield Software Limited), and energy, macro- and micronutrient intakes were automatically computed for most foods using McCance and Widdowstone's 'The Composition of Foods (Food Standards

Agency)'24. These were supplemented, when recipes or foods were not available, by the Ugandan Food Tables (UFT)25 which are derived from the United States Department of Agriculture National Nutrient Database for Standard Reference. For food items, such as milk, meat and flour, where composition may vary geographically, both UFT and The Composition of Foods values were compared, and generally the lower of the two values used. Some foods such as oil, and maize and wheat flours are fortified in Uganda with vitamin A, and iron respectively, however this does not appear to be consistent26. Since the current study is concerned with method validation only and as such, unfortified values have been used.

We could find no data of direct nutrient analysis of food in Uganda or East Africa therefore some uncertainty remains regarding the accuracy of food composition data in this setting. It is recognised that neither US based UFT values25, nor the UK Composition of Foods24 may reflect actual nutrient composition of Ugandan foods.

Statistical analysis

Weight-for-age z-scores (WAZ) were calculated with WHO Anthro using the WHO reference population27 and compared to the Uganda Demographic and Health Survey (UDHS), which use the median of the National Centre for Health Statistics28, Centres for Disease Control and Prevention (CDC)29, and WHO reference populations27. All other statistical analysis was completed using IBM SPSS Statistics for Windows v22 (IBM). Prior to statistical tests, Kolmogorov-Smirnov statistic and Q-Q plots were used to assess data distribution. Only estimated energy requirements were non-normally distributed, therefore Wilcoxon signed-rank test was used when comparing estimated energy requirements and estimated intakes and variability was assessed using interquartile range (IQR, 25-75th centiles). Bland-Altman analysis was conducted for a range of macro- and micronutrients, to compare each individual assessment of 24hDR (DR1 and DR2) and then to compare these with WFR30. Mean difference and standard deviation of the difference between each DR, and DR and reference method were generated for energy, protein and iron consumption, and reported as mean difference and limits of agreement (i.e. $\pm$ 1.96*standard deviation of mean difference).

The relationship between estimated intakes of energy, protein and iron were explored using intraclass correlation coefficients (ICC) and by quartile cross-classification. ICCs compared absolute agreement of average measures, using a two-way random model. Classification was defined as correct (same quartile), adjacent ($\pm$1 quartile), or grossly misclassified by 2 or more quartiles. Differences between estimated requirements and estimated intakes by WFR, DR1, and DR2 were analysed using paired t-tests. Initial analysis was completed between WFR, DR1 and DR2 in pairs. Statistical significance was defined as $p < 0.05$.

Results

Demographics and anthropometry

Of 24 children recruited (14 in Mbale and 10 in Soroti), two did not complete the dietary assessment and three were excluded due to recurring or new illness. Of the remaining 19, 12

were female (61.9%), mean age (±SD) was 3.4 years (±2.6), and mean weight (±SD) was 14.0kg (±5.6). The mean WAZ score (±SD) was -0.19 (±1.75). Three children were moderately or severely underweight defined as WAZ scores ≤ -2.0. The majority (n=13) had WAZ scores between -2 and 2. Three children had high WAZ scores ≥2. Four children were partially breastfed therefore were not included in comparisons with estimated requirements as determining a reliable 'portion size' was impossible. A post hoc power analysis showed that with 19 participants, this study has 80% power to detect a difference of 16.7% or 1097kJ in energy intake at a significance level of 0.05, using the mean energy consumption of 6563kJ and SD of 1706kJ.

Inter-assessor variation

Figure 1 shows Bland-Altman analysis with mean difference, absolute limits of agreement and percentage (%) between DR1 and DR2 for energy 289.4kJ, -2111.9 to 2690.6kJ (-40.0 to 51.0%); protein 1.3g, -9.93 to 12.6g (-32.8 to 41.7%); and iron 0.2mg, -2.5 to 2.8mg (-48.3 to 55.1%). The intraclass correlation coefficient for the two 24-hour dietary recalls for energy was 0.802 (95% CI, 0.429-0.933), for protein 0.925 (95% CI, 0.779-0.975), and for iron 0.868 (95% CI, 0.618-0.955) suggesting high inter-assessor reliability. Since the estimates by DR1 and DR2 for each of these parameters were comparable as assessed by cross-validation and Bland-Altman analyses, we therefore used the global mean of these estimates to compare with WFR data for conciseness.

