

Inflammation and Immunity in Severe Acute Malnutrition

Kelsey David Joel Jones

**Department of Paediatrics,
Imperial College, London**

Thesis submitted for the degree of Doctor of Philosophy

**Imperial College
London**

Declarations

The work in this thesis is my own except where referenced. Chapters 4 and 6 are based on my first draft of the following papers:

Jones KD, *et al.* Mesalazine in the initial management of severely acutely malnourished children with environmental enteric dysfunction: a pilot randomized controlled trial. BMC Medicine (2014) 12:133.

Jones KD, *et al.* Ready-to-use therapeutic food with elevated n-3 polyunsaturated fatty acid profile, with or without fish oil, to treat severe acute malnutrition: a randomized controlled trial. BMC Medicine (2015) 13: 93

All figures are original, or are reproduced under creative commons licenses (unless otherwise stated)

The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives license. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the license terms of this work

Abstract

Background: Malnutrition is responsible for 45% of child deaths because it is associated with an increased vulnerability to major infectious disease. The mechanisms behind this association are undefined. It occurs despite the fact that malnourished children have increased levels of enteric and systemic inflammatory activation

Methods: Case-control study of inflammatory activation and response to *ex vivo* pathogen challenge determined relationships between existing and potential inflammatory function and nutrition. Randomised controlled trial of a gut-specific immunomodulatory agent assessed for functional redundancy in enteric inflammation. Randomised controlled trial of nutritional rehabilitation strategies with enhanced n-3 polyunsaturated fatty acid provision assessed for potentially beneficial effects on inflammation.

Results: SAM was not associated with abnormalities in systemic cytokine response to innate immune challenge in an *ex vivo* model compared to control children, but more severe intestinal inflammation, and kwashiorkor may be. Children with SAM and stunting have clear evidence of enteric inflammation, and have evidence of cytokine-mediated inhibition of the GH-IGF-1 axis, which may play a role in linear growth failure. Treatment of children with SAM and stunting with mesalazine appeared to be safe, and there was limited evidence of an impact on enteric inflammation. Stunting is associated with LC-PUFA deficiency, but acute malnutrition is not. Production and administration of a ready-to-use therapeutic food with elevated n-3 PUFA was technically feasible, but was not associated with positive outcomes in growth, recovery or immune function compared to standard treatment. Provision of pre-formed DHA in RUTF may be essential to preserve optimal tissue levels during nutritional rehabilitation.

Conclusions: Further strategies targeting inflammatory activation as part of the management of SAM should be trialled. The relationship between systemic and enteric inflammation and nutrition deserves further study.

Table of Contents

DECLARATIONS	2
ABSTRACT	3
TABLE OF CONTENTS	4
ACKNOWLEDGEMENTS	7
ABBREVIATIONS	10
TABLES	14
FIGURES	15
CHAPTER 1: INFLAMMATION AND IMMUNITY IN SEVERE ACUTE MALNUTRITION: AN INTRODUCTION	17
1.1 MALNUTRITION: DEFINITION OF CLINICAL SYNDROMES.	17
1.2 EPIDEMIOLOGY OF ACUTE MALNUTRITION AND STUNTING.	19
1.3 CLINICAL CONSEQUENCES OF SAM AND STUNTING.	22
1.4 IMMUNOLOGIC CORRELATES OF SAM.	25
1.4.I T CELL FUNCTION	27
1.4.II SYSTEMIC INFLAMMATORY ACTIVATION	29
1.4.III EPITHELIAL BARRIER FUNCTION	30
1.5 ENVIRONMENTAL ENTERIC DYSFUNCTION	30
1.6 SAM: THERAPEUTIC PARADIGM.	33
1.7 EED: THERAPEUTIC PARADIGM	36
1.8 IMMUNOLOGIC CONSEQUENCES OF CHRONIC PATHOGEN EXPOSURE.	38
1.9 INNATE IMMUNE FUNCTION AND INFLAMMATORY BOWEL DISEASE.	44
1.10 STATEMENT OF HYPOTHESES.	45
CHAPTER 2: SPECIFIC BACKGROUND AND PILOT WORK	49
2.1 MESALAZINE IN SAM WITH EED	49
2.2 NJUGU PLUS: POLYUNSATURATED FATTY ACIDS IN SAM	52
2.3 INFLAMMATION AND IMMUNITY	57
CHAPTER 3: INNATE PATHOGEN SENSING AND RESPONSE IN SEVERE ACUTE MALNUTRITION: A CASE-CONTROL OBSERVATIONAL STUDY	60
3.1 METHODS	60
3.1.1 STUDY SETTING	60
3.1.2 STUDY PARTICIPANTS	62
3.1.3 SCREENING AND ENROLMENT	64

3.1.4	LABORATORY METHODS	65
3.1.5	STATISTICAL METHODS	68
3.2	RESULTS	70
3.2.1	CLINICAL AND DEMOGRAPHIC CHARACTERISTICS	70
3.2.2	RELATIONSHIP BETWEEN INFLAMMATORY INDICES	73
3.2.3	RELATIONSHIP BETWEEN INFLAMMATORY AND ANTHROPOMETRIC INDICES	75
3.2.4	WHOLE BLOOD STIMULATION DATA PROCESSING	77
3.2.4	WHOLE BLOOD STIMULATION RESULTS	82
3.3	DISCUSSION	90

CHAPTER 4: MESALAZINE IN THE INITIAL MANAGEMENT OF SEVERELY ACUTELY MALNOURISHED CHILDREN WITH ENVIRONMENTAL ENTERIC DYSFUNCTION: A PILOT RANDOMISED CONTROLLED TRIAL

97

4.1	METHODS	97
4.1.1	PARTICIPANTS AND SETTING	97
4.1.2	SCREENING, ENROLMENT AND RANDOMISATION	100
4.1.3	TREATMENT	103
4.1.4	MONITORING	104
4.1.5	SAFETY AND REPORTING	106
4.1.6	OUTCOMES	111
4.1.7	LABORATORY METHODS	112
4.1.8	STATISTICAL METHODS	114
4.1.9	STUDY OVERSIGHT	114
4.1.10	SPECIFIC ETHICAL ISSUES	115
4.1.11	COMMUNITY ENGAGEMENT	118
4.2	RESULTS	121
4.2.1	FOLLOW-UP AND COMPLIANCE	123
4.2.2	ADVERSE EVENTS AND TOXICITY	125
4.2.3	GROWTH AND RECOVERY	130
4.2.4	INFLAMMATORY INDICES	136
4.3	DISCUSSION	140

CHAPTER 5: RELATIONSHIP BETWEEN N-3 POLYUNSATURATED FATTY ACID STATUS AND ANTHROPOMETRIC INDICES IN KENYAN CHILDREN: A MULTI-CENTRE CASE-CONTROL OBSERVATIONAL STUDY

146

5.1	METHODS	146
5.1.1	STUDY SETTINGS	146
5.1.2	STUDY PARTICIPANTS	147
5.1.3	SCREENING AND ENROLMENT	149
5.1.4	LABORATORY METHODS	150
5.1.5	STATISTICAL METHODS	151
5.2	RESULTS	154
5.2.1	CLINICAL AND DEMOGRAPHIC CHARACTERISTICS	154
5.2.2	FATTY ACID COMPOSITION IN SAM	157
5.2.3	DESATURASE AND ELONGASE ENZYME ACTIVITY IN SAM	163
5.2.4	PREDICTORS OF FATTY ACID COMPOSITION: REGRESSION ANALYSES	164
5.3	DISCUSSION	172

CHAPTER 6: READY-TO-USE THERAPEUTIC FOOD WITH ELEVATED N-3 POLYUNSATURATED FATTY ACID PROFILE, WITH OR WITHOUT FISH OIL, TO TREAT SEVERE ACUTE MALNUTRITION: A RANDOMISED CONTROLLED TRIAL	178
6.1 METHODS	178
6.1.1 PARTICIPANTS AND SETTING	178
6.1.2 INTERVENTION	179
6.1.3 SCREENING, ENROLMENT AND RANDOMISATION	183
6.1.4 MONITORING	184
6.1.5 SAFETY AND REPORTING	185
6.1.6 OUTCOMES	186
6.1.7 LABORATORY METHODS	187
6.1.8 STATISTICAL METHODS	191
6.1.9 STUDY OVERSIGHT	192
6.2 RESULTS	194
6.2.1 STABILITY OF THE RUTF	195
6.2.2 BASELINE CHARACTERISTICS:	195
6.2.3 FOLLOW UP AND COMPLIANCE:	198
6.2.4 SAFETY:	200
6.2.5 LIPIDOMIC ANALYSIS:	201
6.2.6 GROWTH:	215
6.2.7 HAEMATOLOGIC INDICES:	217
6.2.8 INFLAMMATORY INDICES:	219
6.3 DISCUSSION	222
 CHAPTER 7: FUTURE RESEARCH DIRECTIONS	 230
7.1 UNDERSTANDING GUT FUNCTION/INFLAMMATION IN SAM	231
7.2 FURTHER TRIALS OF IMMUNOMODULATION IN EED	232
7.3 FURTHER TRIALS OF LC-PUFA IN SAM	234
 REFERENCES	 237

Acknowledgements

Funding: Work described in this thesis was funded by The Wellcome Trust via a Research Training Fellowship to Kelsey Jones (092088). Additional funding was from The Wellcome Trust via a Fellowship to James Berkley (083579), from the NIHR via an Academic Clinical Fellowship to Kelsey Jones, from the NIHR and the Biomedical Research Centre of Imperial College, London via John Warner, and from a grant from The Wellcome Trust to the Centre for Respiratory Infection, Imperial College, London via Kelsey Jones. The trial described in Chapter 6 was funded by The Bill and Melinda Gates Foundation through the Grand Challenges Explorations initiative (OPP1046183). Additional support for collaborators was from The NIHR Respiratory Biomedical Research Unit in Southampton and from The Wellcome Trust via an equipment grant to Anthony Postle (093500). Fish oil capsules used in the trial described in Chapter 6 were donated by Seven Seas (Hull, UK).

Support for work in Kilifi: I am grateful for support provided by staff across the whole breadth of the KEMRI-Wellcome Trust Research Programme, not limited to teams working in Laboratory services, Pharmacy, Stores, ICT, Finance, Human Resources and the Director's office, both at the Kilifi and Nairobi offices. I am grateful to Britta Urban and her group for help and support in setting up my laboratory work. I am grateful to all the field workers, nurses, clinical officers, nutritionists and doctors working at Kilifi County Hospital. I am grateful to my office- and laboratory-mates for discussions and support

Support for work in Nairobi: I am grateful for German Doctors for hosting my work in Mathare. I am grateful for all the staff of the Baraka Health Centre and Feeding Programme for their help and support with research. Every member of staff was involved and contributed. I am particularly grateful for the enthusiasm and support of Barbara Hüntten-Kirsch. I am grateful to the CTX study team at Mbagathi Hospital, especially Molline Timbwa and Jenifer Mikusa for help and support. I am grateful to Barbara Lohman-Payne, Michele Merkel, Bhavna Chohan, and Grace John-Stewart and staff at the Universities of Nairobi and of Washington, for providing laboratory access and support. I am grateful to Charles Magiri and staff at KEMRI Nairobi for

helping with specimen storage. I am grateful to the local Ministry of Health staff, particularly Moses Owino, for support.

Specific acknowledgement for Chapter 4: I am grateful for the help and support afforded by Baraka Feeding Programme Staff, particularly Caroline Wanjiru Munyi, Hussein Samira Ahmed and Eclay Vusi Imbiakha; by members of the Pharmacy team, especially Evans Opar; and by members of the community health volunteer team including Viola Akinyi Omamo, Zilpah Atieno Oduyo, Evans Otieno Opar, Nicholas Ochieng Ogonda, Eclay Vusi Imbiakha, Doreen Mideva Kidagi, Pamelah Akoth Ayieko, Beatrice Wesonga Makachia, Peninah Akoth Obonyo, Elizabeth Adhiambo Ojijo, Julia Odinga Okechi and Caroline Adhiambo Omollo. I am grateful to Rose Omia and her team for helping with community engagement. I am grateful to Jenifer Mikusa and Molline Timbwa for assistance with recruitment and follow-up activities. I am grateful to Ahmed Laving, Simon Murch and Peter Sullivan for advice and assistance. Moses Ngari designed the trial database and assisted with data analysis. Musa Mulongo and Dennis Odera assisted with design of, and performed the HEK293 endotoxin assay and sCD14 ELISA, respectively. I am grateful to members of the DSMB; Stephen Allen (Chair), Trudie Lang, Charles Opondo, and the local safety monitor, Diana Murangu. I am grateful to the Clinical Trials Pharmacy and Pharmacovigilance Department at Imperial College Healthcare NHS Trust for technical assistance, and to Johnstone Thitiri, Jimmy Shangala and the pharmacy team at KWTRP for help blinding the interventional product. I am grateful to the KWTRP Clinical Trial Monitoring team.

Specific acknowledgement for Chapter 5: Annette West, Victoria Goss and Grietel Koster performed most of the lipidomic analysis in Southampton. I am grateful to Anthony Postle and Philip Calder for support and advice.

Specific acknowledgement for Chapter 6: I am grateful to Rehema Ali, who helped to co-ordinate trial activities, Maureen Khasira, who acted as trial nutritionist, and Alison Talbert, who acted as trial clinician. I am grateful to Peter Akomo, Steve Collins, and the Valid International and Valid Nutrition teams in Europe, Kenya and Malawi for assistance with design and implementation of the novel RUTF. Dennis

Odera, Ken Omollo, and Anne Ndungu performed much of the laboratory work for the trial in Kenya. Annette West, Victoria Goss and Grietof Koster performed most of the lipidomic analysis in Southampton. Miguel Garcia-Knight helped to design the flow cytometry panel. Gabriel Mwambingu and the KEMRI-Wellcome Trust Research Programme's Clinical Trials Laboratory team performed laser-assisted optical rotation analyser analysis. Moses Ngari designed the trial database and assisted with data analysis, including writing the first draft of the 'do file'. I am grateful to Johnstone Thitiri and the KWTRP Clinical Trial Monitoring team for assistance. I am grateful to Anthony Postle and Philip Calder for support and advice. I am grateful to the independent trial steering committee Andrew Prentice (Chair), Caroline Jones, Patricia Njuguna, and the local safety monitor, Anna Seale.

I am grateful to staff of the Imperial College, London Department of Paediatrics and Wellcome Centre for Clinical Tropical Medicine for support.

I am grateful to Alan Jackson and Gary Frost, my examiners, for helpful discussion and thoughtful suggestions in my *viva voce* examination.

I am grateful to my supervisors James Berkley, Jane Mitchell, Andrew Prentice and John Warner. I am particularly grateful to Jay, for directly supervising all the work in this thesis and for providing vision and direction, and to John for his tireless support. I am grateful to Greg Fegan for providing statistical support and advice throughout.

I am grateful to all the participants, their families, carers and communities for agreeing to take part in this work.

I am grateful to my friends, family, for all their support. I am especially grateful to my fiancée Claire for supporting and believing in the work, even when it was so far away.

Abbreviations

5-ASA	5-aminosalicylic acid (mesalazine)
AA	Arachidonic acid
AE	Adverse event
AIDS	Acquired immunodeficiency syndrome
ALA	Alpha-linolenic acid
ALT	Alanine transaminase
ANOVA	Analysis of variance
AST	Aspartate transpeptidase
B2M	Beta-2 microglobulin
BCG	<i>Bacillus Calmette-Guérin</i>
BD2	Beta-defensin 2
BHC	Baraka Health Centre
BSPGHAN	British Society for Paediatric Gastroenterology, Hepatology and Nutrition
CHV	Community health volunteer
CI	Confidence interval
CRP	C-reactive protein
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosopentaenoic acid
DSMB	Data, safety and monitoring board
EED	Environmental enteric dysfunction
EEN	Exclusive enteral nutrition
ELISA	Enzyme-linked immunosorbent assay
EndoCAb	IgG anti-endotoxin core antibody

EPA	Eicosapentaenoic acid
ESR	Erythrocyte sedimentation rate
ET	Endotoxin tolerance
ETA	Eicosatetraenoic acid
FA	Fatty acid
FBC	Full blood count
FLA	Flagellin
Gar	Gardiquimod
GBP	Pound sterling
GCLP	Good Clinical Laboratory Practice
GDP	Gross domestic product
GGT	Gamma-glutamyl transferase
GH	Growth hormone
HAZ/LAZ	Height/length-for-age z-score
Hb	Haemoglobin
HIV	Human immunodeficiency virus
IBD	Inflammatory bowel disease
Ig	Immunoglobulin
IFN	Interferon
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IP-10	IFN- γ -induced protein 10
IU	International units
KCH	Kilifi County Hospital
KEMRI	Kenya Medical Research Institute
KSH	Kenyan shillings
KWTRP	KEMRI-Wellcome Trust Research Programme
L:M	Lactulose:mannitol dual sugar absorption test

LA	Linoleic acid
LC-PUFA	Long-chain PUFA
LLD	Lower limit of detection
LPS	Lipopolysaccharide
LSM	Local safety monitor
LTA	Lipoteichoic acid
MAM	Moderate acute malnutrition
MH	Mbagathi Hospital
MHC	Major histocompatibility complex
MTB	<i>Mycobacterium tuberculosis</i>
MUAC	Mid-upper arm circumference
NEFA	Non-esterified fatty acid
NEMO	NF- κ B essential modulator
NGO	Non-governmental organisation
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerisation domain
OTP	Outpatient therapeutic feeding programme
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PCSK	Pam3CSK4
PIC	Poly(I:C)
PMA	Phorbol 12-myristate 13-acetate
PMN	Neutrophils (polymorphonuclear leukocytes)
PPD	Purified protein derivative
PRR	Pattern recognition receptor

PUFA	Poly-unsaturated fatty acid
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RR	Relative risk
RSV	Respiratory syncytial virus
RUSF	Ready-to-use supplementary food
RUTF	Ready-to-use therapeutic food
SAE	Serious adverse event
SAM	Severe acute malnutrition
SGA	Small for gestational age
SIBO	Small intestinal bacterial overgrowth
sSA	Sub-Saharan Africa
SUSAR	Suspected unexpected serious adverse reaction
TAG	Triacylglycerol
Th	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UC	Ulcerative colitis
ULN	Upper limit of normal
UNICEF	United Nations Children's Emergency Fund
USA	United States of America
USD	Dollars (US)
WASH	Water, sanitation and hygiene
WAZ	Weight-for-age z-score
WCC	White cell count
WHO	World Health Organisation
WHZ/WLZ	Weight-for-height/length z-score
Zy	Zymosan

Tables

1.1	Prevalence of stunting and severe wasting	20
3.1	Whole-blood stimulation conditions	66
3.2	Clinical and demographic characteristics	71
3.3	Stimulant/cytokine pairs taken forward for further analysis	79
3.4	Univariate regression analyses against cytokine release	86
3.5	Multivariate regression analyses against cytokine release	87
4.1	Baseline characteristics	122
4.2	Outcomes	124
5.1	Baseline characteristics of the participants	155
5.2	Percentage of fatty acid species in erythrocyte fractions and plasma phospholipid between children with SAM and controls	158
5.3	Predictors of individual fatty acids' relative abundance in erythrocytes	165
5.4	Predictors of individual fatty acids' relative abundance in plasma PL	167
5.5	Fatty acids in severely malnourished children (<i>previous study characteristics</i>)	173
6.1	Measured RUTF fatty acid composition	181
6.2	Baseline characteristics of the participants	197
6.3	Compliance, Safety and Growth	199
6.4	Erythrocyte fatty acid composition	203
6.5	Plasma phosphatidylcholine fatty acid composition	210
6.6	Plasma phosphatidylcholine/erythrocyte fatty acid correlation	212
6.7	CD3+ (T cell) phosphatidylcholine fatty acid composition	213
6.8	CD3+ (T cell) phosphatidylethanolamine fatty acid composition	214
6.9	Inflammatory indices	216
6.10	T cell functional indices	220

Figures

1.1	Global burden of stunting	21
1.2	Increased odds-ratio for mortality from infection is not confined to SAM	23
1.3	Vicious cycle of acute malnutrition and infection	26
1.4	10 steps approach to inpatient management of SAM	34
1.5	NF- κ B signalling pathway	40
1.6	Innate immune training	42
1.7	Conceptual framework	48
2.1	Pilot work on PUFA in SAM	55
3.1	Map showing location of Mathare 4A and BHC	73
3.2	Relationship between chronic inflammatory indices	74
3.3	Relationship between SAM and inflammation	76
3.4	Associations between inflammatory and anthropometric variables	77
3.5	Crude cytokine concentrations in stimulated/unstimulated conditions	78
3.6	Subsection of the scatter plot matrix	80
3.7	Scree plot	81
3.8	PCA loading plot	81
3.9	Stimulated cytokine release: impact of SAM	83
3.10	EndoCAb/calprotectin and PC1 – component-plus-residual plots	88
3.11	SAM and cytokine response to PAMP challenge	89
4.1	Screening and enrolment procedures	102
4.2	Choo Chart	112
4.3	Age distribution of SAM at Baraka	117
4.4	Trial flow diagram	121
4.5	Timing of adverse events	126
4.6	Stool frequency and consistency	126
4.7	Hepatic toxicity	128
4.8	Creatinine	129
4.9	Haemoglobin	130
4.10	Anthropometry	131

4.11	MUAC in kwashiorkor	132
4.12	Insulin-like growth factor-1	133
4.13	IGF-1 and inflammation	135
4.14	Inflammatory indices	137
4.15	Cytokines/chemokines	138
5.1	Family-level consumption of food items between cases and controls	157
5.2	Fatty acid composition according to SAM syndrome	160
5.3	Fatty acid composition according to SAM/control and location	162
5.4	Desaturase and elongase enzyme activity in SAM	163
5.5	Fatty acid composition for oedematous participants compared to others	169
5.6	Association of HAZ/LAZ with fatty acid content	171
5.7	Fatty acids in severely malnourished children (<i>previous study results</i>)	174
5.8	Anthropometric characteristics of the study cohort	177
6.1	Gating strategy	189
6.2	Trial flow diagram	194
6.3	Erythrocyte fatty acid composition	202
6.4	Enrolment LC-PUFA by breastfeeding status	204
6.5	Erythrocyte DHA by breastfeeding status	205
6.6	Desaturase and elongase activity	206
6.7	Erythrocyte DHA/EPA stratified by baseline status	207
6.8	Impact of baseline PUFA status on response	208
6.9	Correlation between plasma phosphatidylcholine and erythrocyte fatty acids for key species	211
6.10	Haematologic indices	218
6.11	T cell compositional/functional correlations	221

CHAPTER 1

Inflammation and immunity in severe acute malnutrition: an introduction

1.1 *Malnutrition: Definition of clinical syndromes.*

‘Malnutrition’ is a term used to describe clinical syndromes that result from inadequate or inappropriate nutritional intake. In its broadest sense it includes nutritional excess. However, it is most often used, sometimes interchangeably, with ‘undernutrition’ in the context of dietary nutrient insufficiency to meet metabolic and growth demands.^{3,4} Day-to-day functioning for mammals requires a continuous nutrient supply to fulfil the metabolic requirements of homeostasis, account for routine cell and tissue turnover, and provide the substrate for growth and development. Additional nutrient demand may result from environmental stressors such as infection or exposure to temperature extremes, or via as a result of increased utilisation due to exercise. Nutrient supply to tissues involves a complex interplay of mobilisation from internal stores, biosynthesis from precursors, and exogenous intake in the diet. This means that accurate determination of ‘deficiency’ requires an understanding of short and long term dietary intake, utilisation characteristics, size and type of stores, and future needs. A single snapshot assessment of an individual seldom provides sufficient information by which to define and interpret their nutritional status. However, in resource-limited settings, snapshot evaluation of anthropometry or visible clinical features is the way in which malnutrition syndromes are most commonly defined.^{10, 11} Because of this,

terminology is open to misinterpretation. The term 'malnutrition' implies a causal relationship between the clinical syndrome and nutritional intake but this may not be the case: it is perfectly possible for a child to meet any or all anthropometric criteria for 'malnutrition' without inadequate dietary intake being the primary cause of their body habitus. They may, for example, have cachexia secondary to unrecognised malignant disease. In order to minimise confusion, in this thesis I have avoided the terms 'malnutrition' or 'undernutrition', and have systematically used the following syndromic diagnoses:

- **Severe Acute Malnutrition (SAM):** Clinical diagnosis based on one or other of weight-for-height/length z-score (WHZ/WLZ) < -3 , mid-upper-arm circumference (MUAC) $< 11.5\text{cm}$, or bilateral lower limb oedema in a child aged 6-60 months. Where SAM is diagnosed due to lower limb oedema the condition may be referred to as '**kwashiorkor**' whether or not the other classical clinical features (hepatomegaly, dermatoses, hair changes, withdrawn affect) are present. SAM diagnosed on the basis of WHZ/WLZ or MUAC may be referred to as '**severe wasting**'. Because children's height or length are difficult to measure in community settings, WHZ/WLZ is only used as an entry criteria for studies presented herein where recruitment has taken place from within a hospital.¹² The terms 'protein-calorie' or 'protein-energy' malnutrition and 'marasmus' have been avoided.
- **Acute Malnutrition:** Includes SAM and **moderate acute malnutrition (MAM)**, which is a clinical diagnosis based on WHZ/WLZ < -2 but > -3 , or MUAC $< 12.5\text{cm}$

but $\geq 11.5\text{cm}$. **Wasting** is used to describe children with $\text{WHZ/WLZ} < -2$ (incorporating MAM and many cases of SAM but excluding kwashiorkor)

- **Stunting:** This is a clinical diagnosis based on height/length-for-age z-score (HAZ/LAZ). Children with $\text{HAZ/LAZ} < -2$ are 'stunted' and those with $\text{HAZ/LAZ} < -3$ are 'severely stunted'. Although 'chronic undernutrition' has sometimes been used interchangeable with stunting, I have avoided this description throughout for the reasons described above.
- **Growth failure:** Linear growth failure is the process that results in stunting; ponderal growth failure results in acute malnutrition.