Comparability of WFR and 24-hour dietary recall methods

Figure 2 shows the mean difference for energy was -149.1kJ with limits of agreement of -1619 to 1321kJ (-30.4 to 24.8%), mean difference for protein was -2.9g with limits of agreement of -12.6 to 6.7g (-40.4 to 21.6%) and mean difference for iron was -0.4mg with limits of agreement of -3.1 to 2.3mg (-60.2 to 43.7%). Mean differences with associated upper and lower limits of agreement comparing WFR and combined DR1 and DR2 are displayed for all nutrients included in Supplementary Table 1.

Intraclass correlation coefficients for WFR and combined 24-hour dietary recall estimates of nutritional intake were 0.979 (95% CI, 0.899-0.984) for energy, 0.972 (95% CI, 0.903-0.990) for protein, and 0.936 (95% CI, 0.837-0.975) for iron, summarized in Table 2.

Classification into quartiles of intake and assessment of this agreement by Cohen's Kappa (κ) statistic is displayed in Table 3. This showed that in the majority of cases WFR and dietary recalls agreed on classification, in 79% of cases for energy and 89% for protein and iron. The remainder were classified adjacently, with none being grossly misclassified. Agreement of classification in quartiles was substantial (κ 0.61-0.80) or almost perfect (κ 0.81-1.00) for all nutrients tested34.

Estimated requirements and intake

The median estimated requirement for energy was 4602kJ/day (IQR 25-75th centile = 3836-5208kJ), and intake was estimated at 6544kJ (IQR 25-75th centile = 5330-7448kJ) by the WFR, showing a significant surplus of 1942kJ (p=0.001). Mean estimated protein requirement was 14.2g (±5.1), while WFR-estimated intake was 40.0g (± 12.9)/day, 26.8g in

excess of requirements ($p<0.001$). Mean iron requirement was 8.3mg (±1.6)/day, while WFR-estimated intake was 6.6mg (± 2.6) ($p=0.004$).

Discussion

The 24-hour multi-pass recall method described compared favourably to a weighed food records, with regards to energy, protein and iron intakes. Bland-Altman analyses showed overall agreement for energy, protein and iron intakes between two separate interviewers, suggesting high inter-assessor reproducibility, which is further reinforced by high intraclass correlation coefficients. Classification of energy intake into quartiles showed substantial agreement for energy and almost perfect agreement for protein and iron intakes.

High intraclass correlation coefficients, and low mean differences for energy, protein and iron with weighed food records suggest the triple-pass 24 hour recalls are comparable for assessing daily intakes. The method suggested by Gibson & Ferguson16 was adapted to the local setting and validated in this pilot. Using play-dough and volume displacement generally worked well, and was intuitive for both researchers and subjects. The estimated nutrient intakes must be interpreted with caution owing to wide limits of agreement; in the case of iron particularly, only gross differences in intake can be inferred. For iron one extreme outlier was noted with 4.99mg lower estimated intake by recalls compared to WFR. The cause of this large discrepancy was found to be due to inaccuracy in the portion size estimation of a ready-to-use nutrient-dense nutritional supplement, which contributed over 6mg of iron alone to intake, the only instance in this pilot where this supplement was noted. Studies involving severely malnourished children are likely to encounter ready-to-use feed or calorie enhanced milks, and particular care in estimating the portion size is advised due to nutrient density, for the future study (TRACT), where it is intended to be used the numbers of children with severe malnutrition are anticipated to be few.

Although inter-assessor variability was assessed, this study did not address intra-observer repeatability, which must be borne in mind when the method is used. One limitation of only assessing the preceding 24-hour period is that a habitual identical intake cannot be assumed. Both dietary recalls were undertaken on the same day, which may have introduced bias in parental recall, for example memory of information provided during the first recall may have been reinforced for the second recall, whether accurate or not, thus artificially reducing the inter-assessor variability. Noteworthy, is that whilst the results presented using the mean of two 24-hour dietary recalls will technically reduce the observed variability, the inter-assessor variability was low, therefore conducting a single recall should not have a substantive effect.

Although method validation was the main aim of this study, it is prudent to comment on the intakes observed. Energy intake was higher than in previous reported studies, at 6563kJ compared to 5606kJ31. Intakes of energy and protein were also in excess of requirements by a factor of 1.39 for energy and 2.97 for protein. Similar high protein intakes of 41.0g/day have been reported in children in other regions of Uganda31. Another potential reason for the difference is variation over the week in energy consumption, which is not reflected on a single day recall assessment. Two reasons are suggested for this observation. Firstly, although it was explained to participants that the priority was to observe the children's

intake unbiased, the effect of the researchers' presence is difficult to estimate. Secondly, while these were healthy children, all had had recent contact with healthcare services, and as such may be experiencing catch-up growth and provided with additional food for recuperation. Indeed, WAZ scores observed showed that 16% were severely or moderately underweight, and is similar to the most recent UDHS 2011 census for the Eastern Uganda region32, where prevalence was 15.4%. In contrast to the UDHS results which showed only 0.1% had WAZ scores >2, compared to 15.8% (n=3) of subjects in this pilot.

The methods we have described and validated in children in Uganda appear consistent and correlate satisfactorily with quantitative assessment of dietary intake. A study comparing a single pass 24DR to assess dietary intake with a subsequent 7-day weighed food record in Sri Lankan adults found that 24DR tended to underestimate mean energy levels and macronutrients however the difference in the energy percentages were not statistically different33. Underestimation using single pass 24DR has been previously reported and is improved by triple-pass 24DR17 18. We consider that the method we have assessed to be valid for an on going a factorial treatment trial of African children presenting to hospital with severe anaemia (TRACT trial)19. The method will be used to assess nutritional intake as a surrogate marker of general wellbeing and the association of acute nutritional intake with severity of anaemia, impaired gut barrier function and susceptibility to infection. The TRACT study combines sequential dietary intake assessment using the multi-pass method at each follow-up visit to estimate macro and micro nutrient intake and will be subsequently linked to biomarkers of gut barrier function, gut microbiome, immunity and hormonal appetite control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

24hDR	24 hour dietary recall
CDC	Centres for Disease Control and Prevention
DR	dietary recall
FAO	Food and Agricultural Organisation of the United Nations
ICC	intraclass correlation coefficient
IQR	interquartile range
LOA	Level of Agreement

SD	standard deviation
TRACT	Transfusion and Treatment of severe anaemia in African children: a randomised controlled Trial
UDHS	Ugandan Demographic and Health Survey
UFT	Ugandan Food Tables
UNU	United Nations University
WAZ	weight-for-age z-score
WFR	weighed food record
WHO	World Health Organisation

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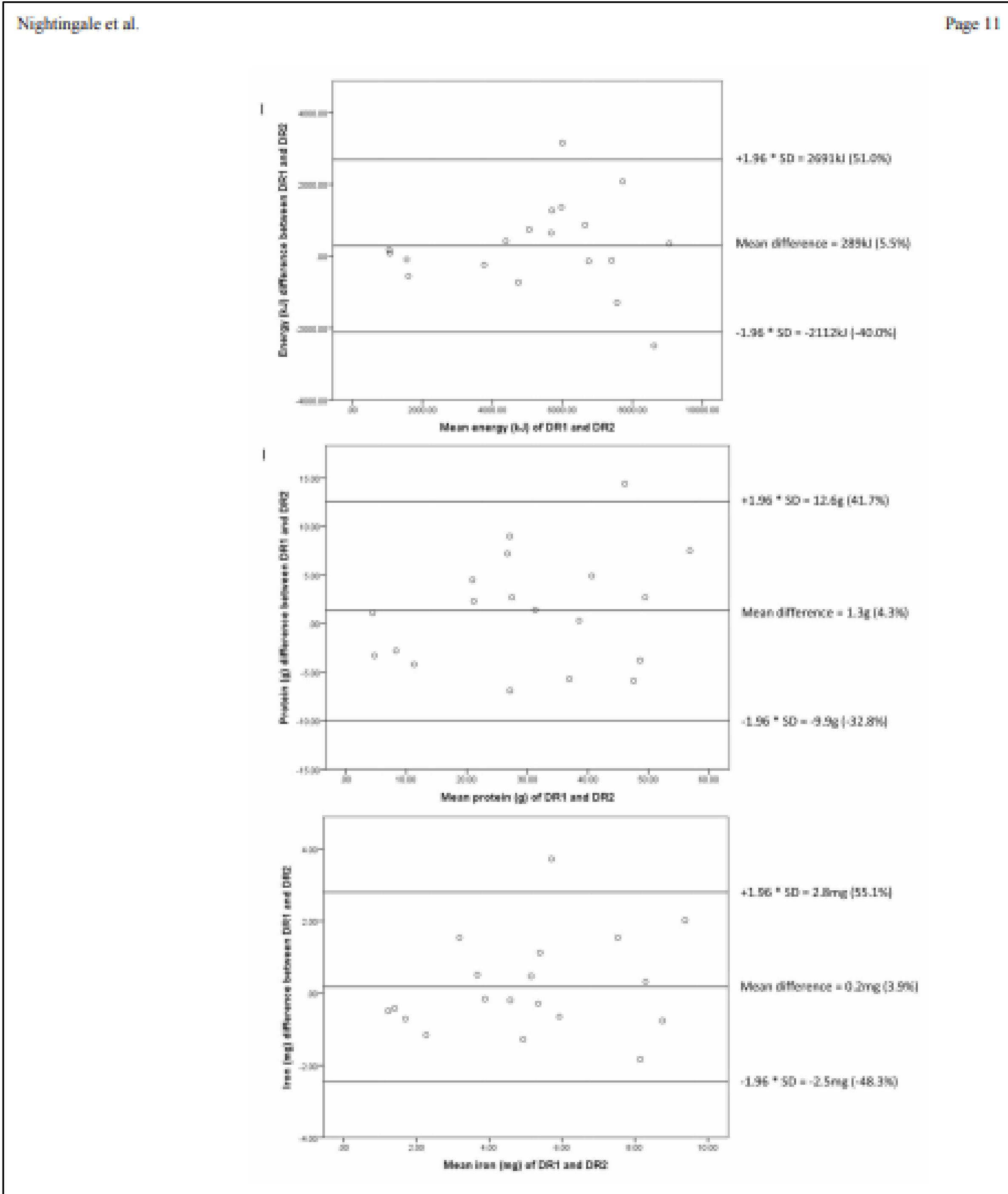