1.2 Epidemiology of acute malnutrition and stunting.

The global burden of acute malnutrition has been surmised on the basis of population-based estimates of wasting prevalence measured as WHZ/WLZ, which suggest that 8% of the world's children aged 6-60 months, some 52 million individuals, are wasted, and 2.9% are severely wasted.⁴ In fact, the burden is almost certainly higher than these figures suggest. Exclusive reliance on WHZ/WLZ fails to count children with $\text{MUAC} < 11.5\text{cm}$ but $\text{WHZ} > -3$, a very vulnerable group who are severely wasted and but also stunted. Kwashiorkor is not counted, although it comprises up to 50% of SAM cases in some settings.¹³ Although acute malnutrition in children aged younger than six months has recently become a focus of attention, high quality data on the scale of the problem are lacking and acutely malnourished infants have been neglected in international prevalence estimates.¹⁴ Furthermore, since episodes of acute malnutrition and SAM tend to last less than a year,

prevalence data underestimate its true impact.^{15, 16} Measuring incident SAM would involve active surveillance programmes. The resource requirements inherent in such monitoring have not been met outside of relatively small-scale cohort studies, and the ethical requirement to treat cases of MAM prior to progression to SAM complicates interpretation.

Although the global prevalence of wasting is decreasing over time, in Africa it is static. In Africa in 1990, 8.7% (95% Confidence Interval 7.4 - 10.0) of children under-5 were wasted compared with 8.5% (7.4 - 9.6) in 2011. The absolute number of children affected had increased from 9.6 million (8.2 - 11.0) to 13.4 million (11.6 - 15.2).³

Table 1.1: Prevalence of stunting and severe wasting

	Stunting		Severe wasting (WHZ/WLZ <-3)	
	Proportion	Number (millions)	Proportion	Number (millions)
Africa	35.6 (33.3-38.0)	56.3 (52.5-60.0)	3.5 (2.9-4.1)	5.5 (4.5-6.4)
Asia	26.8 (23.2-30.5)	95.8 (82.8-108.8)	3.6 (2.4-4.8)	12.9 (8.4-17.3)
Latin America & Caribbean	13.4 (9.4-17.7)	7.1 (4.8-9.4)	0.3 (0.2-0.4)	0.2 (0.1-0.2)
Oceania	35.5 (16.0-61.4)	0.5 (0.2-0.8)	0.7 (0.5-1.1)	0.0 (0.0-0.0)
Total LMICs	28.0 (25.6-30.4)	159.7 (145.9-173.4)	3.3 (2.5-4.0)	18.5 (14.0-23.1)
High income countries	7.2 (4.1-12.6)	5.1 (2.9-8.9)	0.3 (0.0-1.3)	0.2 (0.0-0.9)
Global	25.7 (23.5-27.9)	164.8 (150.8-178.8)	2.9 (2.2-3.6)	18.7 (14.2-23.2)

Table 1.1: Prevalence and number of children 6 to 60 months with HAZ <-2 or WHZ/WLZ <-3 by UN region. Note that severe wasting does not include MUAC, for which global prevalence estimates do not exist (see above). LMICs = low and middle income countries. Data are mean and 95% CIs. Data from Black *et al.*^{3, 4}

Stunting affects more than one in four children living in low- and middle-income countries. Although there have been substantial global reductions in the burden of stunting from an estimated 253 million children in 1990, to 165 million in 2011, again Africa lags behind. It has seen only modest reductions in prevalence and an increase in the absolute number of affected children (Figure 1.1).^{3, 4}

Figure 1.1: Global burden of stunting

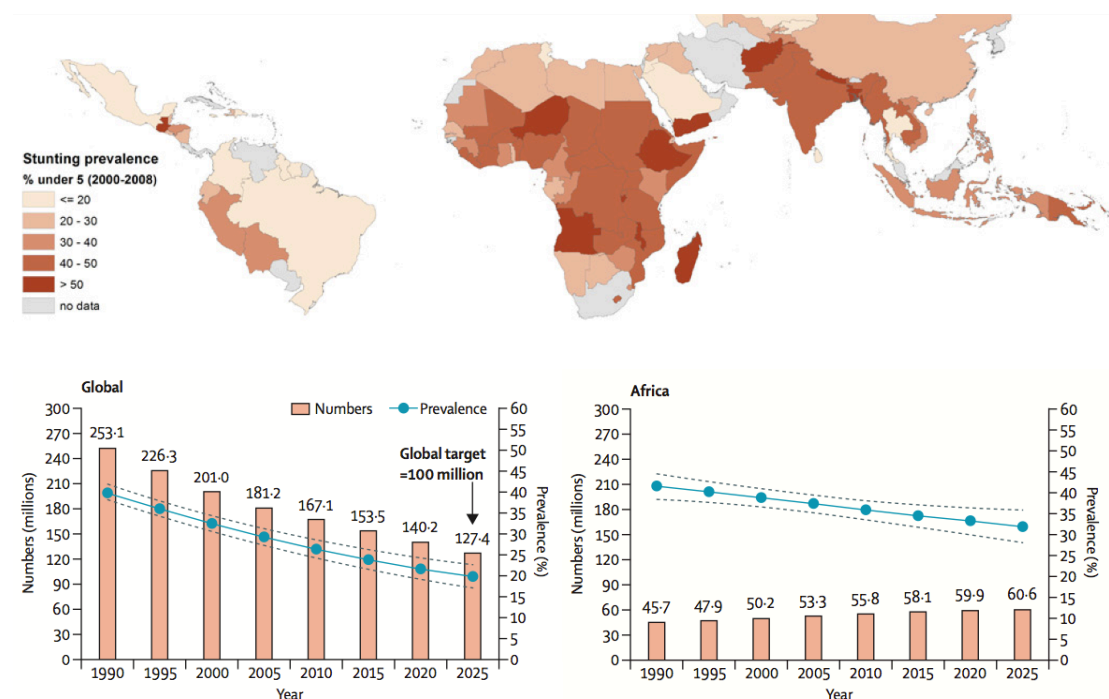


Figure 1.1: Top shows the global distribution of stunting prevalence (from Ericksen *et al.* reproduced under a creative commons license).^{3, 8} Bottom shows stunting trends – Africa (bottom right) lags behind global improvements (from Black *et al.* reproduced with permission).⁴

The reasons for sub-Saharan Africa's slow progress on nutritional health relative to the rest of the world are complex. A key factor is that while prevalence of both stunting and wasting are strongly correlated with per-capita gross domestic product (GDP) at a national level, there has been no relationship between change in GDP

with time (macroeconomic growth) and change in stunting/wasting prevalence.¹⁷ In sub-Saharan Africa (sSA), there has been a positive association between macroeconomic growth rate and population-level inequality as measured by the Gini index (the difference in wealth between the top, and bottom, population deciles), and when wealth creation is associated with increased inequality, those living in poverty will always struggle to see the benefits.¹⁸ Relying on economic growth alone to realise further significant decreases in acute malnutrition and stunting is a risky strategy. Interventions designed to tackle these conditions directly will need to be designed, tested, and rolled out, if healthy growth of children living in poverty is to be optimised.

1.3 Clinical consequences of SAM and stunting.

Tackling acute malnutrition and stunting is a global priority because the conditions have important short- and long-term consequences for children's health and wellbeing. In the short term, both are associated with increased vulnerability to infectious disease-related morbidity and mortality. SAM is associated with particularly increased odds-ratios for mortality from common infections like diarrhoea and pneumonia.¹ The most sensitive estimates of acute malnutrition-dependent mortality have used logistic regression models to measure the fraction of disease/mortality burden that could have been avoided if the acute malnutrition exposure in the population had been reduced to zero (the population attributable risk fraction). Such analyses provide a way to account for the fact that children with acute malnutrition experience increased infectious disease-related mortality across

the spectrum of severity (Figure 1.2). Because moderate acute malnutrition (MAM) is commoner than SAM, deaths due to MAM comprise a significant fraction of all acute malnutrition-attributable mortality.¹⁹

Figure 1.2: Increased odds-ratio for mortality from infection is not confined to SAM

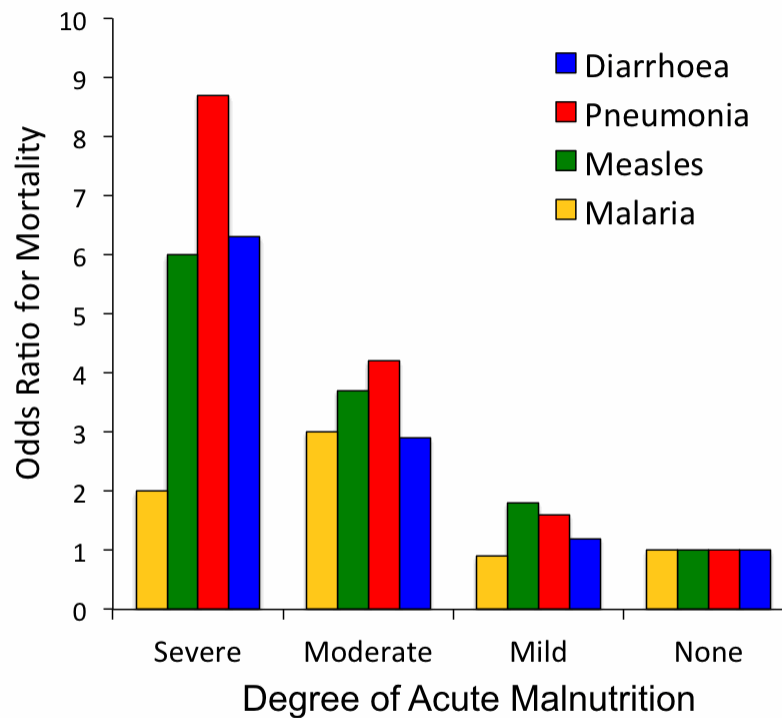


Figure 1.2: Odds ratio for mortality with common infectious disease syndromes shows the importance of MAM. Redrawn from data reported by Black *et al.*¹

In total, 14.7% of global mortality in children under-5 is attributable to stunting, and at least 12.6% is attributable to acute malnutrition (7.4% due to WHZ/WLZ < -3). A composite of foetal growth restriction, suboptimum breastfeeding, stunting, wasting, and micronutrient deficiencies (all considered nutritional exposures) is attributable for 44.7%.⁴ The impact of kwashiorkor on global mortality is undefined,

but is likely to be substantial, since it is associated with extremely high mortality rates in the context of infection.^{4, 20}

In addition to short-term risks from infectious diseases, acute malnutrition and stunting in early childhood are associated with long-term loss of growth and developmental potential.

- **Growth:** Growth faltering occurs during the first two years of life and once established appears to be extremely difficult to reverse except in the context of major and sustained environmental change, such as relocation to a developed country.²¹⁻²⁴
- **Education and development:** Improved linear growth in early childhood is associated with longer time spent in school, improved educational attainment and better performance in reading and nonverbal cognitive tests.^{22, 23, 25, 26}
- **Economic productivity as an adult:** A positive association between height and income is recognised in a wide variety of settings, including Europe and the USA.²⁷ In areas where stunting is prevalent, its associations with reduced final adult height, educational achievement and neurocognitive ability are likely to at least partly underlie a durable association with income.²²
- **Cardiovascular disease risk in adulthood:** Although studies from many settings have indicated that small-for-gestational age (SGA) increases cardiovascular risk profile in adulthood, independent effects of early childhood growth as they relate to low-income settings are less obvious, and if they exist at all are likely to be more modest (at least for linear growth) than previously suspected.^{23, 28}

- **Adverse pregnancy outcome and small for gestational age in the next generation:** Both maternal and paternal short stature and low educational achievement are independently associated with SGA in their children, providing a pathway by which growth failure during infancy may impact on the next generation, thereby entrenching and perpetuating poverty.^{29, 30}

1.4 Immunologic correlates of SAM.

The mechanisms by which undernutrition syndromes appear to potentiate infection remain unclear. While stunting and mild/moderate acute malnutrition are associated with increased infection-driven mortality at the population-level, the main research drive has been to understand the situation in SAM, where the association is strongest.

In 1959, Nevin Scrimshaw and colleagues published an article (expanded to a WHO monograph in 1968) entitled 'Interactions of nutrition and infection', which concluded:^{31, 32}

“Infections are likely to have more serious consequences among persons with clinical or subclinical malnutrition, and infectious diseases have the capacity to turn borderline nutritional deficiencies into severe malnutrition. In this way, malnutrition and infection can be mutually aggravating and produce more serious consequences for the patient than would be expected from a summation of the independent effects of the two.”³¹

Figure 1.3: Vicious cycle of acute malnutrition and infection

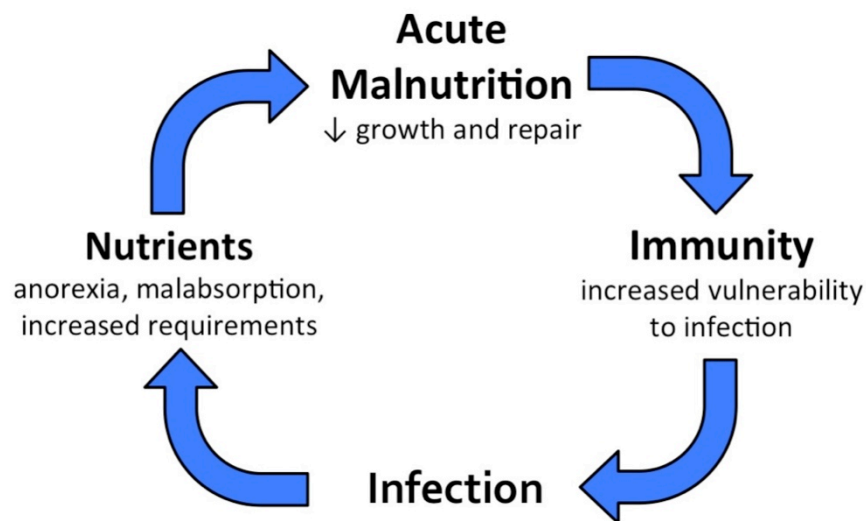


Figure 1.3: Scrimshaw's vicious cycle describes a pattern whereby acute malnutrition exacerbates infectious disease-driven morbidity, which in turn jeopardises nutritional status and growth.

This recognition of a synergistic and cyclical relationship between 'malnutrition' and infection has proved durable and highly influential (Figure 1.3). Mechanisms by which clinically manifested infectious diseases could worsen nutritional status are immediately apparent: unwell children lose their appetite; diarrhoeal diseases reduce nutrient absorption and increase zinc turnover; malaria results in anaemia and alters iron utilisation, for example.^{33, 34} However, despite significant research efforts, defining the precise way(s) that acute malnutrition impacts on host response to, and control of, infection has proved difficult.

The immunologic correlates of acute malnutrition in man have recently been the subject of a systematic review by Rytter *et al.*³⁵ The authors identified 245 papers reporting data on immune function amongst children with SAM, encompassing wide

diversity in study setting, design, case definition, control ascertainment and immunologic methodology.³⁶ The contemporary relevance of much of the work described must be interpreted in the light of several important methodologic and reporting caveats. Firstly, while modern syndromic definitions distinguish between acute malnutrition (wasting) and stunting, older studies frequently use weight-for-age z-scores to define 'malnutrition' which may encompass both. Only 21% of the studies used a definition of SAM that corresponds to the current WHO definition, and only two used the latest WHO growth standards. Secondly, most of the studies were performed in the 1970s and 1980s, at a time when many of the 'malnourished' children may in fact have been suffering from advanced HIV disease, either prior to its discovery or before routine diagnostic testing was practical or ethical. Thirdly, the clinical description of cases in most studies is insubstantial, and failure to properly account or control for other coincident acute or chronic infections (for example, tuberculosis, which may be directly associated with T cell anergy) may confound the immunologic data.³⁷ While keeping these caveats in mind, previously published work has been based around assessing for impact of SAM in three main domains of immunity: T cell function, systemic inflammatory activation, and epithelial barrier integrity.

1.4.i T cell function

Studies in the 1970s and '80s found that children with SAM have reduced cutaneous delayed-type hypersensitivity reactions to tuberculin following BCG (*Bacillus Calmette-Guérin*) vaccination, *Candida* antigen inoculation and chemical stimuli.^{35, 38-}

⁴⁰ This corresponded with reduced *in vitro* T cell proliferation upon mitogen stimulation,⁴¹⁻⁴³ which appeared to improve during nutritional rehabilitation.^{44, 45} However, the delayed-type hypersensitivity response involves concerted function of antigen-specific T lymphocytes and antigen-presenting cells, and a reduced response could result from functional abnormality of either. A focus on the T cell reflected the dogma of adaptive immune primacy of the time but a more recent study, one of the only ones to utilise modern immunologic techniques, has suggested that peripheral T cell effector function is in fact constrained by reduced number and maturation efficiency of dendritic cells (DCs). In the study, by Hughes *et al.*, (T cell) interferon- γ (IFN- γ) production after stimulation of peripheral blood mononuclear cells (PBMCs) with PPD (mycobacterial purified protein derivative) was reduced in SAM, but in an equivalent co-culture experiment with autologous pre-primed DCs, IFN- γ production was restored.⁴⁶

The clinical significance of these findings is unclear. Children with isolated deficits in T cell number or function have markedly increased severity of infection with common pathogens such as herpes simplex virus and varicella zoster virus, which are not characteristic of SAM.⁴⁷ Less severe primary T cell loss-of-function abnormalities are frequently characterised by paradoxical immune dysregulation with autoimmune disease, allergy and elevated immunoglobulin E (IgE) production, prominent. These features are also not characteristic for SAM.⁴⁸ In addition, children with SAM are able to mount robust immune responses to responses to a diverse array of vaccines, which represent the summation of numerous innate and adaptive cellular processes.⁴⁹

1.4.ii Systemic inflammatory activation

Several small, but methodologically sound, studies have indicated that children with SAM have elevated inflammatory and Acute Phase proteins even without demonstrable or suspected clinical infection. Such observations are particularly susceptible to bias introduced by incomplete clinical categorisation, and studies without clear descriptions of how freedom from infection was ascertained must be treated with caution.⁵⁰ In urban Ghana, children hospitalised with SAM but without overt infectious complications had elevated C-reactive protein (CRP) as well as decreased albumin, prealbumin and transferrin compared to healthy controls.⁵¹ Similar findings were reported from southern Nigeria.^{52, 53} In rural Kenya, children with SAM without infection had elevated IL-6 and soluble tumour necrosis factor receptor levels, especially in the context of kwashiorkor.⁵⁴ In a study from Jamaica, on the other hand, concentrations of CRP were similar between children with SAM without infection and controls.⁵⁵

Chronic immune activation is an increasingly well-recognised consequence of HIV infection and independently predicts progression to AIDS.⁵⁶ It also plays a role in the pathophysiology of obesity, in the ontogeny of malignancy, and has a direct negative impact on growth.^{57, 58} As described below, it may have functional consequences of clinical relevance to SAM.

1.4.iii Epithelial barrier function

Children with kwashiorkor develop a characteristic exfoliative dermatosis with epidermal atrophy, which represents a clear potential site for pathogen entry.⁵⁹ Skin breakdown does not appear to be immune mediated, since leukocyte infiltration was not evident in the few studies that examined biopsies, and cellular infiltration at a site of local trauma was similar between children with SAM and controls.³⁵

In terms of surface area, the largest epithelial barrier between internal milieu and external environment is the gastrointestinal epithelium. Detailed investigation of children with SAM has contributed greatly to our understanding of an inflammatory enteropathy, Environmental Enteric Dysfunction (EED), that is likely to be universal in SAM, but also extremely common in non-acutely malnourished children in resource-limited settings. While there is some debate about whether enteropathy associated with SAM and EED represent the same clinic-pathologic process, in the absence of evidence to the contrary it seems prudent to regard them as part of the same broad spectrum of enteropathies prevalent in the context of poverty.⁶⁰

1.5 *Environmental Enteric Dysfunction*

Environmental enteric dysfunction (EED) refers to an acquired clinical syndrome of reduced intestinal barrier and absorptive function associated with intestinal mucosal inflammation that was previously known as environmental or tropical enteropathy.⁶⁰⁻⁶² The first descriptions of EED were made in adults, and the concept that it represented an acquired and environmentally-mediated phenomenon

emerged in a 1971 study of Peace Corps volunteers.⁶³ Residence in India or Pakistan for 18-24 months was associated with weight loss, subclinical malabsorption (defined via xylose absorption and Schilling tests), and jejunal villous atrophy that recovered following return to the USA. Other than weight loss the volunteers were largely asymptomatic and did not have parasites or amoebae in their stool, in contrast to tropical sprue, which is associated with diarrhoea and malabsorption.⁶⁴ Histologic findings of small intestinal villous atrophy and crypt hyperplasia with lymphocytic infiltration are characteristic of EED, but are non-specific, and resemble other chronic inflammatory enteropathies.⁶⁰ Technical and ethical impediments to widespread gastrointestinal endoscopy in asymptomatic individuals means that EED has usually been inferred on the basis of non-invasive tests of intestinal function, the commonest of which is the lactulose-mannitol (L:M) dual sugar absorption test.⁶⁵ Epidemiologic studies using this approach have revealed extremely high prevalence of impaired enteric mucosal barrier function associated with poverty, to the extent that some degree of EED can be considered virtually ubiquitous amongst children living in settings where food security, safe water and facilities for hygienic waste disposal are absent or erratic.⁶⁶⁻⁶⁹

EED is associated with growth failure and stunting. Studies using different techniques to quantify mucosal barrier function from diverse ecological settings around the world have revealed a consistent inverse relationship between EED activity and linear growth.⁷⁰⁻⁷³ While it had previously been supposed that stunting was primarily a manifestation of chronic undernutrition due to dietary inadequacy in conditions of poverty, well-conducted controlled clinical trials of nutritional

supplementation, micronutrient fortification or nutrition-specific education have demonstrated only modest impacts on linear growth.⁷⁴ A direct effect of EED on growth is plausible:⁷⁵ As described previously, the histologic lesion of EED resembles other inflammatory enteropathies and products of Th1 (T helper cell type-1) signalling and neutrophil extravasation are detectable in stool.^{73, 76-78} It is hypothesised that systemic inflammatory activation results from translocation of exogenous antigens, luminal bacteria and their products consequent to breakdown in epithelial barrier function.^{68, 79} A similar triad of growth failure, intestinal inflammation and systemic inflammation due to bacterial translocation is also observed in other inflammatory enteropathies of childhood, for example Crohn's disease. It is well recognised that intestinal and systemic inflammation in Crohn's drives linear growth failure through a direct effect of inflammatory mediators on the Growth Hormone – Insulin-like Growth Factor-1 (GH-IGF-1) axis and via IL-6 mediated repression of chondrocyte function at growth plates.^{80, 81} Similarly, a recent study from Zimbabwe demonstrated inverse correlation between acute phase proteins and IGF-1 in a population with high prevalence of EED.⁷⁹

The cause or causes of EED are currently undefined but have recently become the focus of significant research interest. The prevailing theory is that chronic ingestion of faecal material, resulting from water contamination and suboptimal sanitation/hygiene practices (so-called 'WASH' exposures) is critical in establishing the condition. This theory has been supported by cross-sectional comparisons of EED activity in reference to WASH exposures.^{72, 82} It is now being tested in three large cluster-randomised controlled trials of high-intensity WASH improvements, which

are due to report in the next two years.^{83, 84} The concept that microbial pressure plays a causal role is further supported by a meta-analysis of the effect of antibiotics on linear growth in children living in low- and middle-income countries, which showed that they increased height by 0.4mm per month (0.0mm to 0.7mm), and by the fact that small intestinal bacterial overgrowth (SIBO) is associated with decreased absorptive function amongst children living in poverty.^{85, 86} However, it is possible that modulation of the composition or content, rather than the total biomass, of intestinal bacterial exposure is most important, since evidence is accumulating of significant alterations in the gut-resident microbiota in the context of growth failure.⁸⁷ It remains possible that EED is caused or perpetuated by a single intestinal pathogen. One potential candidate is *Giardia intestinalis*, which has been variously associated with increased mucosal permeability, malabsorption and linear growth faltering in young children.⁸⁸⁻⁹⁰ The importance of *Giardia* may have been systematically understated because it can be difficult to diagnose. Recent studies using PCR (polymerase chain reaction)-based diagnostic methodologies have found it to be far more prevalent than previously indicated.⁹¹ *Giardia* causes synergistic growth failure in the context of undernutrition in a murine model of EED.⁹²

1.6 SAM: therapeutic paradigm.

The clinical management of SAM involves a programme of interventions designed to treat, ameliorate or minimise complications and metabolic disturbance during nutritional rehabilitation, and to promote anthropometric and neurodevelopmental recovery.¹¹ Inpatient care is structured around a '10 step' approach, divided into

initial stabilisation and subsequent rehabilitation phases, the implementation of which has been associated with dramatic reduction in mortality compared to historical controls (figure 1.4).⁹³