Figure 1.

Bland-Altman plots of first and secondary dietary recalls: (I) energy, (II) protein, (III) iron

Legend: DR, dietary recall; SD, standard deviation

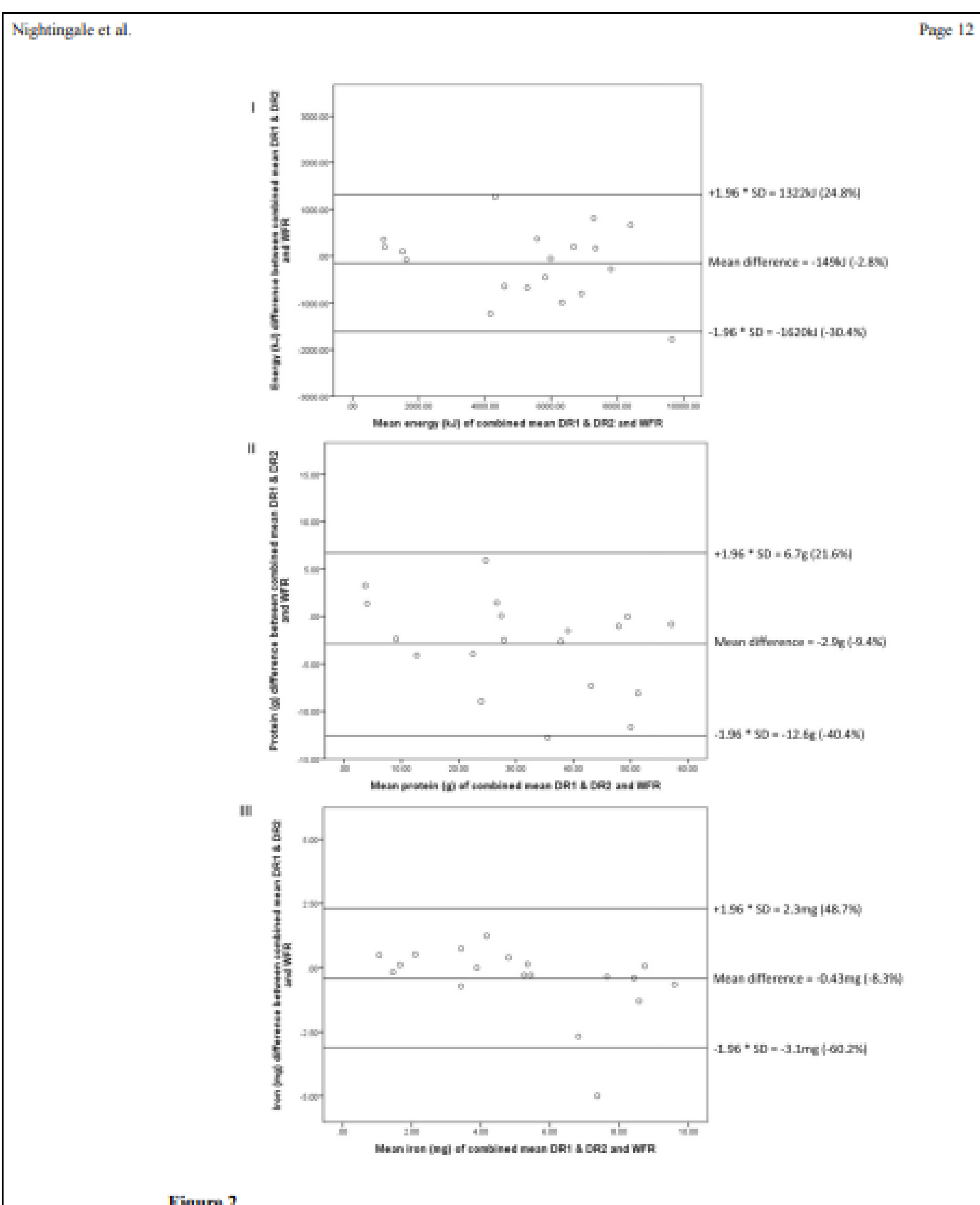


Figure 2.

Bland-Altman plots of combined dietary recalls and weighed food records: (I) energy, (II) protein, and (III) iron

Legend: DR, dietary recall; SD, standard deviation; WFR, weighed food record

Table 1
Methodology of dietary data collection and portion size estimation

Stage	Methodology	Person conducting	Portion Size Estimation
1	Weighed food record	First researcher	Weighing
2	24-hour dietary recall	Second researcher	Volume of play dough *
3	24-hour dietary recall	Third researcher	Household measures **
			Standardised food item size †

* for foods eaten by hand;

** cups, bowls, table- and teaspoons of water or dry uncooked rice;

† for example 1 egg, half of 1 medium onion

Table 2

Intraclass and bivariate correlation coefficients comparing estimated intakes by weighed food records and 24-hour dietary recalls[†]

Nutrient	Weighed Food Record		Combined Dietary Recalls			
	Mean	SD	Mean	SD	ICC (95% CI)	*r* (p-value)
Energy (kJ)	6563	1706	6335	1537	0.98 (0.90-0.98)	0.96*
Protein (g)	40.0 [#]	12.9	36.4 [#]	11.4	0.97 (0.90-0.99)	0.985 [#]
Iron (mg)	6.6	2.6	6.0	2.0	0.94 (0.84-0.98)	0.91 [#]

SD, standard deviation; ICC, intraclass correlation coefficient; CI, confidence interval; *r*, correlation coefficient

[#] $p<0.001$

*$p=0.02$

[†] ICCs compared absolute agreement of average measures, using a two-way random model

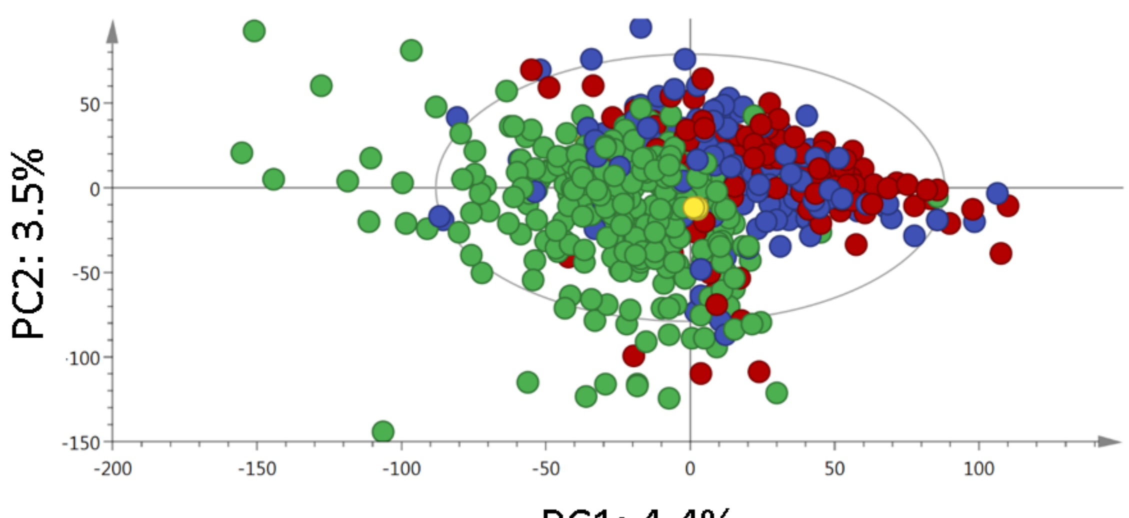
Table 3

Cross-classification of children to quartiles according to intake estimates

Nutrient	Classified correctly (%)	Classified adjacently (%)	Grossly misclassified (by ≥2 quartiles) (%)	Cohen's Kappa κ (p-value)
Energy	15 (79)	4 (21)	0	0.719 (<0.001)
Protein	17 (89)	2 (11)	0	0.859 (<0.001)
Iron	17 (89)	2 (11)	0	0.859 (<0.001)