Figure 1.4: 10 steps approach to inpatient management of SAM

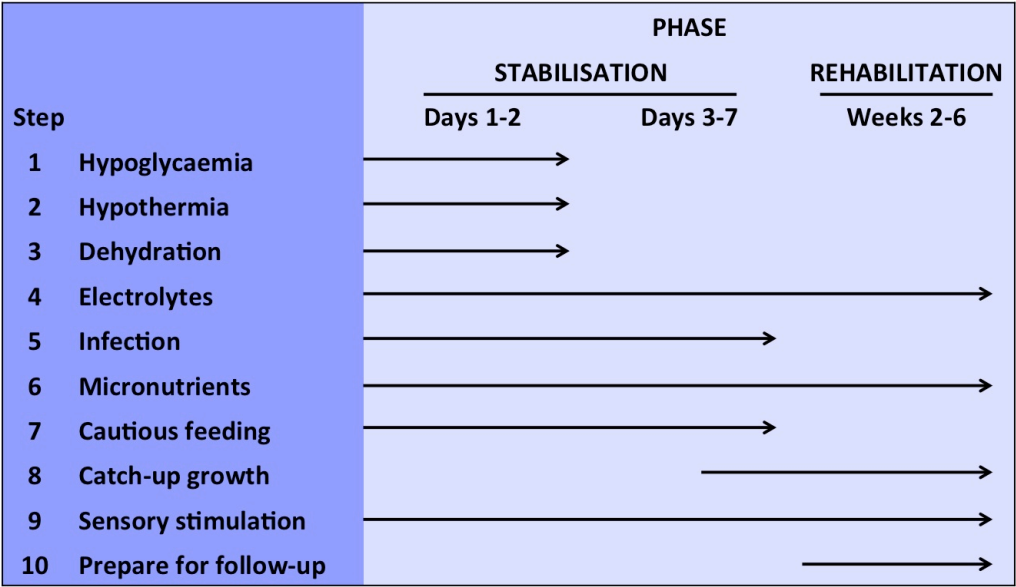


Figure 1.4: Consideration of 10 key clinical priorities provides a framework for the modern inpatient management of SAM. The ‘10 steps’ have been widely adopted by WHO and national guidelines.²

However, inpatient care of SAM is associated with substantial opportunity costs for families already (usually) suffering the effects of poverty, and acute malnutrition is an important risk factor for nosocomial infection.⁹⁴ Therefore, where safe and practicable, children with SAM should be treated in outpatient or community-based settings, with inpatient care reserved for those with medical complications or who lack appetite.^{95, 96} A key enabling factor in the implementation of community management of SAM has been the development of ready-to-use therapeutic foods

(RUTF). RUTF are lipid-enveloped pastes comprising either a peanut or legume plus cereal base, mixed with oils, sugar, electrolytes and micronutrients to a formula designed for recovery from SAM. As a consequence of their low water activity, RUTF are microbiologically stable with a long shelf life and can be used at home, unlike milk-based formulas that can only be prepared safely in hospital.⁹⁷ The technical specification for nutritional composition of RUTF is almost identical to that for 'F-100' therapeutic milk, which is the standard of care for nutritional rehabilitation of children with SAM and was based, for the most part, on published specifications for infant formula manufacture.⁹⁸ There have been no major changes to the composition of F-100/RUTF since they were initially designed.⁹⁹ Although clinical trial-based evidence of efficacy is relatively weak, this is a reflection of the fact that the clear practical benefits of RUTF, its very rapid and widespread adoption by organisations like Médecins Sans Frontières, and the lack of viable alternatives means that a situation of equipoise for the testing of RUTF was lost very soon after its development.¹⁰⁰ RUTF is currently provided to 1.96 million children annually, at a cost of \$200 million.¹⁰¹ The main manufacturer of RUTF is Nutriset (Malaunay, France) who make Plumpy'nut, but local production is possible and would provide a more equitable framework for further scale-up of its use.^{102, 103} Such a scale-up is certainly necessary, since just 10% of children with SAM have access to lipid-based RUTF.¹⁰¹

Ancillary treatments in the early management of SAM include broad-spectrum antibiotics targeted at small intestinal bacterial overgrowth as well as unrecognised systemic infection, and antihelminthic treatments.¹⁰⁴ A week of amoxicillin reduced

mortality amongst children with SAM receiving treatment in community settings in Malawi by a third, and it significantly reduced referral for inpatient care in a trial from Niger.^{105, 106} A trial of six months' co-trimoxazole prophylaxis for children with complicated SAM has recently been completed and will report in 2016 (NCT00934492). A trial of different parenteral antibiotic schedules for the management of complicated children with SAM has been funded (James Berkley, personal communication).

Despite substantial decreases in mortality associated with SAM when WHO guidelines are implemented effectively, it is important to recognise that there remains room for improvement. In-hospital mortality rates remain high in many settings, and post-discharge mortality rates indicate a failure of SAM management to normalise risk.¹⁰⁷⁻¹⁰⁹ In Kenya, 20% of children who had been admitted to hospital with WAZ < -4 and who survived to discharge died in the subsequent 6 months.¹¹⁰ In Malawi, 42% of children with SAM died in the 18 months following presentation, including 10% after discharge from SAM treatment.¹¹¹ In Bangladesh, amongst children with SAM and pneumonia who were admitted to hospital but survived to discharge, 8.7% had died within three months.¹¹²

1.7 EED: Therapeutic paradigm

In contrast to SAM, there are currently no specific treatments available for EED, although a number of approaches have been tried:

- **Micronutrients:** In two small studies from India, there was a suggestion that a single vitamin A supplement (200,000 IU, which is the Integrated Management of Childhood Illness standard dose) was associated with a greater decline in L:M than placebo amongst children hospitalised with diarrhoea or respiratory disease, whereas daily lower-dose vitamin A (16,700 IU, which is still supra-physiological) in the community was not.¹¹³ Two trials of alanyl-glutamine, which is conditionally essential for rapidly dividing cells such as those in the gut epithelia, showed that supplementation was associated with modest decrease in L:M amongst Brazilian children.^{114, 115} A trial of n-3 (omega-3) polyunsaturated fatty acid (PUFA) supplementation amongst infants in The Gambia showed no effect on L:M or markers of enteric inflammation,¹¹⁶ and a further recent trial of micronutrient and fish oil supplementation in Malawian children also failed to show any impact.¹¹⁷ A two week course of zinc supplementation at 20 mg per day limited deterioration of L:M over time in another study from Malawi.¹¹⁸
- **Probiotics:** A trial of *Lactobacillus GG* treatment for 30 days in Malawian children with EED failed to impact on intestinal permeability.¹¹⁹ An efficacy trial of a synbiotic preparation, containing a number of probiotic species and prebiotics, in the management of SAM also failed to show any measurable impact.¹²⁰
- **Anti-parasitic approaches:** A single dose of albendazole prevented deterioration of L:M over time in a study from Malawi.¹¹⁸ A trial from Bangladesh that evaluated three-monthly albendazole (anti-helminthic) along

with monthly secnidazole (anti-amoebic and anti-protozoal) also failed to demonstrate any impact on gut integrity.¹²¹

- **Rifaximin:** Rifaximin is a gut-specific broad-spectrum antibiotic with a complex mode of action that is thought to involve modulation of microbial virulence and has been associated with reduction in endotoxaemia in patients with hepatic encephalopathy.¹²² A short course of rifaximin in healthy Malawian children did not impact on L:M.⁶⁷

Gut mucosal integrity improves during nutritional rehabilitation for SAM, but it is unclear which of the components of treatment are most important.¹²³ The modest improvements seen with some of the micronutrient interventions have not translated to improvements in growth, so at present there appears to be no specific treatment for EED over and above treating SAM if it is coincident.

As noted above, in view of the fact that chronic exposure to faecal material is considered to play an important part in the pathogenesis of EED, large cluster-randomised trials of WASH interventions are currently underway in Bangladesh, northern Kenya and Zimbabwe. Further trials of all of the nutritional interventions listed above have also been registered.

1.8 Immunologic consequences of chronic pathogen exposure.

The human immune system has been classically divided into two arms. The innate immune system mounts rapid non-specific antimicrobial responses driven by

exposure to 'danger' signals. The adaptive immune system provides antigen-specific immunity and is capable of anamnestic 'memory' responses, where immune responses are amplified in the context of prior exposure to a specific antigen.

The prototypical examples of innate danger sensors are the Toll-like receptors (TLRs). TLRs form part of an extensive network of microbial pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs); restricted non-protein motifs that have been highly conserved in pathogen evolution.^{124, 125} They are expressed on the surface of immune cells resident at mucosal surfaces, the main potential sites of pathogen challenge, and function as sentinels. Pathogen challenge and TLR ligation results in activation of antimicrobial immunity via a complex set of local and distal cellular and hormonal pro-inflammatory processes. Innate immune cells with direct antimicrobial activity are recruited from the circulation. Arriving neutrophils engulf (phagocytose) and kill extracellular pathogens, and myeloid cells begin to trigger and establish the adaptive response. The fact that this activation is not antigen-specific allows it to develop rapidly and function both as a trigger and an organising influence on subsequent immune responses. As such, microbial recognition and activation is a critical early step in the handling of all pathogens, providing early warning and stimulation to all arms of the antimicrobial immune response. More recently, the discovery of sensors of endogenous products of tissue damage (damage-associated molecular patterns, DAMPs), and cytosolic PRRs (especially the nucleotide-binding oligomerisation domain (NOD) receptors, NLRs) has underscored the diversity in molecular danger sentinels.¹²⁶⁻¹²⁸ While PRRs have been considered to function via a relatively

restricted array of intracellular response pathways, the most well understood of which involves activation of the transcription factor NF- κ B (figure 1.5), in fact they are capable of orchestrating highly distinct responses to different pathogen exposures, challenging the concept of a broadly 'non-specific' innate immune response.¹²⁹

Figure 1.5: NF- κ B signalling pathway

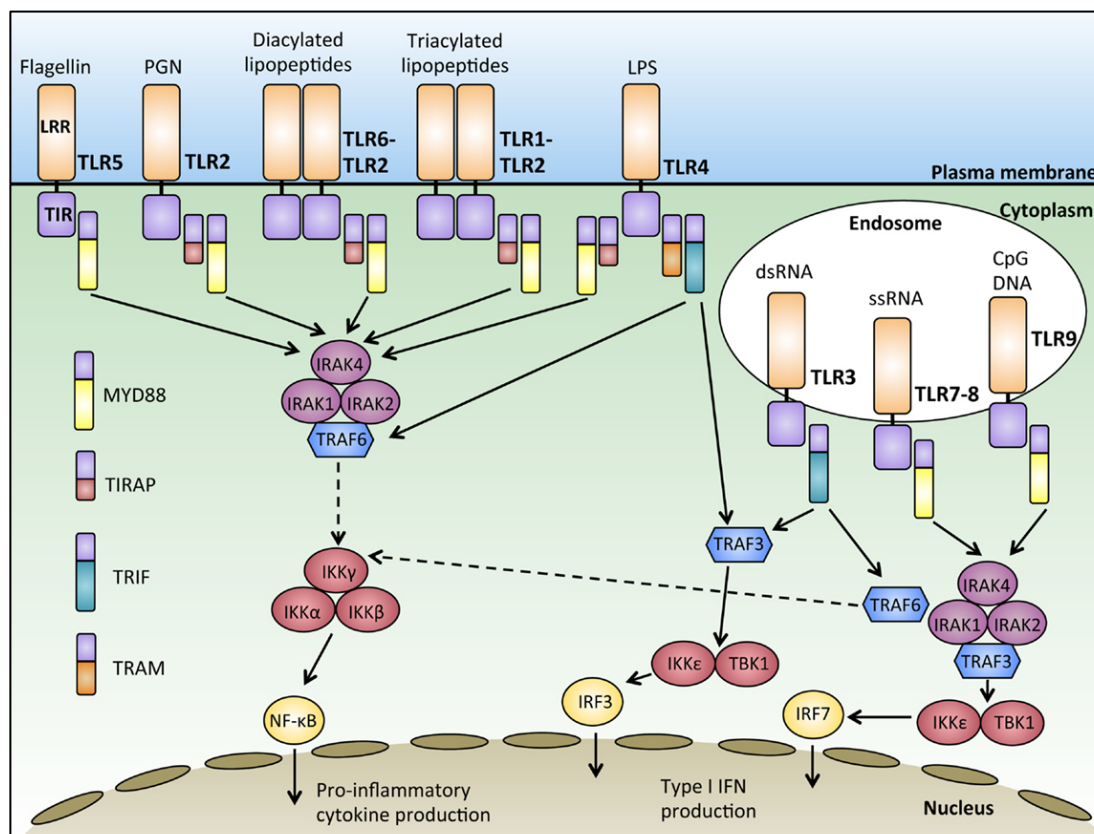


Figure 1.5: NF- κ B is a critical regulatory protein complex that integrates information from a diverse range of pattern recognition receptors. Figure from Wang *et al.* reproduced under a creative commons license.⁹

In the last 15 years it has become increasingly well recognised that the quality and intensity of innate immune responses are shaped by past, as well as concurrent,

microenvironmental exposures, indicating that the innate immune system is capable of a form of immunologic memory. BCG is an attenuated *Mycobacterium bovis* strain that provides protection against disseminated tuberculosis (caused by *Mycobacterium tuberculosis*, MTB). It is administered intradermally and elicits a vigorous cell-mediated inflammatory response, due in part to its high-level endogenous adjuvanting activity. In a number of studies in resource-poor settings BCG vaccination, or the presence of a BCG scar, has been shown to be protective against mortality in early childhood. The magnitude of the protective effect on BCG on all-cause mortality is probably too large to be explained by protection against tuberculosis alone.¹³⁰ In a landmark study from Guinea-Bissau, it was shown that the absence of a BCG scar was associated with respiratory syncytial virus (RSV)-mediated acute lower respiratory tract infection, suggesting a non-antigen-specific or heterologous protective effect of BCG vaccination.¹³¹ This phenomenon has been termed 'trained immunity'.¹³² A wealth of data from murine and other experimental models have indicated that BCG is able to modulate myeloid-derived cellular function in certain circumstances, but until recently equivalent data from human studies has been lacking.^{133, 134} In 2015, Jensen *et al.* found that early BCG administration to low birthweight infants was associated with increased pro-inflammatory cytokine production in a whole blood stimulation assay in response to purified protein derivative (PPD, a mycobacterial protein mix, providing an index of homologous immunity) and several TLR-ligands four weeks after vaccine administration.¹³⁵ TLR ligands are not protein-based and hence cannot be presented on MHC or be subject to adaptive anamnestic immune responses – strongly suggesting an impact on innate cellular function. Healthy adult individuals receiving

BCG exhibited long-lasting changes in monocyte phenotype, with increased expression of PRRs and enhanced pro-inflammatory cytokine release following challenge with lipopolysaccharide (LPS, also known as endotoxin, a Gram –ve bacterial cell wall component that is the ligand for TLR4) up to a year after vaccination.¹³⁶ A series of recent papers have shown that trained immunity is achieved via epigenetic modification of monocytes/macrophages, accompanied by a shift towards cellular glycolytic metabolism, and partly mediated via NOD2.^{5, 137, 138} Crucially, epigenetic modulation of monocyte function was bidirectional: prior exposure to certain stimuli (beta-glucan, a component of fungal cell walls) potentiated or ‘trained’, cells, whereas other stimuli (LPS) had the opposite effect, of ‘tolerising’ them (figure 1.6).

Figure 1.6: Innate immune training

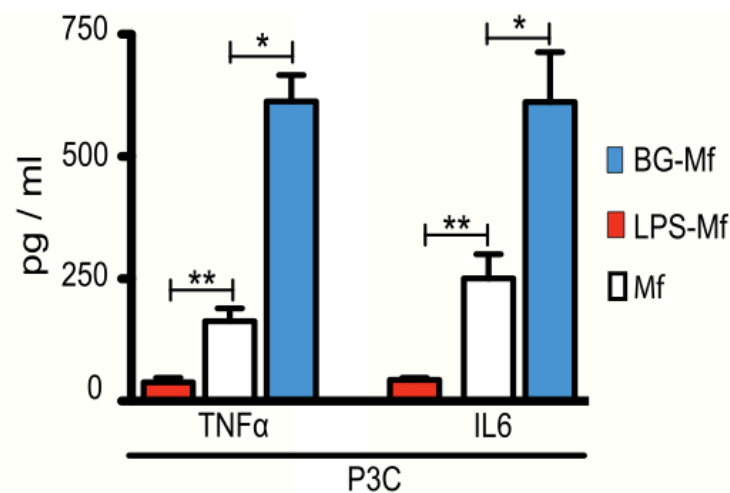


Figure 1.6: Saeed *et al.* showed that preincubation of monocytes with tissue culture media containing beta-glucan (BG) or lipopolysaccharide (LPS, a TLR4 ligand) had differential impact on cytokine expression following stimulation with the TLR2 ligand, Pam3Cys (P3C).⁵ Figure reproduced with permission.

Just as innate training has a clinical correlate in live vaccine-induced heterologous immunity, so innate tolerising is evident in Endotoxin Tolerance (ET), which refers to a phenomenon whereby repeated exposure to LPS imparts a refractory phenotype to monocytes upon further homologous and (sometimes) heterologous innate immune challenge.¹³⁹ ET has been demonstrated in a wide variety of *in vitro* and whole organismal models, including human challenge studies.¹⁴⁰ It is thought to occur to a clinically relevant degree in settings of sepsis, multi-organ dysfunction, and critical illness from a wide variety of causes.^{139, 141, 142} ET is an adaptive phenomenon and its dysregulation may contribute to the pathogenesis of some chronic inflammatory conditions.^{143, 144}

The recognition that the innate immune system may be sensitive to recent or distal prior exposure may provide a framework through which to address an apparent paradox in the pathology of SAM:

Children with SAM often have evidence of chronic inflammatory activation, yet their observed vulnerability to major infection implies a difficulty in mounting effective immune responses to pathogen challenge.

This is true both in the systemic circulation (where children with SAM have hypercytokinaemia but are vulnerable to severe malaria – which is a systemic pathogen), and in the gut (where children have inflammatory EED but remain vulnerable to diarrhoeal disease and infection with mucosal pathogens).^{145, 146}

1.9 *Innate immune function and inflammatory bowel disease.*

Application of systems biologic approaches to infectious disease has revealed that a diverse array of genomically-encoded disorders in innate immune function exist, and may be responsible for increased susceptibility to infectious diseases.¹⁴⁷ The majority of these are due to loss-of-function mutations in key pathways involved in immune sensing. For example, primary immune deficiencies with increased susceptibility to invasive bacterial disease occurring due to mutation in almost every molecule in the TLR4 signalling pathway have been described.¹⁴⁸ Similar work has been undertaken in inflammatory bowel disease (IBD), for whilst IBD is traditionally thought of as a polygenic disease partly mediated by as-yet-undefined environmental exposures, it may also result from an array of monogenic disorders.¹⁴⁹ Importantly, several strands of research indicate that although IBD results from an inflammatory process, it may also arise from effective loss-of-function in inflammatory pathways:

- **Monogenic IBD:** Primary immune defects in granulocyte function and trafficking present with colitis that may strongly resemble Crohn's disease. For example, chronic granulomatous disease (which occurs due to absent neutrophil oxidative burst function) is a clinically important differential diagnosis to consider in the work-up of early onset IBD.¹⁵⁰ Deficits in innate danger sensing or in adaptive immune cell number or function, e.g. hypomorphic NEMO (NF- κ B essential modulator) disease or Common Variable Immunodeficiency Disease, may also present with colitis as part of multi-system inflammatory syndromes.^{149, 151}

- **Functional studies in IBD:** In pioneering work utilising a highly experimental approach, Smith *et al.* showed delayed clearance of radiolabelled bacteria after intradermal administration in Crohn's along with reduced neutrophil chemotaxis to the site of pathogen challenge resulting from an impairment in cytokine secretion from tissue-resident macrophages.¹⁵² Genome-wide association studies of Crohn's have implicated the NLR, NOD2, in its pathogenesis.¹⁵³ Impaired sensing of muramyl dipeptide (a component of Gram +ve bacterial cells walls and the ligand for NOD2) has been demonstrated in peripheral blood-derived monocytes from Crohn's patients.¹⁵⁴
- **HIV and intestinal inflammation:** Although advanced HIV disease is characterised by global CD4+ T cell depletion, it is also associated with an inflammatory enteropathy that is histologically indistinguishable from EED.⁶⁰

1.10 Statement of hypotheses.

1. Previous studies suggest that severely acutely malnourished children have high-level chronic exposure to pathogens and their products via impaired skin and mucosal barrier function. I hypothesise that chronic exposure to PAMPs is associated with innate immune training in the systemic circulation. Particularly, I hypothesise that a tolerising effect of chronic exposure to LPS across a leaky gut epithelium leads to a state of long-term endotoxin tolerance that renders children highly susceptible to invasive infection with enteric-derived Gram –ve bacteria.

2. I hypothesise that local inflammation perpetuates this effect via bystander immune activation, particularly in the gut. Therefore, I propose that broadly anti-inflammatory therapeutic agents may be used to promote mucosal homeostasis and encourage normalisation of innate immune pathogen-sensing pathways during recovery from SAM (see Box 1).

Box 1: Reducing inflammation to promote immunity in SAM

Chronic innate immune activation (chronic inflammation, see section 3.3) can lead to impaired innate immune responses to subsequent pathogen challenge. The prototypical example of this effect is endotoxin tolerance, described in section 1.8. It can also, at least theoretically, lead to impaired adaptive immune responses. For example, the production of retinoic acid by dendritic cells is essential for the induction of effective T cell responses, but is dependent on the activity of retinaldehyde dehydrogenase (RALDH), which is potently inhibited by local prostaglandin E2 (PGE2) production.^{6, 7} SAM is associated with chronic intestinal and systemic inflammation, which might impair inflammatory and immune responses to pathogens and predispose to major invasive infection. Some anti-inflammatory treatments might dis-inhibit other pro-inflammatory and immune pathways in a manner that may help to restore mucosal immune homeostasis and address observed vulnerability to infectious diseases.

I will address these hypotheses across three distinct studies:

- a) Cross-sectional study relating PRR function to acute nutritional status, as measured by MUAC, and systemic and enteric inflammatory activation (Chapter 3).

- b) A pilot randomised controlled trial of mesalazine, a gut-specific anti-inflammatory agent, in children with SAM and evidence of EED (Chapter 4).
- c) A pilot randomised controlled trial of different strategies of provision of n-3 polyunsaturated fatty acids (PUFA), which have anti-inflammatory activity, in children with complicated SAM requiring hospital treatment (Chapter 6).

In addition, I will describe the results of a further study that has direct relevance to the hypotheses under investigation.

- d) A cross-sectional study of n-3 PUFA status across a spectrum of acute nutritional status in two contrasting ecological settings (Chapter 5)

Figure 1.7: Conceptual framework

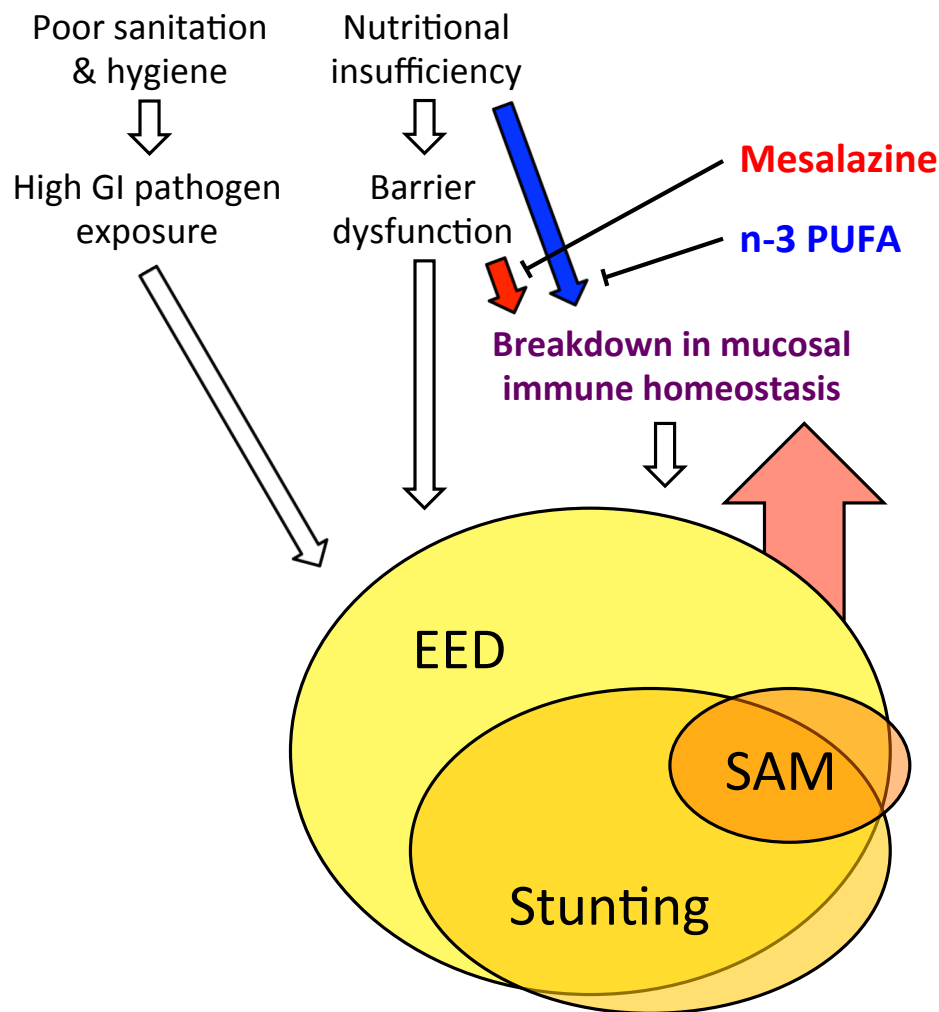


Figure 1.7: Nutritional insufficiency is associated with intestinal epithelial barrier dysfunction. In the context of dysbiosis and high pathogen burden, an inflammatory enteropathy ensues. Innate inflammatory activation results in tissue damage and further impairs barrier function. Relative innate tolerance to microbial products impedes immune clearance of translocating bacteria from the lamina propria, and establishes a chronic inflammatory state with breakdown in immune homeostasis. Impaired absorption and inflammation-driven growth failure promote development of stunting and SAM. The nature of this homeostatic breakdown (purple) will be interrogated via determination of innate functional immune responsiveness in SAM. Two potential therapies are proposed. Mesalazine will promote immune homeostasis by acting to rebalance the host immune activation in favour of resolution rather than chronic inflammation. n-3 PUFA will have a similar effect, and additionally ameliorate the potentially pro-inflammatory impact of RUTF.

CHAPTER 2

Specific background and pilot work

2.1 *Mesalazine in SAM with EED*

It has been suggested that gastrointestinal luminal contents drive the mural inflammatory activation of EED via specific enteric pathogens, a global increase in pathogen burden (due to high faeco-oral transmission), or altered microbiota.^{75, 155-}

¹⁵⁷ However, in all other inflammatory intestinal diseases of childhood, aberrant or inappropriate host response plays a critical role in the pathogenesis, even where an intraluminal trigger is evident.¹⁵⁸ In coeliac disease for example, although antigen-specific T cell activation is the hallmark, bystander induction of innate and other immune components contributes to tissue damage and sustained pathology.¹⁵⁹

A key unanswered question is whether intestinal inflammation in EED is an appropriate, adaptive response to excessive pathogen exposure that mitigates the impact of acute and chronic enteric infections; or if it is maladaptive, with host-driven immunopathology contributing to mucosal barrier breakdown and growth failure.

Mesalazine is an aminosalicylate derivative (5-aminosalicic acid, 5-ASA) with gut-specific anti-inflammatory and immunomodulatory activity. It has a complex mechanism of action that includes oxygen free radical scavenging, reduction in cyclooxygenase 2, inducible nitric oxide synthase and NF- κ B activity, and induction of peroxisome proliferator-activated receptor-gamma.¹⁶⁰⁻¹⁶³ It is commonly used to

induce remission of mild to moderate paediatric ulcerative colitis and for maintenance of remission.¹⁶⁴⁻¹⁶⁶ Mesalazine is only modestly efficacious for these indications. Meta-analysis of studies in adults showed that when used for induction of remission in UC, 72% of patients receiving mesalazine failed to remit compared to 85% of those receiving placebo (relative risk (RR) 0.86, 95% confidence interval (CI) 0.81 to 0.91).¹⁶⁷ When used for maintenance of remission, 41% of patients receiving mesalazine relapsed compared to 58% on placebo (RR 0.69, 95% CI 0.62 to 0.77).¹⁶⁸ Mesalazine has not proved to be efficacious in Crohn's.¹⁶⁹ However, mesalazine remains widely used, partly because it has a very favourable safety profile compared to other members of its class, with less frequent renal, hepatic and gastrointestinal toxicities. In all reported clinical trials of its use in children with IBD there have been no serious drug-related adverse events.¹⁶⁴ Meta-analysis of its safety profile in clinical trials as a treatment for UC found that *"the incidence of adverse events and withdrawals due to the 5-ASA formulations did not significantly differ from that associated with placebo"*.¹⁶⁷ The British Society for Paediatric Gastroenterology, Hepatology and Nutrition (BSPGHAN) consider it to be *"safe and effective in the induction and maintenance of remission of active ulcerative colitis"*.¹⁶⁶ The most commonly reported mild adverse effects include headache and gastrointestinal disturbance. The most important potential known toxicity is renal. Nephrotoxicity is idiosyncratic rather than dose dependent, but is extremely rare (0.26% per patient year) and usually responds well to withdrawal of the drug.¹⁷⁰ BSPGHAN recommend renal and hepatic function monitoring every 6 months for those on treatment.¹⁷¹

Two preparations of mesalazine are available as child-friendly enteric-coated granules designed for easy oral administration. Salofalk is produced by Dr. Falk Pharma, and Pentasa is produced by Ferring Pharma. Additionally, olsalazine capsules (which may be opened and the contents sprinkled on food) are produced, as Dipentum, by UCB Pharma. Mesalazine is no longer patent protected, and a number of generic formulations are available. Because mesalazine is commonly used against ulcerative colitis, most delivery mechanisms have aimed to release the majority of active drug in the colon. Pentasa, on the other hand, begins releasing active drug in the duodenum and is targeted against small- and large-intestinal pathology.¹⁷² Current mesalazine formulations do not require cold-storage.

Treatment of inflammatory bowel disease is conventionally divided into induction of remission and maintenance phases. The most efficacious therapeutic strategies for induction (particularly in Crohn's) are provision of systemic corticosteroids, or exclusive enteral nutrition (EEN) with a polymeric or elemental feed. EEN requires the utilisation of liquid milk-based feeds, and widespread community-based implementation in settings where EED is common would be highly impractical. The avoidance of a necessity to provide liquid-based feeds has been the cornerstone of RUTF's success and it is not immediately clear how or whether an elemental RUTF could be formulated. The use of systemic immunosuppressants in children with acute malnutrition, stunting or EED is contraindicated because *M. tuberculosis* is an important pathogen in such children and infection is extremely difficult to rule out using current diagnostic techniques.¹⁷³ Mesalazine has minimal, if any, systemic immunosuppressant activity.¹⁷⁴

In Chapter 4, I report the results of a pilot clinical trial with a primary objective of investigating the safety and acceptability of mesalazine as an adjunctive treatment for children with severe acute malnutrition (SAM). The trial was designed with intensive follow-up in order to detect any indication that the strategy was interfering with a beneficial, adaptive response. Secondary objectives were to collect hypothesis-generating data relating to inflammatory activation and growth.

2.2 Njugu Plus: Polyunsaturated fatty acids in SAM

The n-3 and n-6 families of polyunsaturated fatty acids (PUFA) are biologically important molecules with a wide variety of structural and functional roles. They are immunologically active as precursors to the eicosanoid family of inflammatory mediators and as a result of their characteristic physical properties when incorporated in the lipid membranes of leukocytes.^{175, 176} Short chain n-3 and n-6 family members cannot be synthesised *de novo* and must be provided by dietary intake. They act as precursors for the biosynthesis of longer chain fatty acids (LC-PUFA) that are formed via the sequential action of desaturase and elongase enzymes. LC-PUFA may also be consumed pre-formed in the diet. Immunomodulatory activities of the n-3 (which generally downregulate inflammation) and n-6 PUFAs (which are pro-inflammatory) and their derivatives are mutually antagonistic, partly because they compete in the same set of metabolic pathways, rendering their dietary intake ratio of particular importance. LC-PUFA are also essential to visual development, brain growth and neuronal modelling.¹⁷⁷

Specifications for F-100 and RUTF permit broad ranges of PUFA composition, with n-6 and n-3 fatty acids comprising 3 to 10%, and 0.3 to 2.5% of total energy content, respectively.⁹⁶ There is no stipulation for provision of pre-formed LC-PUFA. Short and long chain n-6 PUFA are abundant in peanuts and many of the vegetable oils normally used in manufacturing RUTF. Conversely, n-3 PUFA (especially n-3 LC-PUFA) are present in a limited range of source ingredients, principally in oily fish. RUTF composition therefore typically has a high n-6:n-3 PUFA ratio with a very low absolute n-3 LC-PUFA content.

Several previous studies have investigated fatty acid status in malnourished children. Analyses of erythrocyte and plasma lipids have proved highly inconsistent, but relatively low levels of arachidonic acid (AA) and docosahexaenoic acid (DHA) are most frequently reported findings.¹⁷⁸⁻¹⁸⁵ Composition of plasma and erythrocytes should only be regarded as proxy measures of sufficiency, since it is possible that preservation of their composition is achieved by diversion away from other tissue compartments: deficiency might be occult if, for example, measurable levels in the blood were maintained at the expense of optimal deposition in neuronal tissue. There is little doubt that children with SAM are at high risk for having very low recent n-3 LC-PUFA intake. For young children, the major dietary source of n-3 and n-6 PUFA is breast milk, the composition of which is critically dependent on maternal intake.¹⁸⁶ A systematic review of studies measuring breast milk composition revealed that breast milk from sub-Saharan African mothers has consistently high AA content but very variable DHA content, probably driven by large local discrepancies in access to fish.¹⁸⁷ Access to dietary sources of fish and n-3 PUFA is strongly related to gross

domestic product on a per-country basis,¹⁸⁸ and a detailed analysis of fatty acid intake by Gambian children revealed a steep decline in n-3 PUFA and preformed LC-PUFA at the point of weaning.¹⁸⁹ In conditions of moderate or intermittent food insecurity far from sources of affordable fish, intake of n-3 LC-PUFA is likely to be marginal, it is reasonable to assume that decreases in food security would be associated with reduced fish intake for both breastfeeding mothers and their children in many circumstances.

Prior to undertaking the work described in Chapter 6, I conducted a small cross-sectional pilot study assessing PUFA status in children with SAM (unpublished). I recruited children with SAM (n = 16) and non-acutely malnourished hospital controls (n = 16) from a rural hospital in Kilifi, Kenya, and measured erythrocyte fatty acid content by gas chromatography. I found that children with SAM had markedly decreased n-6 and n-3 PUFA compared to controls, with an elevated n-6:n-3 ratio suggesting severe deficiency in n-3 PUFA.

Figure 2.1: Pilot work on PUFA in SAM

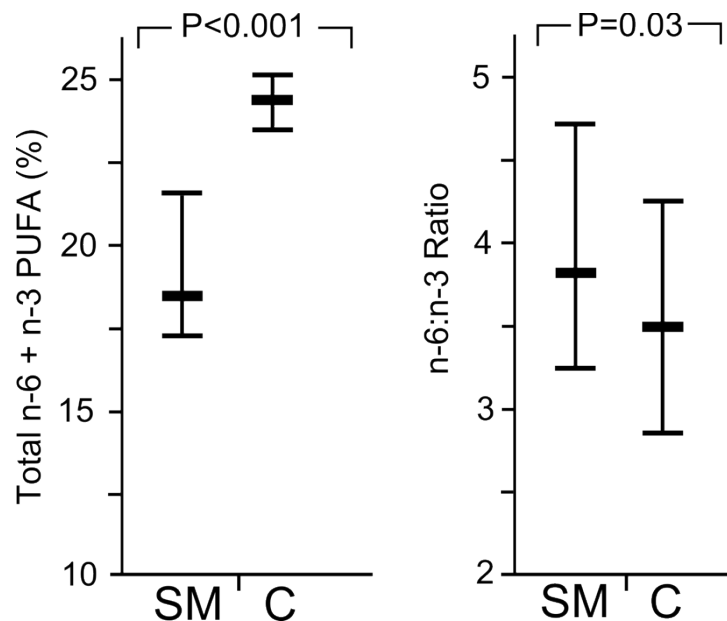


Figure 1.8: Total n-6 and n-3 fatty acids as percentage of erythrocyte membrane phospholipid comparing severely acutely malnourished children (SM), to healthy controls (C) indicates marked association between malnutrition and PUFA deficiency. (16 severely malnourished children and 16 controls, at Kilifi District Hospital in 2010, median age 22 months for both groups.)

In this context, I hypothesised that the provision of RUTF with very high n-6:n-3 PUFA ratio would, at best, fail to adequately address underlying n-3 PUFA deficiency and, at worst, might exacerbate inflammatory activation and adversely affect recovery. Provision of differential PUFA intake during recovery from SAM had been attempted in one previous trial. Smit *et al.* randomised 17 Pakistani children with low WAZ to 500 mg/day of fish oil alongside standard care (n = 10), which consisted of multivitamin provision and parental nutritional education, or standard care alone (n = 7) for up to 12 weeks.¹⁹⁰ They demonstrated a marked increase in erythrocyte composition of all n-3 LC-PUFA in the intervention arm but no change from baseline

in the controls. Similarly, Koletzko *et al.* demonstrated an increase in n-3 LC-PUFA in plasma phospholipids from baseline in a cohort of 8 Nigerian children with SAM after two weeks of follow-up, when provided a rehabilitation diet that contained fish.¹⁷⁹ No previous studies had assessed the impact of RUTF on fatty acid status, but this is especially important because RUTF is intended to be the only foodstuff consumed apart from breast milk during nutritional rehabilitation of SAM, and therefore must truly be nutritionally 'complete'.

Importantly, generation of RUTF with elevated n-3 PUFA content is likely to be compatible with local production using materials sourced from within East Africa. Nile Perch (*Lates niloticus*), whose oil is rich in n-3 PUFA, were introduced to Lake Victoria, bordering Kenya, Uganda and Tanzania, in the 1950s.¹⁹¹ They are one of the largest freshwater fish and a fierce predator. Whilst this has had a profoundly negative effect on the Lake's ecosystem (causing the extinction or near-extinction of more than a hundred native species), it has stimulated fishing operations and Nile Perch is widely available.¹⁹² A potential alternative source of n-3 PUFA is flax seed oil, which is very rich in the short chain n-3 PUFA alpha-linolenic acid (ALA). Flax has been successfully cultivated in East Africa, and Ethiopia is the fifth largest global producer of flax.¹⁹³

In Chapter 6, I describe the Njugu Plus trial (Njugu is the Swahili word for peanut, and is the local named used for peanut-based RUTF). The objectives of this trial were to determine whether a formulation of RUTF with a lower n-6:n-3 PUFA ratio is practical, safe and acceptable in the management of children with SAM; and whether

its use has an impact on children's LC-PUFA status as measured by erythrocyte fatty acid composition. In order to determine an absolute requirement for pre-formed LC-PUFA, the new formulation was tested with or without fish oil supplementation in addition to RUTF. Because LC-PUFA are immunologically active, secondary objectives included assessment of impact on structure and function of leukocytes.

2.3 Inflammation and immunity

Throughout this thesis, the following terminologies are used:

- **Inflammation** is a process that is initiated by activation of the innate immune system and results in the accumulation of fluid, plasma proteins and leukocytes within tissues at a site of local injury.
- **Immunity** refers to lymphocyte-mediated responses to particular proteins or pathogens and incorporates both primary and anamnestic memory responses.
- **Systemic inflammation** occurs when innate inflammatory activation occurs in the bloodstream rather than within tissues. In extreme cases this may be associated with the systemic inflammatory response syndrome (SIRS). Because of cross-talk between the innate and adaptive immune systems, this may be associated with polyclonal T and/or B cell activation or modulation in specific antigen-response phenotype. The former may also be referred to as chronic immune activation.
- **Infection** is present where microbial replication in tissues or at a mucosal surface leads to a local inflammatory response.

- **Mutualism** is present at mucosal surfaces (especially the gut) where microbial replication and inflammatory activation exist in homeostatic balance.

Each of the laboratory assays employed in the various studies is designed to interrogate one area of inflammation/immunity (see Box 2, below). Where multiple markers are used (e.g. the large variety of markers measuring systemic inflammation), this is because the character as well as amplitude of response is of hypothesised importance. Inflammatory and immune functions are interrogated through the employment of sets of *ex vivo* or *in vitro* model systems as described in Chapters 3 and 6. Note that many of the systemic inflammatory markers are often used clinically as markers of infection, however in most cases children with overt infections at point of presentation were not eligible for inclusion in the studies presented herein (see specific inclusion/exclusion criteria for more details).

Box 2: Laboratory assays - hypothesised domain of interest

Inflammation: tissue specific (gut)

- Faecal calprotectin
- Faecal β -defensin 2

Inflammation: systemic

- C-reactive protein
- Soluble CD14
- β -2 microglobulin
- Total Immunoglobulin G
- Erythrocyte sedimentation rate
- Peripheral white cell count
- Peripheral platelet count
- Peripheral T cell activation/exhaustion phenotype
- Cytokines/chemokines (CXCL10, Eotaxin, GRO α , IFN α , IL-1 α , IL-1RA, IL-7, IL-8, IL-10, IL-15, IL-17a, IL-22, IL-31, MCP-1, MIP-1 α , MIP-1 β , SDF-1)

Inflammatory function

- Peripheral whole blood cytokine stimulation assays

Immune function

- T cell response to PHA (polyclonal) stimulation
- T cell response to tetanus Toxoid (antigen-specific) response

Downstream markers of chronic systemic inflammatory activation

- Anaemia
- Low insulin-like growth factor-1 (IGF-1)

Gut barrier function

- Serum endotoxin
- Anti-endotoxin core IgG (EndoCAb) titres α

CHAPTER 3

Innate pathogen sensing and response in severe acute malnutrition: a case-control observational study

3.1 *METHODS*

This was a single-centre case-control observational study. The study recruited severely acutely malnourished children from the Baraka Clinic, Mathare Valley, Nairobi, and controls without SAM from the local community between November 2012 and April 2013.

The primary objective was to assess for association between innate pathogen sensing and response characteristics (measured in whole-blood stimulation models) and either of two independent axes; nutritional status (measured using anthropometry), and chronic inflammatory activation (measured using a variety of laboratory readouts). The hypothesis was that chronic exposure to pathogens and their products via impaired skin and mucosal barrier function would lead to innate immune training in the systemic circulation: reduced PAMP response would be seen with more severe acute malnutrition and greater systemic inflammatory activation.

3.1.1 Study Setting

Mathare Valley (Mathare) is an informal settlement, or slum^{*, 194} that is home to an estimated 200,000 people who live in an area of 0.88 km², giving a population density of 228,000 people per km², or 5 m² per person, well short of the 30 m² minimum recommendation employed in the organisation of emergency refugee camps.^{195, 196} It is one of the largest slums in Nairobi, lying approximately 3 km east of the central business district between the affluent suburb of Muthaiga, and Eastleigh, which is predominantly inhabited by Somali immigrants. The average monthly household income is less than 8,500 KSH (approximately \$80 USD, or £55 GBP), and average monthly expenditures (on food, water, rent, healthcare, education etc.) exceed 9,100 KSH.¹⁹⁵ By any index, extreme poverty is endemic.¹⁹⁶

The Baraka Health Centre (BHC) has been run by “German Doctors”, a registered non-governmental organisation, since 1997.¹⁹⁷ BHC provides low-cost health care for more than 65,000 patients per year, 22,000 of whom are new attenders. Children aged under-five have been treated for free since 2011, and more than 12,000 consultations took place in 2013 (Barbara Hüntten-Kirsch, personal communication). Care is provided by volunteer clinicians from Germany, supported (at the time this work was carried out) by two full-time resident German doctors and a local support team comprising clinical officers, nurses, pharmacists, laboratory technicians, social

* UN-HABITAT defines a slum as a contiguous settlement where inhabitants have inadequate housing and basic services, which is often not recognised nor addressed by public authorities as an integral or equal part of the city, and where residents living under the same roof lack one or more of the following: access to safe water; access to sanitation; secure tenure; durability of housing and sufficient living area.

workers, a nutritionist and ancillary staff. Nutritional support is administered at a dedicated outpatient therapeutic feeding programme (OTP) that provides treatment for more than 300 children with SAM annually. In 2013 cure rate was 79% and mortality was 3%, compared to internationally recognised standards (from the Sphere Project) of > 75% and < 10%, respectively.¹⁹⁸ Defaulter rate was relatively high at 16% (< 15% Sphere standard), reflecting a highly mobile population who frequently travel between rural and urban areas in search of work. The work of the OTP was complemented by treatment programmes for MAM (although this was inconsistent in 2012-13 due to irregular supply of fortified cereals and ready-to-use supplementary food (RUSF) by central government) and rickets. In addition, a 'wet-feeding' programme provided short-term free nutritious meals for the most severely impoverished local families following a needs assessment by the community and social work teams.

Prevalence of HIV-infection in Mathare is not known. In 2013, 8% of almost 20,000 routine provider-initiated HIV tests (for adults and children) at BHC were positive (Barbara Hüntten-Kirsch, personal communication).

3.1.2 Study Participants

Formal inclusion and exclusion criteria were as follows:

Inclusion criteria, CASES:

- Children aged 6 months to 5 years old.

- Provision of informed consent.
- Severe acute malnutrition (either of mid-upper arm circumference < 11.5cm or bilateral pedal oedema (weight-for-height/length z-score < -3 on its own was not an inclusion criteria, reflecting the fact that this was a community setting)).
- Eligible for outpatient management of SAM (i.e. no evidence of acute infection, and passes 'appetite test' according to national guidelines).

Exclusion criteria, CASES:

- Known HIV disease or tuberculosis. (All participants received provider-initiated HIV testing and referral in the event of a positive test).^{*199}
- Recent (last two months) treatment for SAM.

Inclusion criteria, CONTROLS:

- Children aged 6 months to 5 years old.
- Provision of informed consent.
- No severe acute malnutrition (any of mid-upper arm circumference < 11.5cm, weight-for-height/length z-score < -3, or bilateral pedal oedema).

Exclusion criteria, CONTROLS:

* Both HIV and active TB disease can cause functional immunosuppression. Their inclusion would have compromised the power of the study in determining nutrition and/or chronic inflammation-specific effects. National guidelines recommend offering provider-initiated testing for HIV for all severely acutely malnourished children, with referral for comprehensive care in the event of a positive test.

- Known HIV disease or tuberculosis. (All participants received provider-initiated HIV testing and referral in the event of a positive test, as above.)
- Recent (last two months) treatment for SAM.

3.1.3 Screening and enrolment

Participants with SAM (cases) were recruited from among those self-presenting to BHC or via a programme of active case-finding in the community conducted by local community health volunteers (CHVs). Information about the study was given to each eligible child's attending parent or carer and informed consent for participation was sought. The research bleed was timed to coincide with a routine provider-initiated HIV test, participants who tested positive for HIV remained in the study but their data was excluded from the analysis.

Controls were identified via opportunistic screening of children attending BHC for vaccination, minor illness, as sibling or child of an attendee, and via screening of children enrolled in the wet-feeding programme (described above). Controls were frequency matched for age and sex.

Baseline clinical and demographic features were recorded, and routine anthropometry was performed according to WHO and national guidelines. Comorbidities were defined according to WHO clinical syndromic diagnoses (diarrhoea and pneumonia) or a positive blood film with clinically apparent disease (malaria).

Blood was drawn by needle and syringe and transferred into appropriate tubes by a member of the study team on the day of recruitment. A stool sample was taken on either the same or the following day, transferring directly from the child's nappy into a sterile Universal container.

3.1.4 Laboratory Methods

3.1.4.i Whole-blood stimulation model

a: Preparation of stimulation plates

Toll-like receptor ligands and controls were prepared from lyophilised stocks according to the manufacturer's instructions and diluted to appropriate concentration (10-fold the concentration in Table 3.1) in RPMI 1640, or sterile RNase-free water in the case of poly(I:C). 11 µl of the diluted stimulants were added to wells of multiple individual polystyrene 96-well plates in triplicate. The plates were sealed and stored at -80°C until the day of use. Stimulant concentrations were determined with reference to previously published protocols and tested with healthy adult donor blood to ensure that the concentrations were not associated with supramaximal responses.²⁰⁰

Table 3.1: Whole-blood stimulation conditions

Target	Stimulant	Source	Concentration
TLR1/2	Pam3CSK4	Synthetic triacylated lipoprotein	1 µg/ml
TLR2	LTA	Lipoteichoic acid from <i>Staph. aureus</i>	10 µg/ml
TLR3	Poly(I:C)	Synthetic analog of dsRNA	10 µg/ml
TLR4	LPS	Lipopolysaccharide from <i>E. coli</i> K12 strain	1 µg/ml
TLR5	FLA	Flagellin from <i>S. typhimurium</i>	5 µg/ml
TLR2/6	FSL-1	Synthetic diacylated lipoprotein	5 µg/ml
TLR7	Gardiquimod	Imidazoquinoline compound	10 µg/ml
TLR7/8	CL075	Thiazoloquinoline compound	10 µg/ml
TLR9	ODN M362	Type C CpG oligonucleotide	1 µM
Dectin-1	Zymosan	Hot alkali-treated cell wall from <i>Saccharomyces cerevisiae</i>	100 µg/ml
+ve Control	PMA/Ionomycin	-	0.1 µg/ml; 1 µg/ml
-ve Control	PBS	-	-

Table 3.1: Stimulants are listed with their final stimulation concentration (after addition of the media/blood mix).

b: Stimulation protocol

Endotoxin-free heparinised blood was prepared by adding 2 ml of whole blood directly to pre-prepared BD falcon tubes containing endotoxin-free sodium heparin solution (final heparin concentration >10 USP/ml), and mixed by inversion. Samples were transported to the joint laboratories of the Universities of Nairobi (Kenya) and Washington (USA) at the University of Nairobi's Kenyatta National Hospital campus

on the same day in boxes cooled with ice packs. Blood was diluted 1 in 5 in a stimulation buffer made up of RPMI supplemented with penicillin/streptomycin, L-glutamine (2 mM), pyruvate (1 mM) and non-essential amino acids. 100 µl blood/media mix was added to each well of a freshly-defrosted stimulation plate, and mixed by pipetting. The plates were incubated at 37°C in 5% CO₂ for 24 hours. Following incubation, the plates were spun down to pellet cells. Supernatants were removed and replicates were pooled prior to freezing at -80°C.

c: Stimulation readout

Production and release of IL-6, IL-8, IL-10 and IP-10 (IFN-γ-induced protein 10) was assessed by ELISA of cell culture supernatants, using kits according to the manufacturers' specifications. Where sample volume was limited, IL-8 and IP-10 were prioritised, as prototypic indices of MyD88-dependent and -independent signalling, respectively.