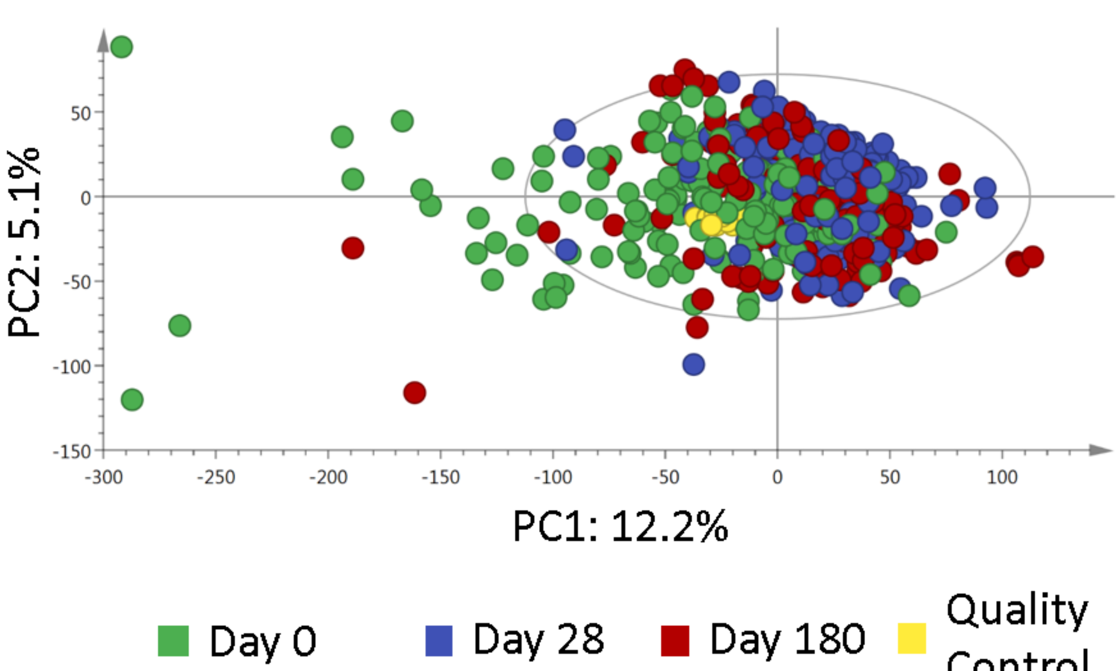
APPENDIX X – ^{1}H NMR EXPERIMENTAL QUALITY CONTROL

Urine (n=657) 4 PCs Q^2 0.161

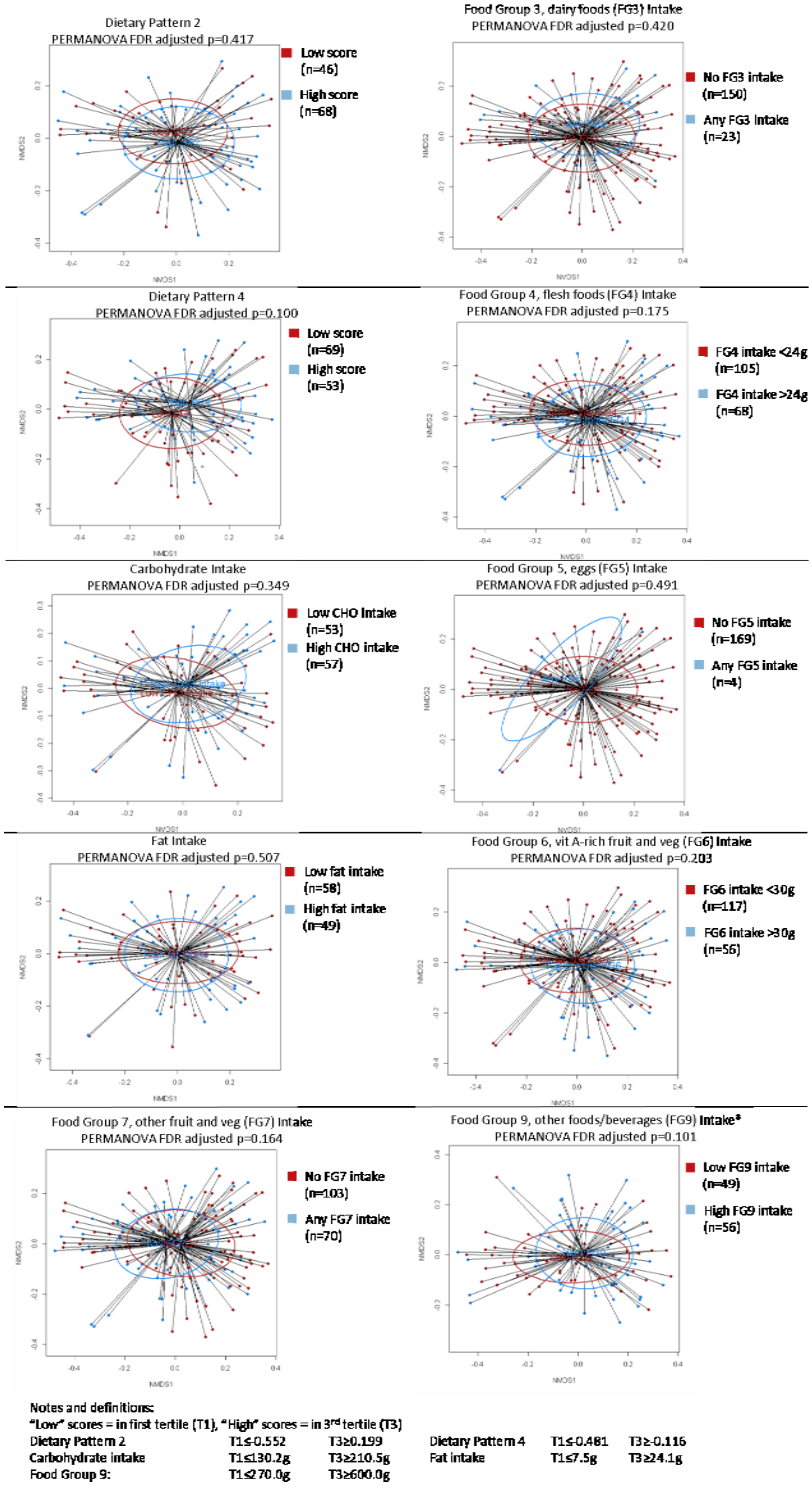


PC1: 4.4%

Plasma (n=605) 3 PCs Q^2 0.184



APPENDIX XI – NON SIGNIFICANT NMDS AND PERMANOVA FOR DIETARY FACTORS



Notes and definitions:
"Low" scores = in first tertile (T1), "High" scores = in 3rd tertile (T3)

Dietary Pattern 2	T1≤0.552	T3≥0.199	Dietary Pattern 4	T1≤0.481	T3≥0.116
Carbohydrate intake	T1≤130.2g	T3≥210.5g	Fat intake	T1≤7.5g	T3≥24.1g
Food Group 9:	T1≤270.0g	T3≥600.0g			

Tertile values for dietary patterns, nutrient and food group intakes used in 16S data analysis

Tertile values	T1	T3		T1	T3
DP1	-0.538	0.220	DP2	-0.469	0.145
DP3	-0.438	0.020	DP4	-0.200	-0.126
Carbohydrate	130.2g	210.5g	Protein	14.4g	28.1g
Fat	7.5g	24.1g	Sugar	17.8g	35.0g
Fibre	1.2g	5.4g	FG1	309.6	595.1
FG8	20.5	58.0	FG9	270.0	600.0