3.1.4.ii Inflammatory indices

In the diagnostic laboratory of the University of Nairobi's Paediatric Department, fresh stool was examined by microscopy (wet mount) for pus cells and ova, cysts and parasites. The presence of faecal occult blood was determined using a commercial rapid diagnostic kit (Hemoccult ICT, Beckman Coulter). Full blood count (FBC) with differential white count, and erythrocyte sedimentation rate (ESR) were measured (Coulter count and Westergren methods, respectively). Aliquots of stool and plasma

were frozen at -80°C, and all subsequent analyses were performed at the KEMRI-Wellcome Trust Research Laboratories, Kilifi, Kenya.

Calprotectin and beta-defensin 2 were measured in stool using commercial kits (Bühlmann and Immunodiagnostik AG) following stool disruption/homogenisation by 30 seconds TissueLyser shaking (QIAGEN, no beads). Plasma total IgG, anti-endotoxin core IgG (IgG EndoCAb), and beta-2 microglobulin were measured using commercial kits according to manufacturers' instructions (ebioscience, Hycult biotech, abcam).

3.1.5 Statistical Methods

Differences between clinical, demographic and inflammatory variables between cases and controls were assessed using Mann-Whitney U test for non-parametric continuous variables or Fishers exact for categorical variables. Sub-group analyses were conducted throughout according to the presence of oedema (kwashiorkor) in the SAM group.

Associations between variables across both cases and controls were assessed using simple linear regression after transformation to normality, or by generation of multivariate regression models via inclusion of all variables with significant association at $P < 0.05$ in univariate analyses.

Because the whole blood stimulation data were expected to produce data with multiple internal correlations, dimensionality reduction was performed by principal

component analysis (PCA). Verification of the data's suitability for PCA was performed as described in the test.²⁰¹ The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was used to assess the suitability of the sample size (where KMO values are characterised: 0.00-0.49 (unacceptable); 0.50-0.59 (miserable); 0.60-0.69 (mediocre); 0.70-0.79 (middling); 0.80-0.89 (meritorious); 0.90-1.00 (marvellous)).²⁰² Batlett's test for sphericity (which should be <0.05) was assessed using the command *factortest* in Stata.²⁰³

P values are presented without correction for multiple hypotheses. $P < 0.05$ is arbitrarily considered statistically 'significant', although all analyses are in fact exploratory, are not powered for any particular outcome, and are designed to be hypothesis-generating. Any positive results would need to be confirmed in subsequent studies. Throughout, analysis was performed in Stata (version 12.0) and graphs were drawn with GraphPad Prism (version 6.0) (except Figures 3.6 and 3.10, which were drawn in Stata). Anthropometric z-scores were calculated using WHO standards (2006).

3.2 RESULTS

3.2.1 Clinical and demographic characteristics

Forty-one children were recruited to the study. Two were diagnosed with HIV-infection following screening and were referred for comprehensive care. Results from 39 participants were analysed in the study. Clinical and demographic characteristics are summarised in Table 3.2.

Table 3.2: Clinical and demographic characteristics

	Kwashiorkor	Severe Wasting	Total SAM	Controls	P (SAM:C)
n	8	16	24	15	-
Age (months)	19 (15 to 24)**	11 (8 to 16)**	15 (10 to 18)	17 (11 to 18)	0.86
Male sex	1 (13)	8 (50)	9 (38)	6 (40)	1.00
MUAC (mm)	-	107 (105 to 112)	107 (105 to 112)	135 (130 to 144)	<0.001
WHZ/WLZ	-	-2.9 (-3.8 to -2.5)	-2.9 (-3.8 to -2.5)	-0.8 (-1.1 to 0.3)	<0.001
Oedema	8 (100)	0 (0)	8 (33)	0 (0)	-
HAZ/LAZ	-3.5 (-3.9 to -3.0)	-2.6 (-3.2 to -1.5)	-3.0 (-3.6 to -1.9)	-2.3 (-2.9 to -0.9)	0.04
Breastfeeding	2 (25)*	12 (75)*	14 (58)	12 (80)	0.30
Monthly h/h income <4000 KES (\$40 USD)	4 (50)	4 (25)	8 (33)	0 (0)	0.14
Monthly h/h income <10000 KES (\$100 USD)	7 (88)	13 (81)	20 (83)	13 (87)	1.00
Number of people living in house	6 (3 to 7)	5 (3 to 7)	5 (3 to 7)	4 (3 to 9)	0.45
Toilet in house/compound	3 (38)	8 (50)	11 (46)	1 (7)	0.013
Water in house/compound	2 (25)	3 (19)	5 (21)	0 (0)	0.38
Mother owns a mobile phone	5 (63)	14 (88)	19 (79)	12 (80)	1.00
Fever	6 (75)	12 (75)	18 (75)	0 (0)	<0.001
Cough	3 (38)	11 (69)	10 (42)	0 (0)	<0.001
Diarrhoea	1 (13)	5 (31)	6 (25)	1 (7)	0.22

Table 3.2: Clinical and demographic characteristics of the participants. Tabulated P values are between total SAM and control groups by Mann-Whitney U tests or Fishers exact. Data are median (inter-quartile range) or number (percentage). * $P < 0.05$ between kwashiorkor and severe wasting sub-groups. ** $P < 0.01$ between kwashiorkor and severe wasting sub-groups. MUAC (mid-upper arm circumference) WHZ/WLZ (weight-for-height/length z score); HAZ/LAZ (height/length-for-age z score); h/h (household).

Children with kwashiorkor were older than those with severe wasting and were consequently more stunted (see below) and less likely to still be breastfeeding. Although participants were recruited on markers of acute malnutrition, they were also extremely stunted, suggesting long-term nutritional inadequacy or ill health. The control group was relatively well matched in terms of age and demographic features. Although almost all participants were living below the international poverty threshold (currently \$1.25 USD per person per day), the number of children living in the most extreme poverty was lower in the controls.²⁰⁴ Interestingly, percentage of participants with access to in-house toilet and drinking water facilities was lower in the control group compared to those with SAM. Most of the controls came from Mathare 4A, the area (or 'village') immediately adjacent to BHC (Figure 3.1). In Mathare 4A there is no infrastructure for single dwelling/compound-level water or sanitation provision. It is likely that many of the children with SAM travelled further for their care from areas (still, without exception, within Mathare) with different local infrastructure. In Mabatini village, for example, some of the poorest people live in roughly-built tower blocks. Although these provide compound-level water and sanitation facilities, the living area and conditions are exceptionally poor and insecure.

Figure 3.1: Map showing location of Mathare 4A and BHC

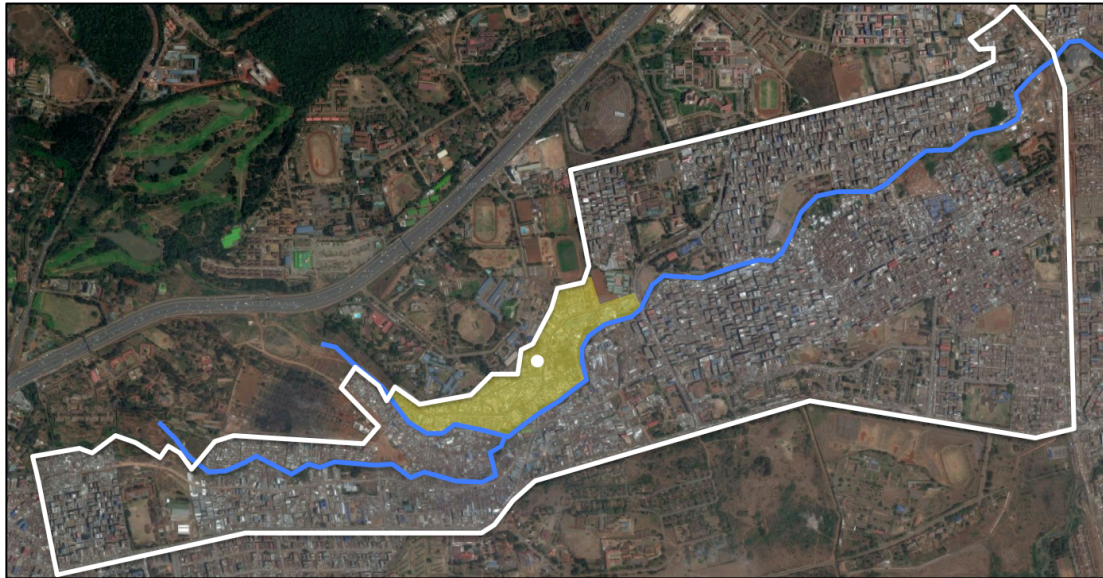


Figure 3.1: Map shows boundary of Mathare (white line), Mathare 4A (yellow shaded area), and location of BHC (white circle). Nairobi river shown in blue. Mabatini is south of the river just opposite BHC. Satellite imagery downloaded from Google Maps (www.google.co.uk/maps), boundary data adapted from content supplied by Map Kibera (www.mapkibera.org).

3.2.2 Relationship between inflammatory indices

Sequential pairwise regression analyses showed no, or weak, relationships between most of the measurements designated as potential chronic inflammatory indices. Exceptions were relationships between total IgG and ESR ($P < 0.001$), and IgG and haemoglobin (Hb) ($P = 0.01$). There was a modest positive correlation between the two faecal inflammatory markers, calprotectin and beta-defensin 2 ($P = 0.07$), see Figure 3.2. There were no significant relationships between IL-8 in plasma (a putative marker of acute inflammatory activation) and any of the chronic inflammatory markers.

Figure 3.2: Relationship between chronic inflammatory indices

	Hb	WCC	Platelets	ESR	Total IgG	EndoCAb	B2M	Calprotectin	BD2
Hb									
WCC									
Platelets									
ESR	-		+						
Total IgG	-			+					
EndoCAb									
B2M									
Calprotectin							-		
BD2								+	

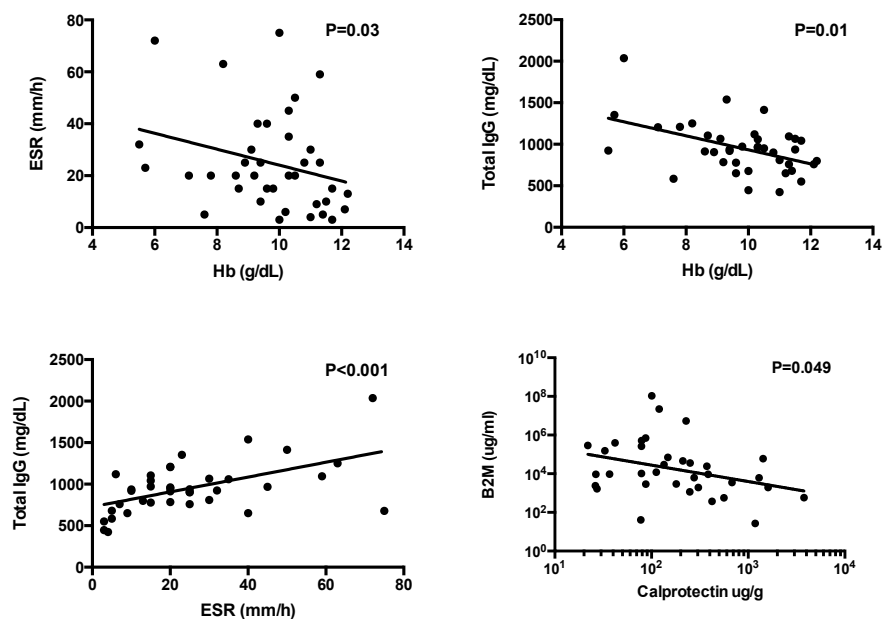


Figure 3.2: Relationship between chronic inflammatory indices (all in plasma save for calprotectin and BD2 in stool) by pairwise regression of transformed (if necessary) values. In the table, + or – indicates the direction of the correlation and colour indicates its statistical significance where red ($P<0.001$), orange ($P<0.01$), yellow ($P<0.05$), white ($P<0.10$). Hb (haemoglobin); WCC (white cell count); ESR (erythrocyte sedimentation rate); IgG (immunoglobulin G); EndoCAb (IgG anti-endotoxin core antibodies); B2M (beta-2 microglobulin); BD2 (beta-defensin 2).

3.2.3 Relationship between inflammatory and anthropometric indices

Comparison of inflammatory indices between the two SAM groups and controls revealed minimal differences. Children with severe wasting, and the SAM group as a whole, had lower platelet counts than controls (Figure 3.3). Additionally, sequential univariate analyses regressing inflammatory indices (and age) against MUAC, WHZ/WLZ, presence of kwashiorkor, or HAZ/LAZ were performed. Where the relationships were significant at $P < 0.05$ the inflammatory indices were combined in a multivariate model. For MUAC, only platelet count was predictive in the multivariate model (ESR was positively associated at $P = 0.03$ in univariate analysis) and platelet count was the only significant univariate association for WHZ/WLZ. For oedema, only age was significantly associated as described, above. For HAZ/LAZ, both age and Hb were associated in the multivariate model (total IgG was inversely related at $P = 0.04$ in the univariate analyses) (Figure 3.4).

Figure 3.3: Relationship between SAM and inflammation

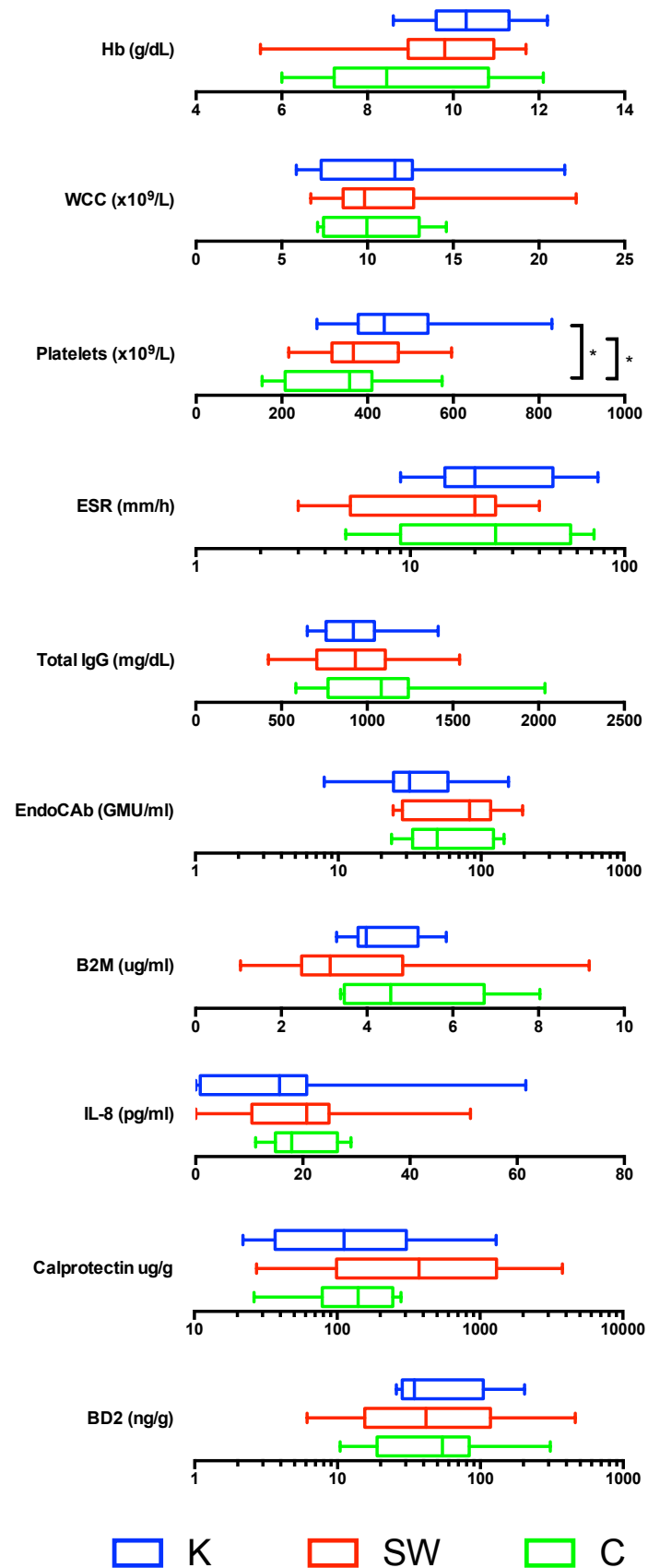


Figure 3.3: Mann-Whitney U tests were used to assess for significant differences between either of the two SAM phenotypes, or the SAM group as a whole, and control children (C). They were also used to investigate for differences between kwashiorkor (K) and severe wasting (SW) themselves. Differences were only apparent for platelet count, which was lower in SW, and in SAM as a whole, than in C ($P < 0.05$ for both tests). Hb (haemoglobin); WCC (white cell count); ESR (erythrocyte sedimentation rate); IgG (immunoglobulin G); EndoCAb (IgG anti-endotoxin core antibodies); B2M (beta-2 microglobulin); BD2 (beta-defensin 2).

Figure 3.4: Associations between inflammatory and anthropometric variables

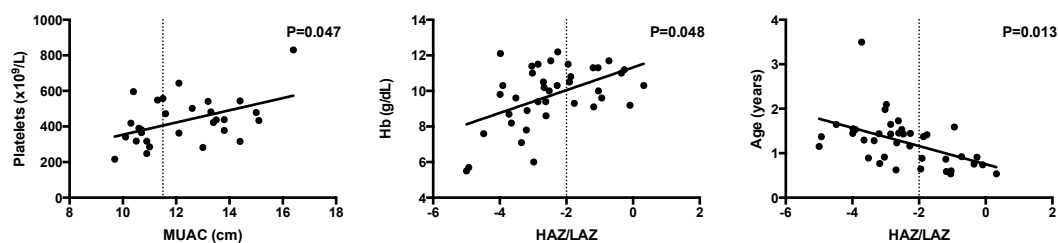


Figure 3.4: Inflammatory variables that were associated with anthropometry in multivariate regression analyses. MUAC (mid-upper arm circumference) WHZ/WLZ (weight-for-height/length z score); HAZ/LAZ (height/length-for-age z score).

3.2.4 Whole blood stimulation data processing

As described in the methods section, I excluded stimulation conditions where the median stimulated cytokine concentration was not more than ten-fold the negative-control concentration. Crude cytokine concentrations are shown in Figure 3.5, and the panel of cytokine responses taken forward for analysis is summarised in Table 3.3. Negative control cytokine concentrations were subtracted from their equivalent stimulated-condition concentrations and the absolute stimulant-induced cytokine concentrations were used in all subsequent analyses.

Figure 3.5: Crude cytokine concentrations in stimulated/unstimulated conditions

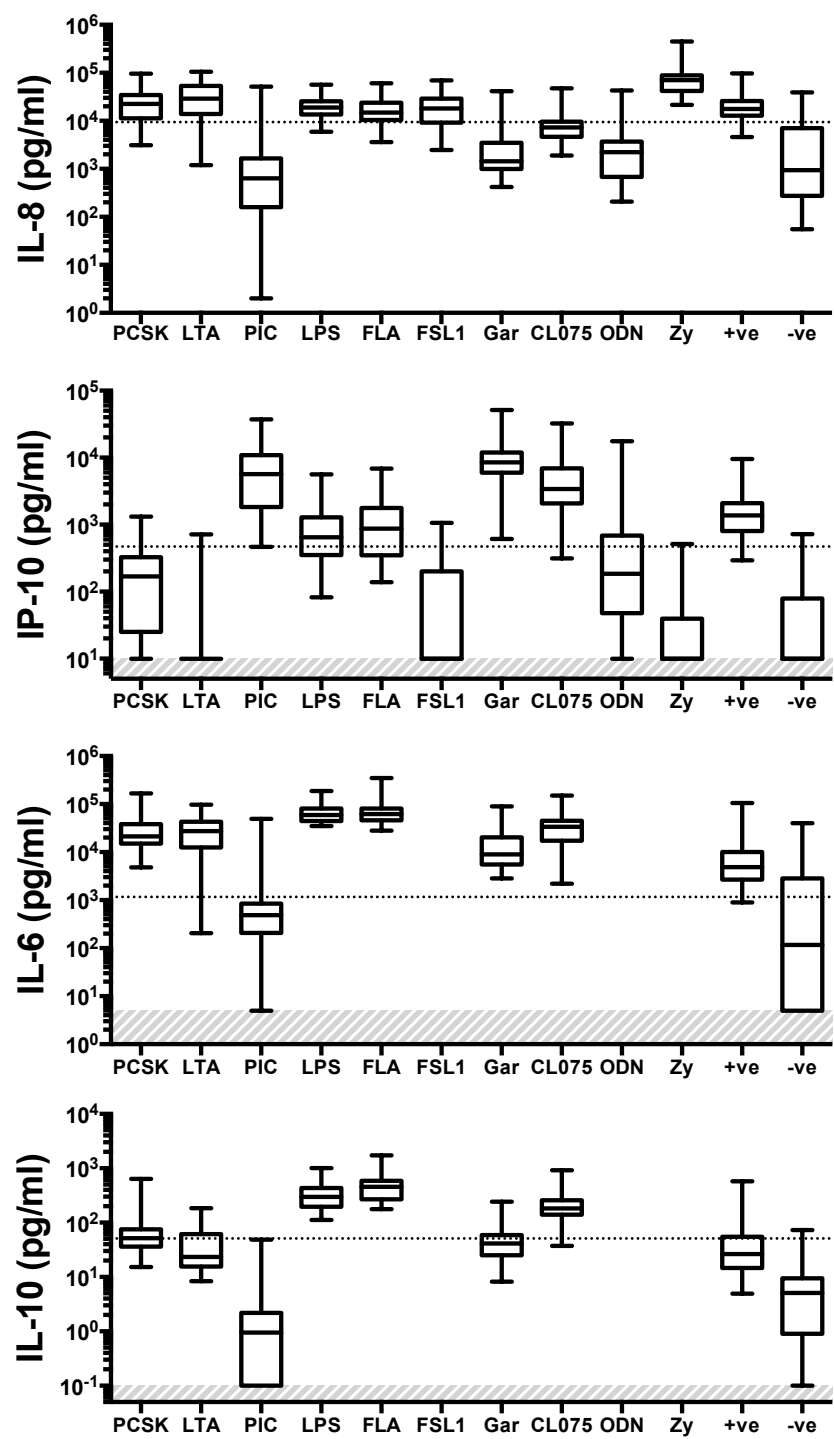


Figure 3.5: Cytokine concentrations measured in tissue-culture supernatant by ELISA. PCSK (Pam3CSK4); PIC (Poly(I:C)); Gar (gardiquimod); Zy (zymosan). Dashed line: 10-fold median cytokine concentration in –ve control wells. Hatched area: below lower limit of detection

Table 3.3: Stimulant/cytokine pairs taken forward for further analysis

Target	Stimulant	Cytokine output
TLR1/2	Pam3CSK4	IL-8; IL-6; IL-10
TLR2	LTA	IL-8; IL-6
TLR3	Poly(I:C)	IP-10
TLR4	LPS	IL-8; IP-10; IL-6; IL-10
TLR5	FLA	IL-8; IP-10; IL-6; IL-10
TLR2/6	FSL-1	IL-8
TLR7	Gardiquimod	IP-10; IL-6
TLR7/8	CL075	IP-10; IL-6; IL-10
TLR9	ODN M362	-
Dectin-1	Zymosan	IL-8
+ve Control	PMA/Ionomycin	IL-8; IP-10; IL-6

Suitability of the data for PCA was ascertained via inspection of the scatter plot matrix for linearity (a subsection of which is provided in Figure 3.6). Although the ratio of cases per variable was very low, the Kaiser-Meyer-Olkin Measure of Sampling Adequacy was 0.68 ('mediocre'). Bartlett's Test of Sphericity gave $P < 0.001$, indicating that the data were suitable for reduction. Outlier was not attempted due to the small number of datapoints, meaning that the results must be regarded as preliminary. The first PC (PC1) explained 57% of the variance in the data, and the second PC (PC2) explained 15% (cumulative 72%, see scree plot Figure 3.7).

Inspection of the PCA loading plot reveals that explanation of the variance of most of the stimulation conditions is provided mainly by PC1. PC2 accounts mainly for IL-8 release (Figure 3.8).

Figure 3.6: Subsection of the scatter plot matrix

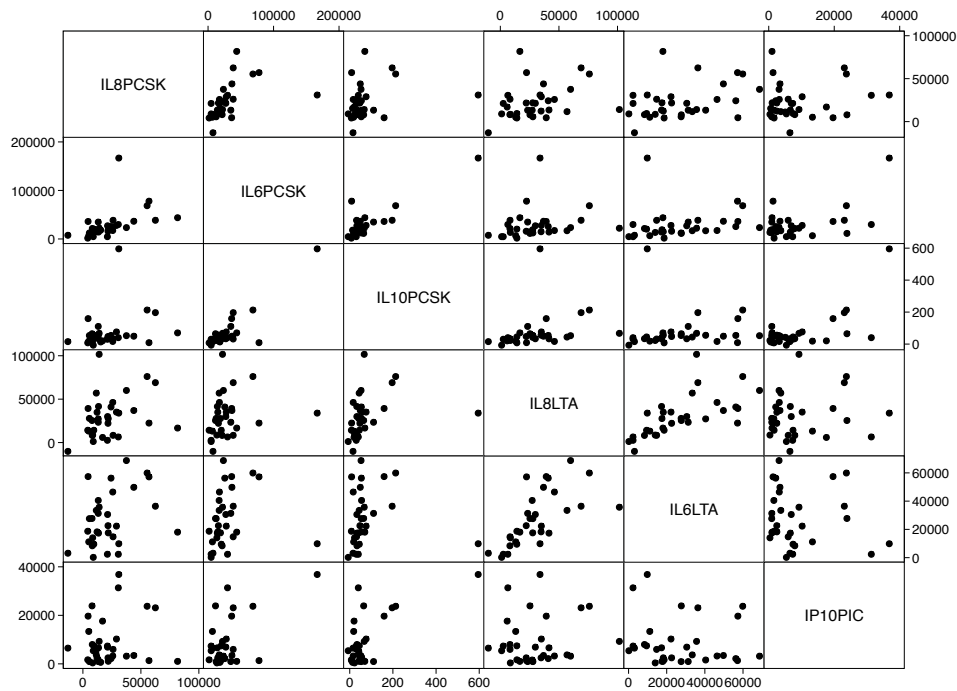


Figure 3.6: Inspection of the scatter plot matrix reveals multiple mostly linear relationships between the variables, indicating their suitability for PCA reduction.

Figure 3.7: Scree plot

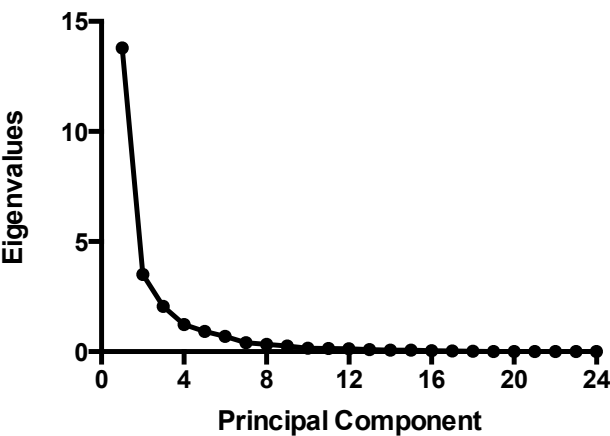
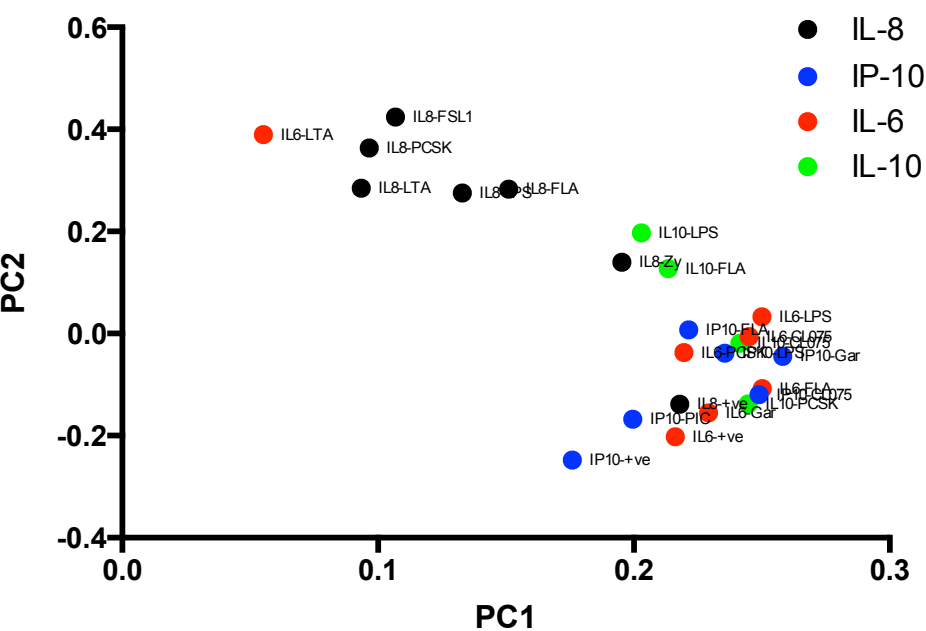


Figure 3.7: Inspection of the scree plot provides justification for the analytic focus on the first two principal components, after which the contribution to variance explanation tails of markedly.

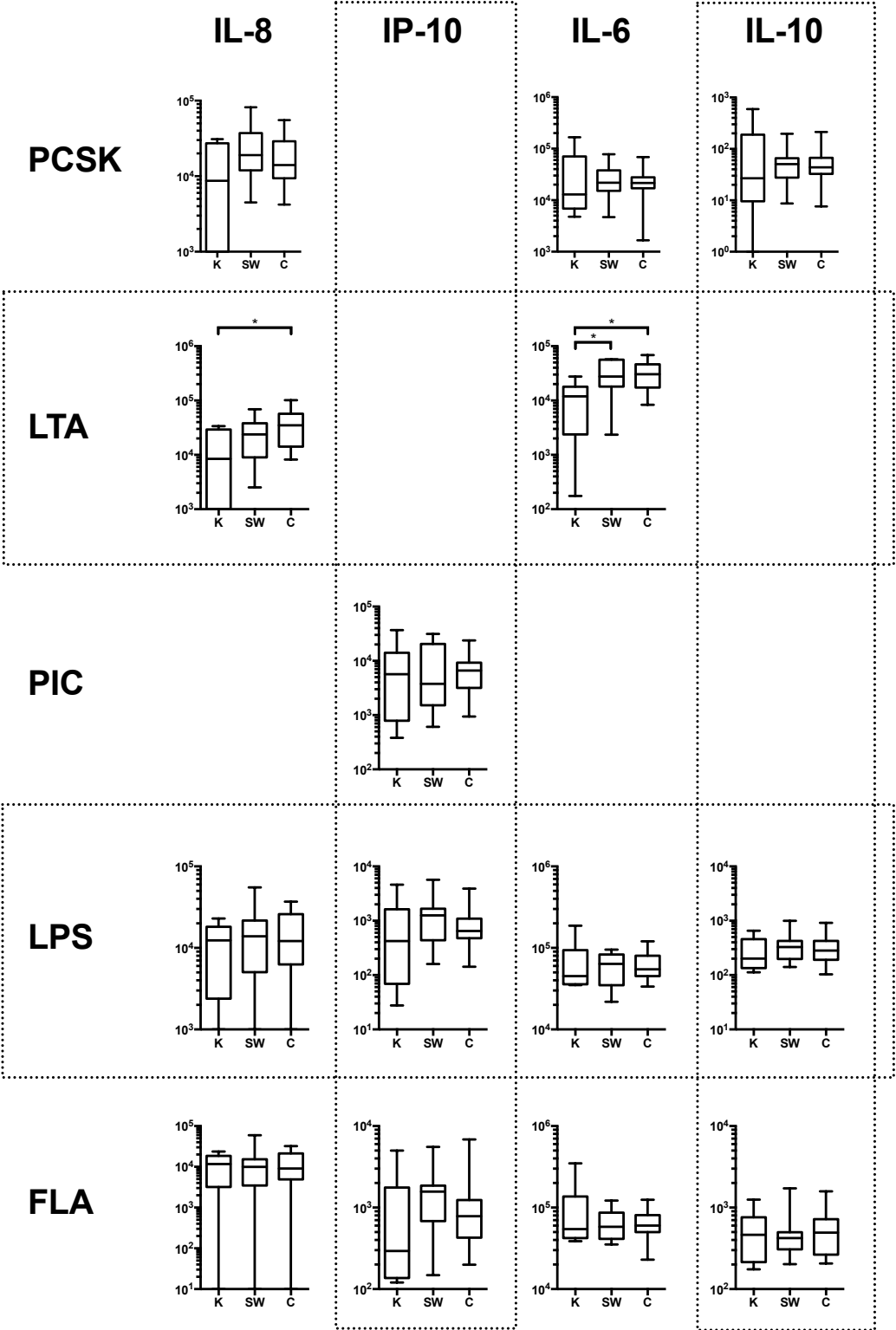
Figure 3.8: PCA loading plot



3.2.4 Whole blood stimulation results

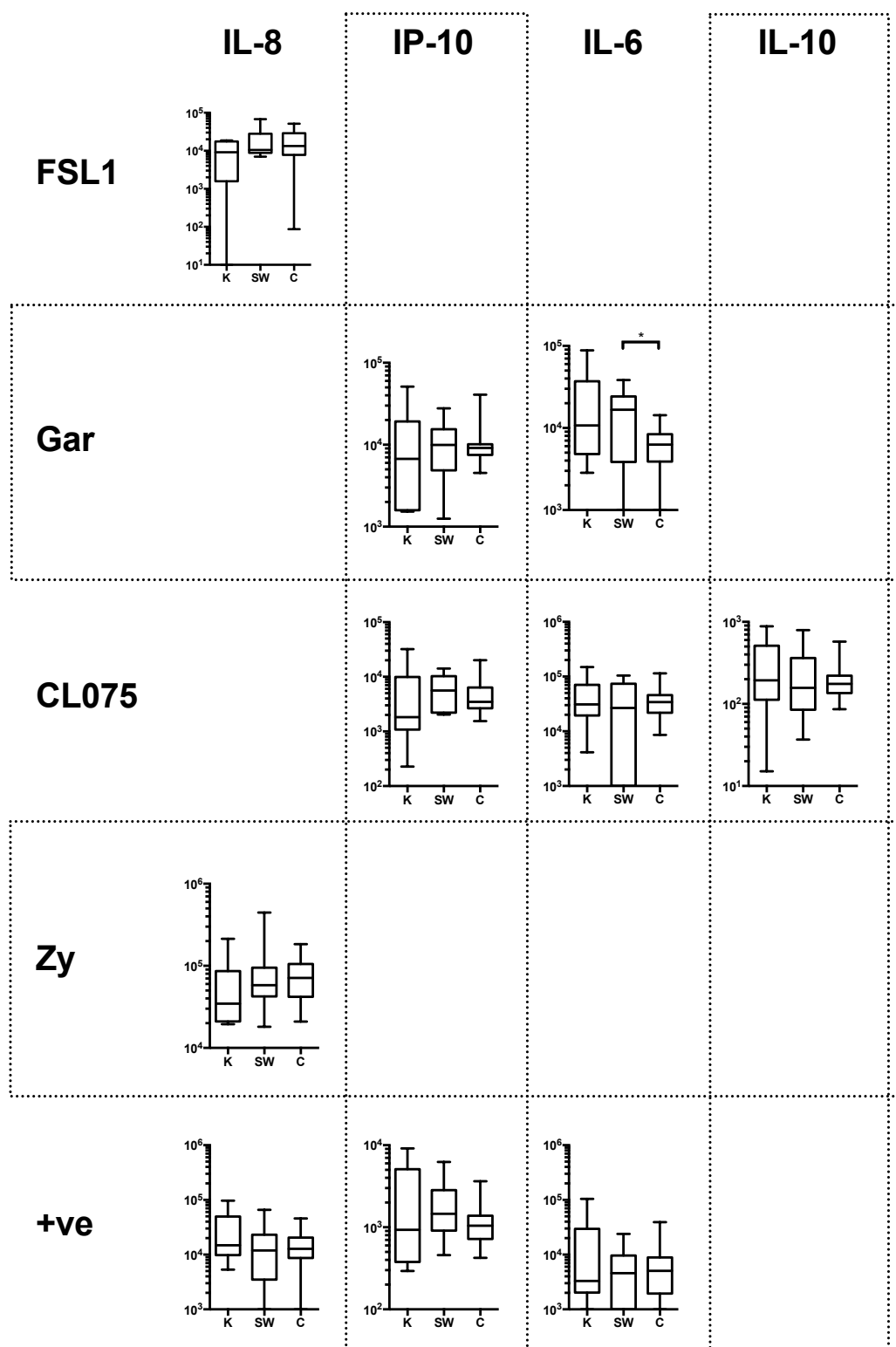
Overall, stimulated cytokine release was very similar between children with SAM and controls. Release by children with kwashiorkor was frequently less than in the other two groups (Figure 3.9). SAM was associated with reduced IL-8 production in response to LTA ($P=0.047$) and increased IL-6 production in response to gardiquimod ($P=0.040$). In subgroup analyses, kwashiorkor was associated with reduced IL-8 and IL-6 production in response to LTA compared to controls, and reduced IL-6 response to LTA compared to children with severe wasting. Severe wasting was associated with increased IL-6 production in response to gardiquimod (Figure 3.9).

Figure 3.9: Stimulated cytokine release: impact of SAM



Cytokine concentration in pg/ml in all cases

Figure 3.9: Stimulated cytokine release matrix. K (kwashiorkor); SW (severe wasting); C (control), other abbreviations as previous. * P<0.05 by Mann Whitney.



Cytokine concentration in pg/ml in all cases

Figure 3.9 (cont.): Stimulated cytokine release matrix. K (kwashiorkor); SW (severe wasting); C (control), other abbreviations as previous. * $P < 0.05$ by Mann Whitney.

Sequential regression analyses were performed against results from each of the stimulation condition and the first two principal components of the reduced data in order to assess for association with inflammatory and anthropometric variables (Table 3.4). The univariate analyses provided strong evidence of a consistent impact of white cell count on cytokine release. Unfortunately due to gaps in the data it was not possible to resolve this further using differential white counts. Additionally, IL-8 concentration was consistently significantly associated with stimulated IL-8 release. Therefore a multivariate analysis was performed, controlling for white cell count and IL-8 concentration in plasma (only for IL-8 release variables and the principal components) (Table 3.5). This revealed a highly consistent positive association between cytokine release (including PC1) and plasma IgG EndoCAb, and a negative association between cytokine release (also including PC1) and faecal calprotectin (PC1 results illustrated in the component-plus-residual plots, Figure 3.10). Kwashiorkor was significantly negatively associated with cytokine release in several conditions, and with PC2 (Figure 3.11).

Table 3.4: Univariate regression analyses against cytokine release

		Hb	WCC	Platelets	ESR	Total IgG	EndoCAB	B2M	IL-8	Calprotectin	BD2	Age	HAZ/LAZ	MUAC	Kwashiorkor
PCSK	IL-8		+												
	IL-6									-					
	IL-10		+							-	-				
LTA	IL-8		+	+		+					-				-
	IL6			+								-	+		-
PIC	IP-10						+								
LPS	IL-8		+						+	-					
	IP-10														-
	IL-6		+												
	IL-10		+								-				
FLA	IL-8		+				+		+	-					
	IP-10														-
	IL-6		+			+									
	IL-10		+												
FSL1	IL-8		+						+						
Gar	IP-10		+												
	IL-6	-				+	+							-	
CL075	IP-10		+												-
	IL-6										-				
	IL-10		+												
Zy	IL-8		+								-				
+ve	IL-8		+				+			-	-				
	IP-10	-					+								
	IL-6	-				+				-					
PC1			+							-	-				
PC2			+	+		-						-	+		-

Table 3.4: P <0.001 (red) <0.01 (orange) <0.05 (yellow) <0.01 (white): +/- (direction of association)

Table 3.5: Multivariate regression analyses against cytokine release

		Hb	Platelets	ESR	Total IgG	EndoCab	B2M	IL-8	Calprotectin	BD2	Age	HAZ/LAZ	MUAC	Kwashiorkor
PCSK	IL-8	-												
	IL-6								-					
	IL-10					+			-					
LTA	IL-8													
	IL6		+								-	+		-
PIC	IP-10					+								
LPS	IL-8					+		+	-					
	IP-10					+	-							-
	IL-6		-											
	IL-10					+				-				
FLA	IL-8	-		-		+		+	-					
	IP-10					+								-
	IL-6				+									
	IL-10													
FSL1	IL-8	-				+		+						
Gar	IP-10													
	IL-6	-				+		-					-	
CL075	IP-10					+	-							-
	IL-6									-				
	IL-10													
Zy	IL-8					+				-				
+ve	IL-8					+			-	-				
	IP-10	-				+								
	IL-6								-					
PC1						+			-					
PC2			+		-						-	+		-

Table 3.5: P <0.001 (red) <0.01 (orange) <0.05 (yellow) <0.01 (white): +/- (direction of association)

Figure 3.10: EndoCAb/calprotectin and PC1 – component-plus-residual plots

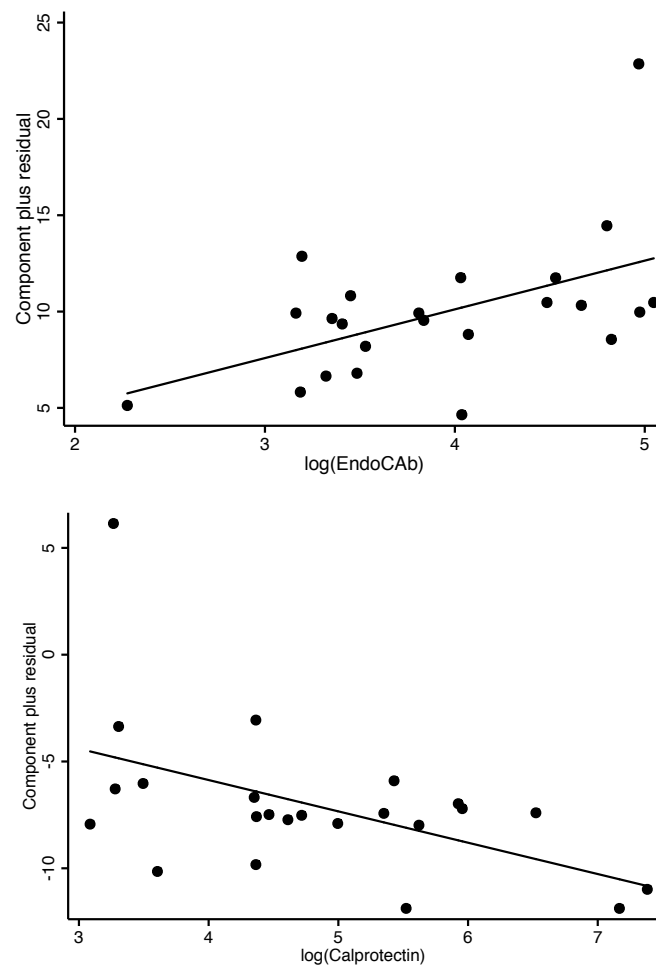


Figure 3.10: Component-plus-residual plots give a graphical representation of the association between PC1 and EndoCAb (top, $P=0.03$), or calprotectin (bottom, $P=0.03$) after adjustment in the regression models detailed above.

Figure 3.11: SAM and cytokine response to PAMP challenge

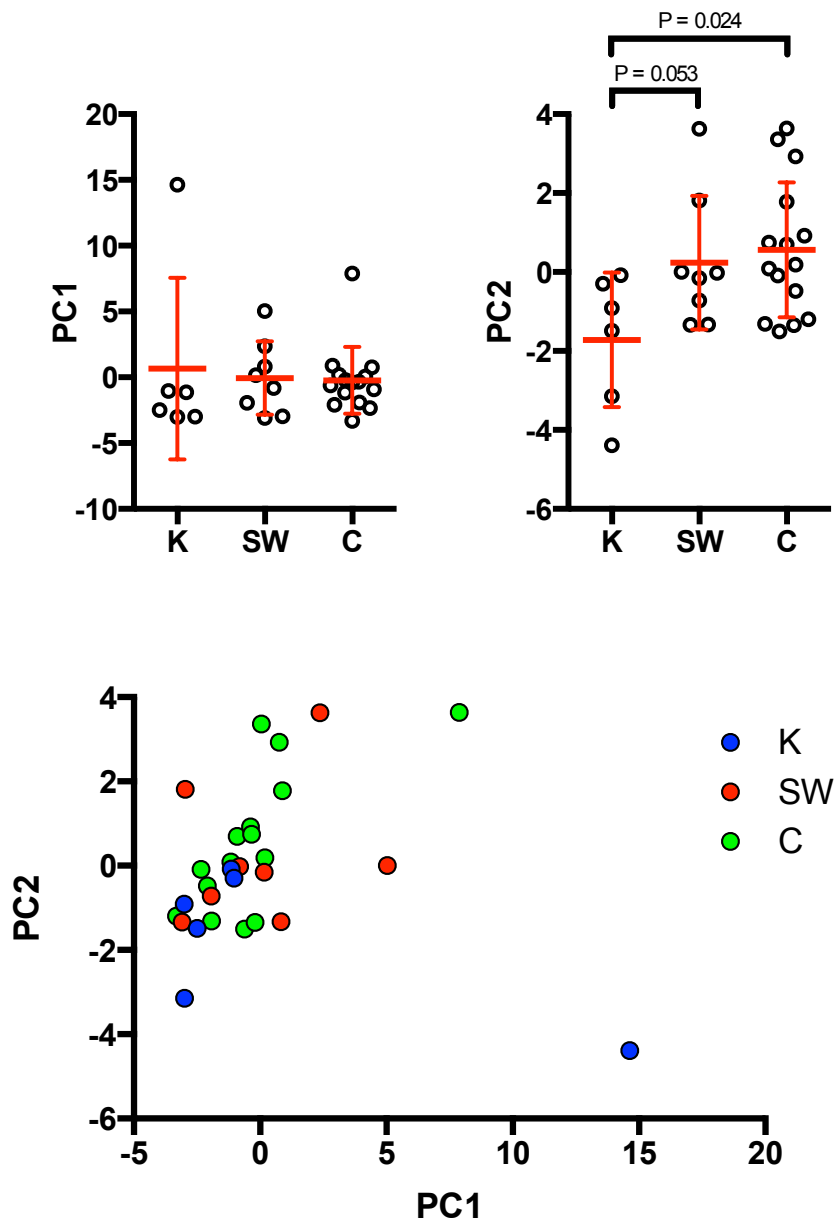


Figure 3.11: PC1 and PC2 are similar in severe wasting (SW) compared to controls (C). PC2 was reduced in children with kwashiorkor (K) compared to C ($P=0.024$) and SW ($P=0.053$) by Mann-Whitney. The PC2/1 identity plot highlights the presence of a major outlier in the K group, discussed later. There is no immediately apparent spatial clustering according to group.

3.3 Discussion

This study was designed to address the hypothesis that children with SAM have chronic enteric and systemic inflammatory activation, that this may have a training/tolerising effect on innate immune response to pathogens, and that consequent immune hyporesponsiveness might account for their greatly increased infection-mediated morbidity and mortality. The study utilised *ex vivo* PAMP challenges to investigate stimulated cytokine production as a primary functional readout. The results do not support the primary hypothesis: SAM had no consistent impact on gut-specific or systemic inflammatory markers, and no impact on release. A possible association between simulated cytokine release and gut functional/inflammatory markers deserves further study

In subgroup analyses there was a possible negative impact of kwashiorkor on stimulated cytokine release. This was seen in several of the experimental conditions and was statistically significant for PC2. Most of the children with kwashiorkor also had low PC1 scores, apart from one outlier, who had the highest PC1 score of all participants. This child had a very distinctive inflammatory profile: In the whole-blood stimulation conditions they produced the highest concentration of cytokine amongst children amongst children with kwashiorkor 22/24 times, and the highest concentration of cytokine amongst all participants 13/24 times. They had the lowest platelet count and second lowest faecal calprotectin of all participants, the highest total IgG, second-highest ESR, and fourth-highest EndoCAb. They were also one of the more anaemic children in the study, with Hb of 6.0 g/dL. It is tempting to

speculate that they may have had some other unique intrinsic or extrinsic factor affecting their immune response. The combination of fever, anaemia and relative thrombocytopenia is suggestive of malaria, which has been previously shown to dysregulate TLR-dependent cytokine responses.^{205, 206} Although Nairobi itself is considered free of malaria transmission, frequent travel back and forth to hyperendemic areas in Western Kenya is not uncommon for Mathare residents and 'imported' malaria is sometimes seen at BHC.²⁰⁷ Unfortunately this child was not tested for malaria at enrolment. They went on to make a full recovery from their kwashiorkor without having been prescribed antimalarial treatment at BHC, though such treatments are very widely available in the informal sector. In the absence of a definitive explanation of their unusual immune phenotype, their results have been included in all analyses.

The findings for children with kwashiorkor are of particular interest, because it has been postulated that this condition may be partly related to a dysregulated and exaggerated inflammatory response to infection.²⁰⁸⁻²¹⁰ Evidence supporting this theory include the fact that kwashiorkor is comparatively rare amongst younger children with SAM or those who are HIV-infected, suggesting a requirement for "*a degree of immunocompetence*" for its development,^{211, 212} that kwashiorkor may (rarely) be precipitated by antiretroviral treatment of very immunosuppressed children with AIDS, potentially as a manifestation of immune-reconstitution inflammatory syndrome,²¹³ and that it been found to be associated with increased circulating inflammatory mediators compared to severe wasting.⁵⁴ In contrast, the present data have suggested that children with kwashiorkor may have a broadly

attenuated cytokine response to innate pathogen challenge, and have found the balance between pro- and anti-inflammatory mediators (e.g. IL-6 and IL-8 versus IL-10) to be preserved. Therefore while the capacity to mount an inflammatory response might be a necessary condition to the development of kwashiorkor, these data do not support the concept of a dysregulated and over-exuberant innate inflammatory response as being a primary cause of the condition.

The inverse associations of faecal calprotectin and plasma IgG EndoCAb with cytokine release are difficult to reconcile. Calprotectin is neutrophil cytosolic protein whose presence in stool is indicative of neutrophil-associated enteric mural inflammation arising from one of a number of infectious or 'inflammatory' (e.g. IBD, graft-versus-host disease) causes.²¹⁴⁻²¹⁶ IgG EndoCAb is thought to be produced in response to intestinal microbial translocation, and higher levels are taken to signify greater intestinal permeability.^{72, 79, 217} A correlation between faecal calprotectin and EndoCAb was anticipated, but was not apparent. Intestinal lamina propria inflammation can be induced in model systems through diverse immune and non-immune pathways, and clinically relevant inflammatory enteropathies can have different immunophenotypes.²¹⁸ It is plausible that both neutrophil-associated and non-neutrophil-associated enteropathies may occur in the context of SAM, and that they have different impacts on local and systemic immune function. Adults with Crohn's disease have defective cytokine secretion by peripheral blood-derived macrophages while those with ulcerative colitis do not.¹⁵² The characteristics of inflammatory inflammation may be affected by such factors as age, dysbiosis or intestinal protozoal infection.^{219, 220} Although the current study is too small to allow

for a detailed exploration of the potential diversity in enteric/systemic immunophenotypes, it provides a clear rationale for further study. An alternative explanation for these observations makes reference to the fact that although elevated EndoCAb has been considered a result of chronic translocation, there is some evidence that severe illness or other causes of high-level translocation may – at least transiently – lead to depletion in detectable EndoCAb (presumably due to utilisation in phagocytosis etc.).^{221, 222} It is possible that elevated faecal calprotectin and low EndoCab define an acute enteropathy with translocation, and that this profile is broadly associated with PAMP tolerance. Such an explanation is *post hoc* and should be investigated in further prospective studies.

Despite the fact that pattern recognition receptors have been recognised since the 1990s and their status as critical players in the immune response to infection and injury is undisputed, only a few previous studies have addressed PAMP sensing in malnourished children, and investigations have mainly been restricted to LPS response. Doherty *et al.*, working in Jamaica, found that release of TNF α and IL-6 following whole-blood stimulation for 2-24 hours LPS 10 μ g/ml increased following nutritional rehabilitation but did not compare children with SAM to healthy controls, which is particularly important when it is well recognised that improvement in resilience to infection lags behind anthropometric recovery.²²³ Muñoz *et al.* performed a similar uncontrolled experiment that demonstrated increasing IL-1 β and TNF α response to 18 hour PBMC stimulation with 1 μ g/ml LPS during nutritional rehabilitation.²²⁴ Palacio *et al.*, working in Chile, found no difference in release of IL-1, TNF α , and IL-6 following 18 hour PBMC culture with LPS 1 μ g/ml between

malnourished children and healthy controls, although the mean WHZ/WLZ of their cases was -1.3 so they mostly did not even meet current criteria for moderate acute malnutrition, let alone SAM.²²⁵ Azevedo *et al.* found increased TNF α production by malnourished children compared to controls in a 24 hour whole-blood stimulation model across a range of LPS concentrations, though their cases were also relatively well nourished, with mean WHZ/WLZ of -1.49.²²⁶ Malavé *et al.* found no difference in IL-6 production between malnourished children and controls following 48 hour PBMC stimulation with 5 μ g/ml LPS.²²⁷ Work in animal models of malnutrition has produced similarly contradictory results. Monk *et al.* demonstrated reduced *in vivo* IL-10 production in response to LPS challenge in two metabolically distinct murine models of undernutrition.²²⁸ Fock *et al.* found the opposite result in a similar system, demonstrated increased IL-10 alongside reduced *in vivo* IL-1 β and TNF α production, with decreased NF- κ B activation *in vitro*.²²⁹⁻²³² Anstead *et al.* showed increased IL-6 production by peritoneal macrophages following *in vitro* stimulation with LPS and IFN γ , similar IL-1 β production but decreased nitric oxide, TNF α and IL-10, with reduced early (< 16 hour) NF- κ B activation.²³³ Reynolds *et al.* showed decreased IL-1 β production by macrophages from malnourished mice in an *in vitro* system.²³⁴ Therefore while there is some evidence of increasing pro-inflammatory cytokine release following LPS challenge during nutritional rehabilitation in humans, in case-control and animal studies there has variously been evidence of positive, negative, or null impact of malnutrition on response to PAMP challenge.

The current study has a number of limitations. The relatively small sample size limits statistical power to detect subtle differences between cases and controls, and means

that results from subgroup analyses, in particular, should be considered preliminary. Positive results should also be treated with caution because the large number of hypotheses under investigation renders the risk of Type 1 error, high. Methods to formally correct for multiple comparisons have not been applied, and *post hoc* hypotheses will need to be tested in future studies. However, the use of PCA has allowed effective integration of the highly correlated data and increased the effective power to detect differences in PAMP response in a small sample size.

Participants in the control group were living in poverty and most were stunted (HAZ/LAZ < -2), suggesting chronic ill-health. They were therefore relatively well matched for the SAM participants in all but acute nutritional status, but should not be considered 'healthy' controls. The temporal, mechanistic and aetiologic relationships between wasting and stunting are incompletely defined. Abnormal inflammatory function and PAMP response might be common to both groups. Certainly, 53% of control children had faecal calprotectin > 100 µg/g, indicating clinically-relevant intestinal inflammation, and suggesting a high prevalence of EED in this setting.²³⁵ Cellular immune function in EED has not been investigated before, and is worthy of future study. Finally, structural and functional diversity in the mammalian immune system means that responses in blood may be qualitatively different to those at mucosal surfaces, where host-PAMP interactions occur. In the gut, for example, epithelial cells play a role in pathogen sensing, and non-antigen-restricted tissue-resident innate lymphoid cells direct immune responses.²³⁶⁻²³⁸ Ethical and logistic difficulties inherent in accessing mucosal specimens from malnourished children for study mean that the overwhelming majority of work done

on understanding their immune function has relied on peripheral blood cells and plasma, and it is unlikely that this will change in the near future. Understanding the relationship between tissue-specific and systemic inflammation in model systems should be a research priority.

In summary, the current study has demonstrated that chronic inflammatory activation and *ex vivo* response of whole blood to a range of PAMP inflammatory stimuli was equal between well-characterised children with uncomplicated SAM and community controls. There was a possible signal of decreased inflammatory response in kwashiorkor that should be investigated in future studies.

CHAPTER 4

Mesalazine in the initial management of severely acutely malnourished children with environmental enteric dysfunction: a pilot randomised controlled trial

This chapter contains data that have been reported previously:

Jones KDJ, Hünten-Kirsch B, Laving AMR, Munyi CW, Ngari M, Mikusa J, Mulongo MM, Odera D, Nassir HS, Timbwa M, Owino M, Fegan G, Murch SH, Sullivan PB, Warner JO, Berkley JA. Mesalazine in the initial management of severely acutely malnourished children with environmental enteric dysfunction: a pilot randomized controlled trial. BMC Medicine (2014) 12(1): 133

4.1 METHODS

This was a single-centre double-blind randomised placebo-controlled trial of the use of mesalazine alongside standard medical and nutritional care in severely acutely malnourished children. Randomisation was balanced 1:1. The hypothesis was that mesalazine might safely reduce enteric and systemic host inflammatory drive suggesting that the inflammatory component of EED is functionally redundant and may worsen growth (analogous to IBD).

4.1.1 Participants and setting

The study took place between June and November 2013 at the nutrition clinic of Baraka Health Centre (BHC), Mathare, Nairobi. Further information on the setting has been provided in Chapter 3.¹⁹⁵

Participants were recruited from among those self-presenting to BHC or via a programme of active case-finding in the community conducted by local community health volunteers (CHVs). Eligible participants were children aged 12 to 60 months with SAM, defined for the purposes of this study as mid-upper arm circumference (MUAC) < 11.5cm or bilateral pedal oedema. They had uncomplicated SAM (i.e. good appetite and no severe clinical illness) and were therefore eligible for outpatient management according to WHO guidelines.¹¹ EED was inferred on the basis of stunting (HAZ/LAZ < -2 using the WHO 2006 growth standards) and chronic inflammation (erythrocyte sedimentation rate (ESR) > 20 mm/hour). Full inclusion and exclusion criteria were as follows:

Inclusion criteria:

- Children aged 12 to 60 months old.
- Provision of informed consent by parent or guardian.
- Stunting (HAZ/LAZ < -2)
- SAM (one or both of MUAC < 11.5cm and bilateral pedal oedema).
- Eligible for outpatient management of SAM (i.e. no evidence of acute infection, and passes 'appetite test' according to national guidelines).
- Evidence of chronic inflammation (ESR > 20 mm/hr) on screening blood tests.²³⁹

Exclusion criteria:

- Known HIV disease or tuberculosis^{* 240}
- Known previous renal disease or asthma[†].
- Recent (previous two weeks) bloody diarrhoea.
- Acute infection requiring treatment (e.g. lower respiratory tract infection or malaria).
- Medical condition precluding normal feeding (e.g. severe cerebral palsy).
- Already receiving treatment for SAM from another centre.
- Biochemical evidence of acute renal or hepatic impairment on screening blood tests: Creatinine > 70 µM,²⁴¹ or abnormal aspartate aminotransferase (AST) or alanine transaminase (ALT) according to KEMRI-Wellcome Trust Clinical Trial Laboratory reference ranges.
- Thrombocytopenia (platelets < 50x10⁹/L).
- Known allergy or hypersensitivity to mesalazine, other salicylate drugs, or any of the product ingredients.
- Concurrent medication known to interact with the study drug (e.g. non-steroidal anti-inflammatory drugs, H2 (histamine receptor 2) antagonists, proton-pump inhibitors)

* The medication regimens used to treat HIV and tuberculosis are frequently complicated by drug interactions, the likelihood or frequency of which would be difficult to predict and would require separate, dedicated investigation. National guidelines recommend offering provider-initiated testing for HIV for all severely malnourished children, and referral for comprehensive care in the case of a positive test.

† Renal problems and asthma are recognised contraindication to the provision of this class of drug.

- Other reason at the discretion of the attending clinician (independent of the trial team).

Exclusion criteria were decided on the basis of contraindications listed in the Summary of Product Characteristics, pre-existing conditions that the investigator group felt increased the risk to participants (e.g. HIV infection, bloody diarrhoea, other overt infection requiring hospital admission), or likely futility of the approach in the presence of other major medical problems (e.g. tuberculosis, cerebral palsy)²⁴². Concurrent or recent viral infection, or administration of a live viral vaccine, were not considered to be contraindications to enrolment: although Reye's syndrome has historically been associated with salicylate (mainly aspirin) use in these circumstances, the investigator group were unable to find even a single report of Reye's associated with mesalazine, and such cautions are not advised when it is used in the context of IBD.

4.1.2 Screening, enrolment and randomisation

Children aged 12 to 60 months old with SAM were referred to the study team at BHC for clinical screening. If clinical eligibility was confirmed and informed consent for participation was provided by the child's parent or guardian, venous blood was taken for HIV testing, full blood count, ESR, creatinine, liver function tests, and film for malaria parasites if required. HIV testing was via point-of-care tests provided by the Ministry of Health (Uni-gold with Determine confirmation).²⁴⁰ Final screening with blood results took place the following day, after which eligible children were

enrolled by assigning the next consecutive study number. In order to minimise unnecessary blood draws, additional blood was taken alongside the screening blood tests for later investigation of baseline inflammatory markers of EED as described later in this chapter. Children who were ineligible due to abnormal screening blood tests were followed up locally (free of charge) or referred to Mbagathi District Hospital (MDH) for ongoing care if required. Costs (including fares) related to their first consultation at MDH were met by the study.

Figure 4.1: Screening and enrolment procedures

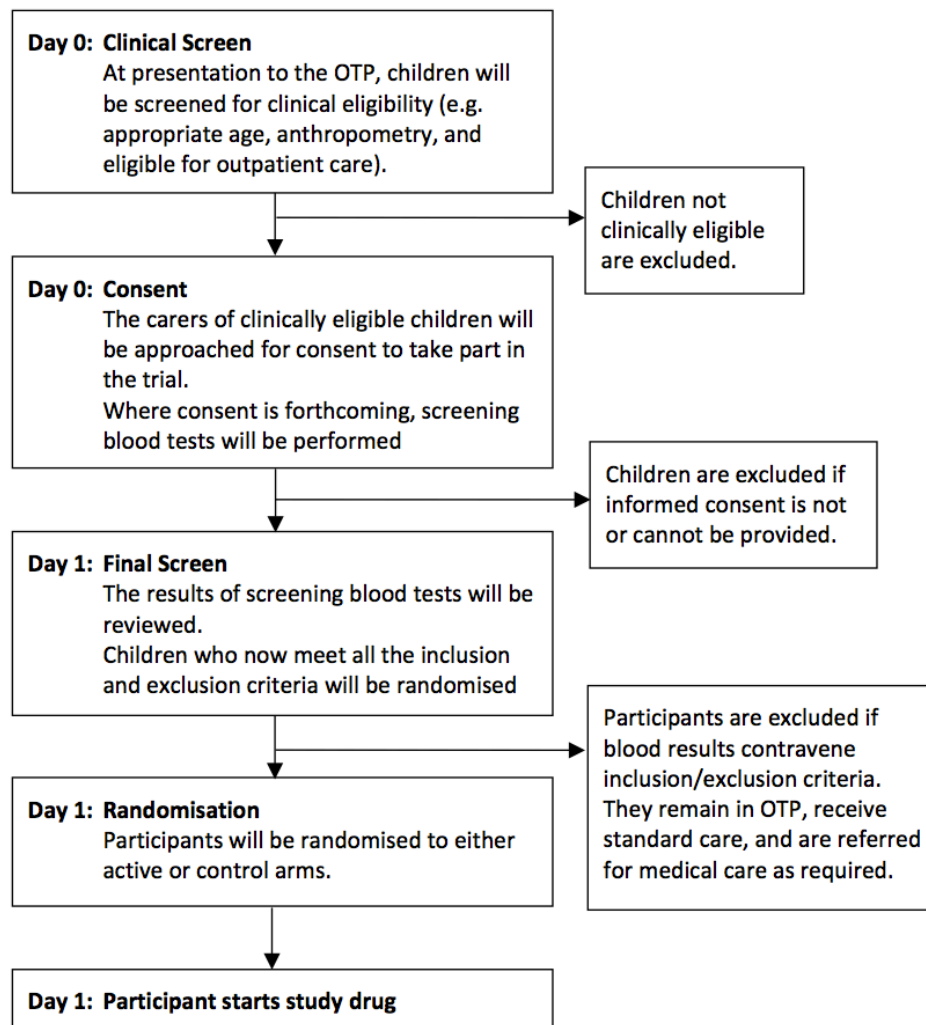


Figure 4.1: Flow-chart outlining screening and enrolment procedures.

A randomisation schedule was developed in STATA (version 12.0) with variable block sizes (2, 4 and 6) using the following code: *"ralloc blknum blksiz Rx, nsubj(44) osize(3) ntreat(2) saving(mys) table"*²⁴³. Allocations were assigned to study numbers by the trial statistician (Greg Fegan). Sachets of mesalazine (Pentasa) granules and matched placebo were purchased from Ferring Pharmaceuticals (Saint-Prex, Switzerland) in 2 g foil sachets that were identical apart from labelling. Prior to initiation of the trial, sachets were disguised by application of opaque 'black-out' labels (Avery) and re-

labelled (with study number) by pharmacy staff independent of the trial team, according to the randomisation schedule.

Dosing of drug and placebo was performed on the basis of weight, which required the contents of the 2 g sachets to be separated into smaller individual doses. Pharmacy technicians at BHC were trained to dispense study drug using an electronic fine-scale balance (TX-323L, Shimadzu). Doses were packed in foil pouches (purchased locally), heat-sealed to render them impermeable to light and air and labelled with the participant's initials, study number and date. Study drug was dispensed weekly in order to minimise deterioration of the active product due to repackaging and to account for changes in participants' weight. Active and placebo granules were indistinguishable.

4.1.3 Treatment

Participants were prescribed 30 mg/kg day of mesalazine or placebo in three divided doses for the first 7 days. Then, if tolerated (see below), the dosage was increased to 45 mg/kg/day for a further 21 days. They were followed up for a further 28 days after stopping the study drug (56 days total). Blood and stool specimens were collected at days 7, 28 and 56. To account for excipients the dose of granules prescribed was 11 mg/kg three times daily for the first week, followed by 16.5 mg/kg three times daily. Because dispensing exactly to the milligram was not possible with a granular product, technicians dispensed in the range of the prescribed dose to prescribed dose +5 mg.

If recognised side-effects of mesalazine occurred in the first week or the day 7 blood tests indicated deterioration in renal or hepatic function or blood dyscrasia (Grade 1-2 toxicity), the dosage was maintained at 30 mg/kg/day without unblinding. Such children were re-assessed on a weekly basis and their dose was escalated if or when it appeared to be safe to do so. The study drug was to be discontinued in the event of Grade 3-4 toxicity. Toxicity grades for biochemical indices were defined according to the US Division of Microbiology and Infectious Diseases' Pediatric Toxicity Tables, 2007, and are detailed later.²⁴⁴ Carers were asked to withhold the study drug if the child developed diarrhoea, blood in stools or unexplained bruising, and to bring the child for assessment as soon as possible. Study drug was suspended during acute watery diarrhoeal episodes, until they had resolved.

All children received nutritional rehabilitation with ready-to-use therapeutic foods (RUTF) conforming to WHO/UNICEF standards until they were nutritionally cured of SAM (MUAC > 11.5 cm and no oedema at two consecutive weekly visits), alongside a seven-day course of amoxicillin and deworming with mebendazole or albendazole according to Kenyan national guidelines.²⁴⁰

4.1.4 Monitoring

Children were to be brought for clinical review after 7 days (day 8). They were seen by the study clinician and assessed for the development of any new symptoms potentially related to the study drug. Routine blood tests were taken to monitor

renal, hepatic, and bone marrow function. Children were to be brought each week thereafter for clinical monitoring and anthropometry. Drug was dispensed at each visit, and delivered to the participant's homestead the same day if they were not able to wait for it. In children with new symptoms potentially related to the study drug the drug was to be discontinued, but the child would stay in follow-up as described below.

After 28 days, within 3 days of completion of the study drug, the child was reviewed again by clinical staff, had anthropometry performed, and a blood test taken for the same set of monitoring tests as at day 8. Plasma was stored for evaluation of EED/inflammatory markers as detailed later, and stool was collected. Children who still required treatment for malnutrition continued to receive full normal clinical care as per national guidelines. A follow-up appointment was scheduled at 56 days after randomisation, when a final review by clinical staff, anthropometry and blood tests were performed.

At all times, any intercurrent illnesses (including recurrence of severe malnutrition) were treated according to Kenyan national and international guidelines.^{10, 11}

The blood volumes removed at each time point were no more than 1 ml/kg (up to a maximum of 7 mls) and less than 3 ml/kg in any 28 day period – well within internationally established limits for research blood draw volume.²⁴⁵ Volumes at each visit were as follows:

Day 0:	5.0 – 7.0 mls blood
Day 8:	2.0 mls blood
Day 28:	5.0 – 7.0 mls blood
Day 56:	5.0 – 7.0 mls blood

In the event of unscheduled visits, participants received a full clinical evaluation by study staff, which was to include blood tests for clinical care only if clinically indicated. In the event of any concern regarding potential medication reactions the child was to be discussed with the local safety monitor who may have recommended unblinding (described later), after discussion with the data, safety and monitoring board. All intercurrent illnesses were treated according to Kenyan national guidelines.

4.1.5 Safety and reporting

All adverse events (AE) occurring in participants during the trial were documented and reported as described below.

4.1.5.i Adverse Event (AE)

An AE was considered to be any untoward medical occurrence, which may occur during or after administration of the investigational product. The AE may or may not have a causal relationship with the product as indicated by physical signs, symptoms, and/or clinically significant laboratory abnormalities that occur. The definition included any intercurrent illnesses, injuries or exacerbation of pre-existing

conditions.

4.1.5.ii Serious Adverse Event (SAE)

Any AE (whether or not considered related to the investigational product) that resulted in any of the following outcomes was defined as an SAE.

- Death (i.e., results in death from any cause at any time)
- Life-threatening event (i.e., the subject was, in the view of the investigator, at immediate risk of death from the event that occurred). This was not to include an AE that, if it occurred in a more serious form, might have caused death.
- Persistent or significant disability or incapacity (i.e., substantial disruption of one's ability to carry out normal life functions).
- Hospitalisation: regardless of length of stay, even if the hospitalisation is a precautionary measure for continued observation. Hospitalisation (including inpatient or outpatient hospitalisation for an elective procedure) for a pre-existing condition that had not worsened unexpectedly did not constitute an SAE.
- An important medical event (that may not cause death, be life threatening, or require hospitalisation) that may, based upon appropriate medical judgment, have jeopardised the subject and/or required medical or surgical intervention to prevent one of the outcomes listed above. Examples of such medical events would have included allergic reaction requiring intensive treatment in an emergency department but not resulting in inpatient admission.

4.1.5.iii Suspected Unexpected Serious Adverse Reaction (SUSAR)

These were reactions that are serious (as defined above) but not expected and considered to be related to the investigational product. This definition captures the important events that are attributed to the product but do not follow known pattern of response to study product.

4.1.5.iv Toxicity

Grade 3 and 4 clinical and laboratory toxicities were pre-specified for renal and hepatic dysfunction, haematological abnormality, allergy and for gastrointestinal and central nervous system symptoms, as these are the most commonly encountered features based on experience using mesalazine in children with IBD. A change in a laboratory variable alone was reported as an AE if classified as grade 3 or 4 toxicity. A clinical toxicity may have been an AE or an SAE depending on severity.

Toxicity	Grade 3	Grade 4
Renal dysfunction ²⁴⁴	Creatinine 1.2-1.5x upper limit of normal (ULN) if 6 months-2 years (84-105uM) Creatinine 1.7-2.0x ULN if >2years (119-140uM)	Creatinine >1.5x ULN if 6 months-2 years (>105uM) Creatinine >2.0x ULN if >2years (>140uM)
Liver dysfunction ¹¹	Transaminases or gamma-glutamyl transferase (GGT) 3-8x ULN Bilirubin 1.5-1.75 xULN if any other LFT derangement, or 2-3x ULN if other LFTs normal.	Transaminases or gamma-glutamyl transferase (GGT) >8x ULN Bilirubin >1.75 xULN if any other LFT derangement, or >3x ULN if other LFTs normal.
Haematologic: Anaemia¹⁰ Thrombocytopenia¹¹	Haemoglobin 4 to 5g/dl, not transfused. Platelets 25 -50 x 10⁹ /L	Haemoglobin <4 g/dl <u>or</u> Haemoglobin <5 g/dl with shock, respiratory distress or cardiac failure. Platelets <25 x 10⁹ /L
Allergy/Cutaneous ¹¹	Generalised urticaria	Anaphylaxis, angioedema
Neurologic ¹¹	Moderate to severe headache (responding to narcotic analgesia)	Intractable headache
Gastrointestinal ^{10, 11}	Diarrhoea with 'some dehydration' (based on clinical features from WHO) Bloody diarrhoea Moderate abdominal pain requiring treatment Severe nausea 4-6 episodes of vomiting/24h	Diarrhoea with 'severe dehydration' (based on clinical features from WHO) Colitis requiring gastrointestinal surgery Severe abdominal pain requiring hospitalisation Unable to ingest fluid >24h >6 episodes vomiting/24h

4.1.5.v Adverse events assessment of causality

An assessment of the relationship of the event to the study drug was to be undertaken by the senior clinical investigators (Kelsey Jones and James Berkley) who would remain blinded to study allocation. This interpretation was to be based on the type of event, the relationship of the event to the time of administration, and the known biology of the intervention. The following were guidelines for assessing the relationship of administration of to the product to the AE:

Relationship	Is there a reasonable possibility that the AE may have been caused by the investigational product?
No	The AE is not causally related to administration of the study drug. There are other, more likely causes.
Yes	The AE is most likely to be causally related to the study drug.

4.1.5.vi Practical handling of adverse events

In the event of an abnormal clinical or laboratory finding, children were to receive appropriate treatment according to Kenya national or WHO clinical guidelines, including admission for assessment and/or treatment where appropriate. Usual clinical practice for suspected reactions to mesalazine was to be followed. Where necessary for patient safety, this may have included unblinding by the local safety monitor (LSM), in communication with the data, safety and monitoring board (DSMB) (Chair's action was sufficient for emergency unblinding)

All SAEs and SUSARs were to be reported to the LSM, DSMB, Sponsor and ethical review committees by email as soon as they occur, and a formal report was to be provided to the above agencies and the Kenya National Pharmacy and Poisons Board within 10 days.

4.1.6 Outcomes

Primary outcomes were frequency of adverse events and compliance with the intervention assessed via interview with the carer and weekly counting of full/empty sachets. Stool frequency and consistency was assessed at each study visit using a Kiswahili translation of the Bristol Stool Form Scale (Figure 4.2).²⁴⁶ Secondary outcomes were time to recovery, growth and a panel of inflammatory markers (see below).

Figure 4.2: Choo Chart

CHOO CHART

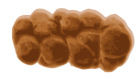
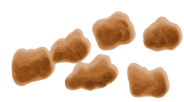
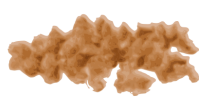
1		Kama NJUGU
2		Kama VIAZI VITAMU
3		Kama MAHINDI
4		Kama NDIZI
5		Kama NYAMA
6		Kama UJI
7		Kama CHAI

Figure 4.2: Swahili translation of the Bristol Stool Form Chart. Developed with the help of the Community Health Worker Team at Baraka Health Centre. Adapted from a representation by Kyle Thompson available at: http://commons.wikimedia.org/wiki/File:Bristol_stool_chart.svg

4.1.7 Laboratory methods

Blood tests designated for safety (full blood count (FBC), ESR, C-reactive protein (CRP), creatinine, alanine transaminase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), bilirubin) and stool for microscopy were

processed in a commercial GCLP-accredited laboratory in Nairobi (Pathologists Lancet Kenya) and results were provided the following day.

Plasma, serum and stool were kept on ice prior to freezing at -80°C pending batched enzyme-linked immunosorbent assay (ELISA) processing. The following were tested by ELISA following manufacturers' recommendations: faecal calprotectin (Bühlmann kits on stool following disruption by 30 seconds TissueLyser shaking (QIAGEN, no beads)), plasma IgG EndoCAb (Hycult Biotech), plasma interferon- γ (Ebioscience) and serum insulin-like growth factor-1 (R&D Systems). Multiplex ELISA (Luminex MAGPIX system) was performed against the following targets in plasma: Eotaxin (chemokine (C-C motif) ligand (CCL)-11), GRO α (growth regulated oncogene- α , chemokine (C-X-C motif) ligand (CXCL)-1), Interferon- α , Interleukin (IL)-1 α , IL-1 receptor antagonist (IL-1RA), IL-7, IL-8, IL-10, IL-15, IL-17a, IL-22, IL-31, IP-10 (interferon- γ induced protein-10, CXCL10), MCP-1 (monocyte chemotactic protein-1, CCL2), MIP-1 α (macrophage inflammatory protein-1 α , CCL3), MIP-1 β (CCL4), SDF-1 (stromal cell-derived factor-1, CXCL12), tumour necrosis factor- β (TNF β) (Ebioscience). Soluble CD14 (sCD14) was measured using an in-house ELISA (capture clone 55-3, detection clone 3-C39 both from BD, recombinant standard from Sigma). Serum endotoxin was measured using HEK-Blue Endotoxin Detection Kit (Invivogen): This test relies on a HEK293 cell line that has been stably transfected with TLR4 pathway genes and a secretory alkaline phosphatase that is transcriptionally regulated by NF- κ B. Heat-inactivated sera (90°C for 30 minutes) were incubated in duplicate with cells for 24 hours in the presence of a detection reagent. Absorption at 620 nm was read against a standard curve.

4.1.8 Statistical methods

Analysis was performed in Stata Version 12.0. I performed Mann-Whitney U tests or t-tests on log-transformed data between the arms at each time point. Growth in height and MUAC across the trial was calculated for individual participants in mm/day and compared using Mann-Whitney U tests. Fisher's exact test was used to compare grouped variables. Comparison of the timing of adverse events was done using logrank. Raw ELISA data was analysed in Graphpad Prism 6.0 prior to import into Stata and z-scores were calculated using WHO Anthro Version 3.2.2 Stata macros. Analyses were performed by intention to treat, except for blood and stool laboratory analyses, which were performed using data from all available specimens (i.e. not including data missed due to withdrawal or failure to get specimens). For this pilot study, no cut-off was considered to indicate 'statistical significance', and P-values are provided throughout. Because the secondary analyses were intended to be exploratory and hypothesis-generating, *post-hoc* correction for multiple comparisons was not performed.

Sample size was set at 22 in each arm with reference to norms in Phase I and early Phase II research. No sample size calculation was performed, and the study was not powered to formally address any outcomes at any given significance level.

4.1.9 Study oversight

All participants enrolled in the study had individual written informed consent provided by a parent or guardian. The study was approved by the Kenya Medical

Research Institute (KEMRI) Ethical Review Committee, the Imperial College, London Ethical Review Committee, and the Kenya Pharmacy & Poisons Board prior to initiation. Imperial College, London was the sponsor. Clinical trials monitoring was performed by staff from the Clinical Trials Facility, KEMRI-Wellcome Trust Research Programme. An independent Data Safety and Monitoring Board (DSMB) was established and an independent consultant paediatrician acted as local safety monitor (LSM). Neither the sponsor nor any other party except the named investigators had any role in the design of the study, interpretation of the results, content of manuscripts or decision to publish. The trial was registered at <http://clinicaltrials.gov/ct2/show/NCT01841099>.

4.1.10 Specific ethical issues

The study was conducted according to guidelines set out in the World Medical Association's Declaration of Helsinki.²⁴⁷ There were two particular aspects of study design that I considered carefully in the development of the protocol: the enrolment of children with SAM as well as stunting, and the age range of children screened for potential enrolment.

While the histologic lesion of EED has been most comprehensively described in children with SAM, it appears to be highly prevalent in children with stunting who are not acutely malnourished. The reason for choosing to conduct the trial with children who were both stunted and had SAM was twofold:

1. The Declaration of Helsinki states that *“medical research is only justified if there is a reasonable likelihood that the populations in which the research is carried out stand to benefit from the results of the research.”*²⁴⁷ I reasoned that a novel (for this indication) pharmacologic treatment like mesalazine would be most practically and safely administered alongside a course of antimicrobials, with nutritional support and close clinical monitoring. Children with SAM receive all of these as part of routine care, and SAM is managed according to a medical model. It therefore seemed likely that any future routine clinical use of mesalazine would most pragmatically occur in the context of SAM.
2. Where a clinical trial potentially involves risk to the participant, it is equitable to ensure that the population involved in the trial represent those with the most to gain from the approach. Because long-term survival from SAM is so poor (as described in Chapter 1) it stands to reason that children with SAM and stunting have more to gain from enhancements in care than those with stunting alone. Again, this balance may change as more evidence accumulates.

In the UK, mesalazine is only licensed for children aged 6 years and older. I believed that it was key to include children as young as 1 year old for the following reasons:

1. The youngest children have by far the greatest prevalence of SAM (figure 4.3), and the greatest mortality as a result of it.¹¹¹ Therefore they represent the population with most to gain from the intervention, as described above.

2. An analysis of anthropometric data from 54 cross-sectional surveys worldwide indicates that stunting overwhelmingly occurs prior to two years of age, and that after two years there is no overall decline in HAZ/LAZ. It is therefore key that any intervention that aims to improve linear growth is targeted at children younger than 2 years of age, because this is the key period of vulnerability.²⁴

Figure 4.3: Age distribution of SAM at BHC

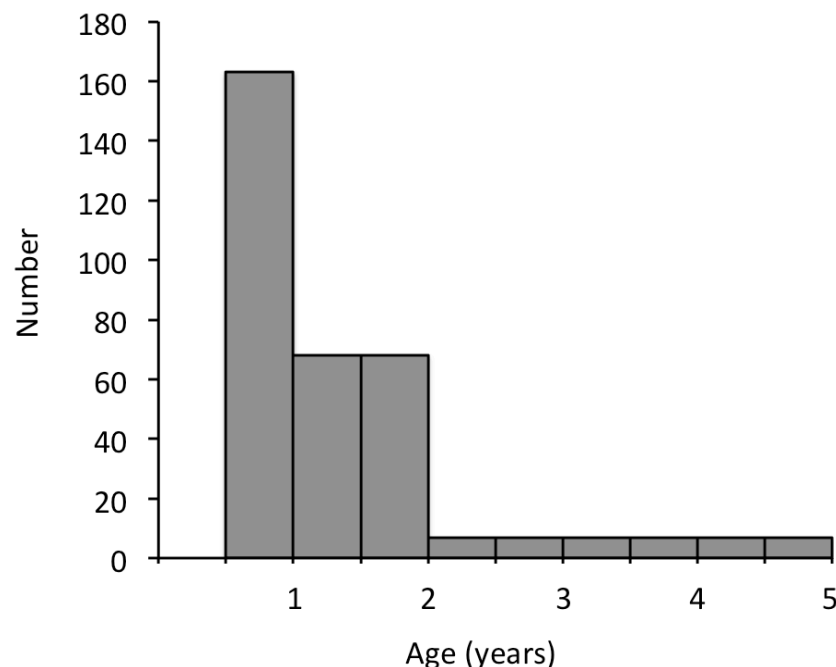


Figure 4.3: Histogram showing age distribution of children aged 6-60 months admitted for outpatient therapeutic feeding at Baraka Health Centre for calendar year 2011. 86% of children were younger than 24 months. Routine data collection by Mrs. Caroline Munyi, Nurse in Charge, Baraka Health Centre Feeding Programme.

Children aged 6 to 11 months were excluded on the basis that I had previously found a positive association between ESR and age, such that very few children in this age group would have met the ESR requirement for enrolment (data not shown). I

reasoned that the requirement for additional blood tests and a visit to clinic the next day could not be justified if the pre-test likelihood of meeting the enrolment criteria was very low. Therefore children aged 12 to 60 months were targeted for enrolment.

4.1.11 Community engagement

A community engagement plan was agreed with the Community Liaison Group at KEMRI-Wellcome Trust Research Programme and Social Services/Community Outreach staff at the Baraka Clinic, at a meeting held at the Baraka Clinic in September 2012. It involved holding three separate meetings for community leaders, community healthworkers, and other stakeholders identified by the Baraka team. The first of these meetings took place at the Baraka Clinic in September 2012 with the following discussion points:

- Who are KEMRI, who are the Wellcome Trust, personal introduction to clinical researchers.
- The idea of 'clinical research', why it is done, and what the benefits may be.
- Concepts around informed consent and ethical issues: autonomy, justice and beneficence.
- The importance of maintaining confidentiality in clinical research.
- What it means in practical terms to be involved in clinical research (screening, additional blood tests, home visits, provision of stool).
- Personal and community risks and benefits of being involved in research.

- What is Severe Acute Malnutrition, why is it important, and why is it an important topic for research.

The meeting was successful, and those attending were positive about the idea of doing research at BHC and in Mathare more generally. The meeting gave an affirmative informal 'vote' as to whether research work should begin.

The next meeting was scheduled to take place before the initiation of the trial in May 2013. The objectives of this meeting were to discuss the following issues:

- Results and insights gained from work already carried out at Baraka
- Scientific rationale for the study
- Eligibility criteria for the study
- Study-specific procedures
 - A description of the packs of study drug that will be used
 - Schedule of follow-up appointments
 - Individual risk-benefit profile for participants
 - What happens if a child becomes unwell during the study
 - Longer-term follow-up
- Description of randomisation and blinding

Community consent was given to begin the trial.

The final meeting took place in June 2014, where results of the trial were presented.

An additional aspect of community engagement was to involve local Ministry of Public Health & Sanitation/Medical Services staff. Dr. Moses Owino, the local District Minister of Health, was aware and supportive of the study, and is a named collaborator on other studies within our group. The proposed work was presented to the two major local NGOs, Concern Worldwide and UNICEF, and to Division of Nutrition (Ministry of Health) staff. All were supportive of the study.

4.2 RESULTS

From June to September 2013, 133 children with SAM and stunting were screened for eligibility. 44 completed screening procedures and were eligible, all of whom were enrolled (Figure 4.4). The arms were well balanced in terms of clinical, anthropometric and demographic characteristics at baseline (Table 4.1).

Figure 4.4: Trial flow diagram

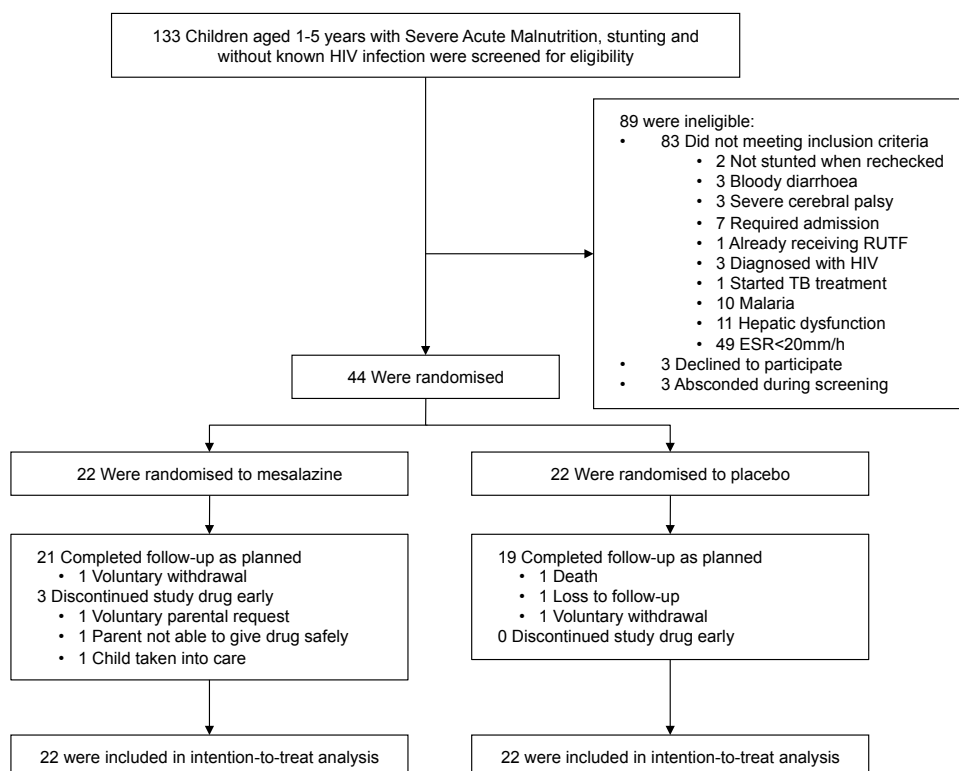


Figure 4.4: Trial flow diagram. *Some participants failed to meet the inclusion criteria on several separate counts.

Table 4.1: Baseline characteristics

Baseline Characteristics	Mesalazine Arm	Placebo Arm
Number enrolled	22	22
Age (months)	19 (14 – 32)	19 (15 – 29)
Sex: Male	10 (45)	11 (50)
Female	12 (55)	11 (50)
Household income category (USD/day)*	\$2-4	\$2-4
Number of people living in the house	5 (4 – 7)	5 (4 – 5)
Access to Improved drinking water source†	100%	100%
Access to Improved toilet facility	50%	41%
MUAC (cm)	12.9 (11.2 – 14.4)	12.7 (11.2 – 13.8)
Height/length-for-age z-score	-3.13 (-3.83 – -2.71)	-3.73 (-4.60 – -2.81)
Nutritional oedema	15 (68)	17 (77)
Current breastfeeding‡	11 (50)	5 (23)
Symptoms: Fever	10 (45)	8 (36)
Cough	9 (41)	6 (27)
Diarrhoea	5 (23)	5 (23)
Biochemistry: Creatinine (umol/L)	18 (17 – 23)	19 (17 – 22)
AST (IU/L)	37 (27 – 52)	40 (32 – 49)
ALT (IU/L)	24 (12 – 31)	17 (15 – 30)
CRP (mg/L)	6.0 (1.3 – 10.8)	6.8 (1.6 – 42.2)
Haematology: Haemoglobin (g/dL)	10.3 (7.9 – 11.2)	9.6 (8.2 – 10.6)
WBC count (x10 ⁹ /L)	12.0 (9.9 – 15.1)	11.8 (9.3 – 13.6)
Platelet count (x10 ⁹ /L)	555 (423 – 666)	536 (282 – 692)
ESR (mm/hour)	32 (24 – 38)	33 (28 – 44)

Table 4.1: Baseline characteristics. Data are medians (inter-quartile range) or numbers (%).

*Median and IQR for both arms all fell within the \$2-4 category. †Mostly communal taps from which water must be purchased. Lack of resources was reported to limit access. ‡P=0.06

4.2.1 Follow-up and Compliance

Following completion of the interventional phase of the study at day 28, one child in each arm was voluntarily withdrawn from the study by their carer (in both cases because the family planned to travel away from Nairobi) and one child in each arm was lost to follow-up. Compliance with the study drug administration schedule was high in both arms (Table 4.2). Early discontinuation of the study drug occurred in three children, in each case for reasons unrelated to the drug itself and all three remained in follow-up: one discontinuation at day 14 was at parental request, another at day 14 was initiated by the study team because the parent was unable to give the drug as prescribed, and the third at day 22 was because the participant was taken into protective custody. All three were in the mesalazine arm.

Table 4.2: Outcomes

	Mesalazine Arm	Placebo Arm	P
Safety and Compliance			
Deaths	0	1	0.32
Hospitalisations*	0	2	0.15
Total adverse events	35	27	0.53
Acute watery diarrhoea	7	8	0.53
Bloody diarrhoea	0	2	0.32
Chronic diarrhoea	1	1	1.00
Gastroenteritis (with vomiting)	5	1	0.15
Giardiasis	6	6	1.00
Oral candidiasis	3	0	0.08
Upper respiratory tract infection	3	5	0.64
Lower respiratory tract infection	3	1	0.30
Tuberculosis	1	1	1.00
Malaria/non-specific febrile illness	4	3	0.43
Rash	2	2	1.00
Total gastrointestinal adverse events	23	16	0.27
Total diarrhoeal adverse events	8	11	0.65
Unable to dose-escalate at day 7	1 (5)	7 (32)	0.05
Compliance (%)	93 (71 – 98)	97 (82 – 99)	0.44
Anthropometry and Recovery			
Recovery: Nutritionally cured at d56	13	12	1.00
Recovery: RUTF-free days at d56	9 (0 – 21)	9.5 (0 – 21)	0.94
Nutritional oedema: baseline	15/22 (68)	17/22 (77)	0.72
d28	7/22 (32)	6/22 (27)	1.00
d56†	3/20 (15)	2/19 (11)	1.00
MUAC (mm): baseline	12.9 (11.2 – 14.4)	12.7 (11.2 – 13.8)	0.56
d28	13.7 (11.8 – 14.7)	13.2 (12.2 – 14.6)	0.82

d56	13.9 (12.5 – 14.8)	13.6 (13.0 – 15.1)	0.59
MUAC growth rate (mm/day): to d28 to d56	0.23 (0.06 – 0.37) 0.12 (0.08 – 0.21)	0.31 (0.18 – 0.61) 0.23 (0.14 – 0.33)	0.15 0.01
Height growth rate (mm/day) to d56	0.31 (0.19 – 0.38)	0.32 (0.21 – 0.38)	0.94
Height-for-age z-score: baseline	-3.13 (-3.83 – -2.71)	-3.73 (-4.60 – -2.71)	0.29
d28	-3.17 (-4.04 – -2.61)	-3.70 (-4.20 – -2.73)	0.42
d56	-3.24 (-4.10 – -2.68)	-3.46 (-4.14 – -2.68)	0.79

Table 4.2: Outcomes. Data are medians (inter-quartile range) or numbers (%) unless otherwise stated. *Includes the one child who died in hospital. †Excluding children who were unable to be assessed.

4.2.2 Adverse events and toxicity

Two children required hospitalisation during the course of the study, both with diarrhoea, dehydration and sepsis, one of whom died. Both were in the placebo arm. One child in each arm was started on tuberculosis treatment on the basis of clinical score and poor response to nutritional rehabilitation. One child in the placebo arm was newly diagnosed with sickle cell disease.

Non-severe adverse events and illness episodes were common, as expected in this vulnerable patient population, but there was no suggestion of excess risk associated with mesalazine treatment either in total adverse events, adverse events involving the gastrointestinal tract, or those associated with diarrhoea (Table 4.2 & Figure 4.5). At day 7 children in the mesalazine arm had firmer stool consistency (Figure 4.6).

Figure 4.5: Timing of adverse events

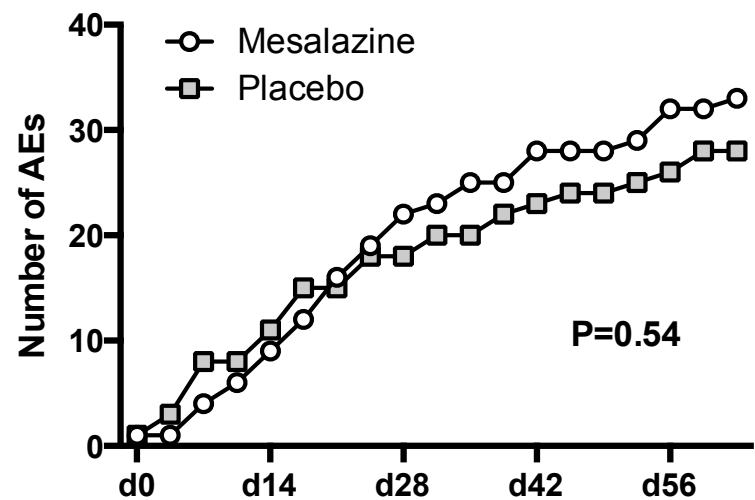


Figure 4.5: Timing of adverse events in the trial: There was no evidence of accumulation of adverse events during the intervention period (the first 28 days).

Figure 4.6: Stool frequency and consistency

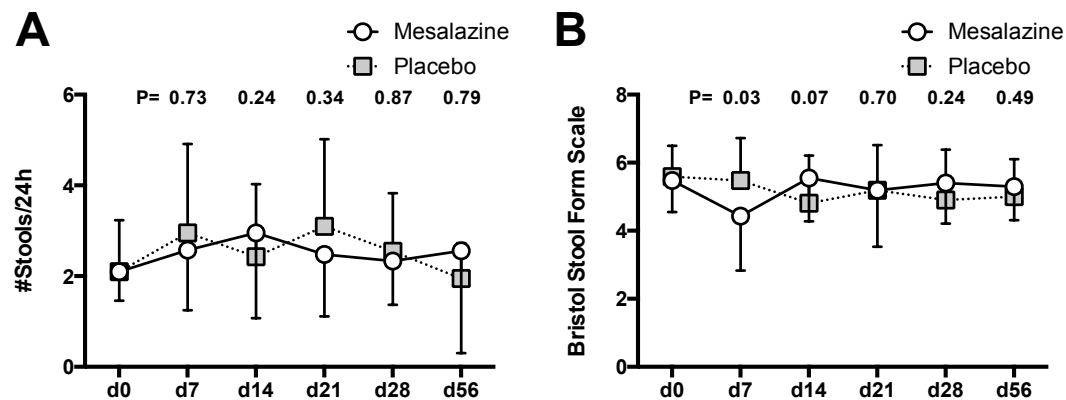


Figure 4.6: Stool frequency (A) and consistency (B) between the arms. Stool was more formed in the mesalazine arm at day 7, but overall there were no major differences between the arms. Mean and standard deviations are plotted but significance tested using Mann-Whitney U tests.

The most common previously reported side-effects of mesalazine, apart from gastrointestinal problems, are headache and rash. Most children were pre-verbal

and would have been unable to report headache but there were no concerns from carers around irritability, restlessness, or problems with sleeping. Four children had an infectious rash during the course of the study but none were considered related to treatment: two had impetigo (both mesalazine arm), one had chickenpox (placebo) and one had scabies (placebo).

Dose escalation at day 7 was delayed in eight participants: seven had developed elevated transaminases compared to their enrolment results and one child was admitted to hospital on the day of their scheduled visit. Of these eight participants, seven were in the placebo arm and one was in the mesalazine arm. Transient elevation of liver enzymes compared to enrolment was common at day 7 and was more pronounced in the placebo arm (Figure 4.7). No Grade 3 or 4 toxicities were encountered in the study.

Figure 4.7: Hepatic toxicity

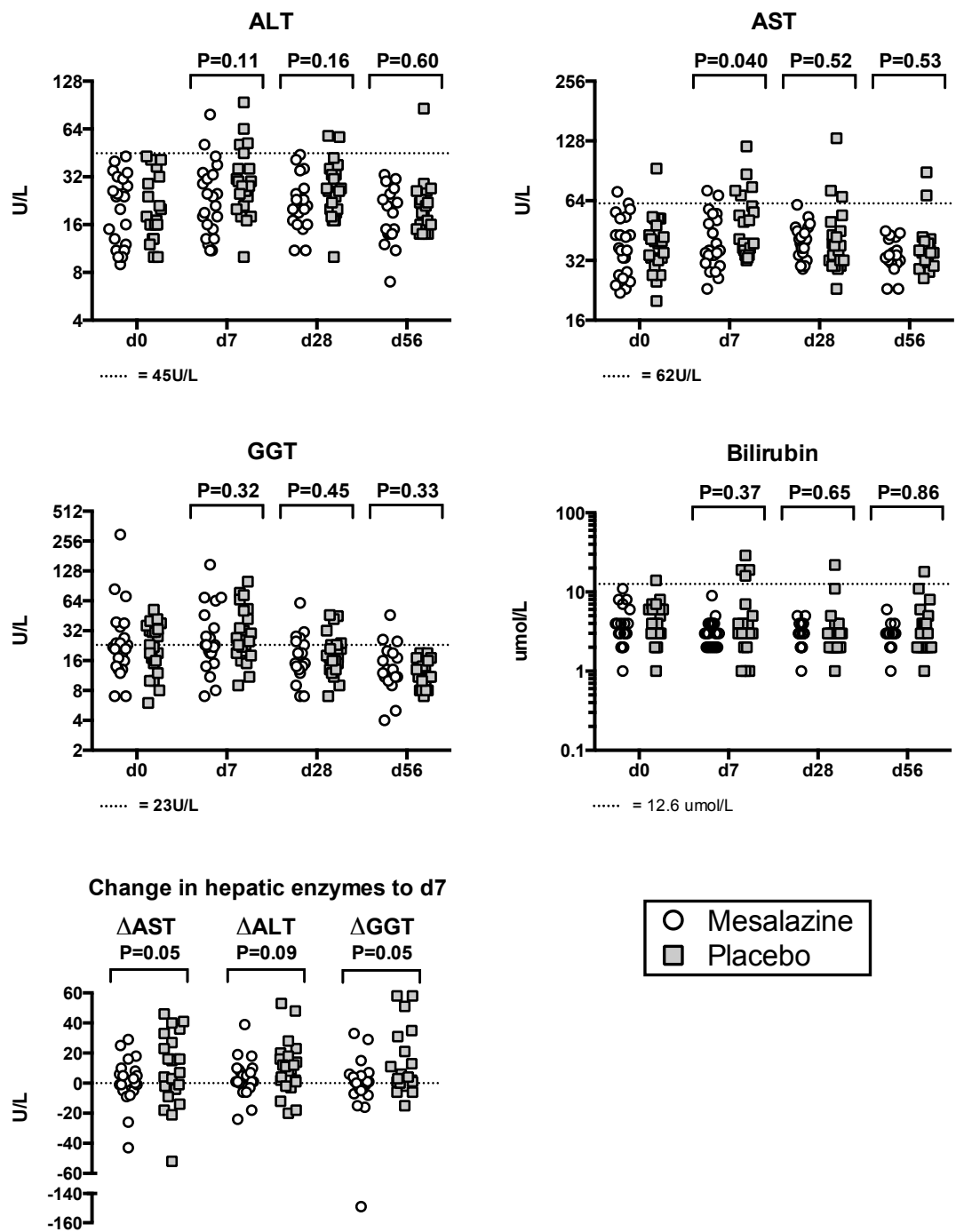
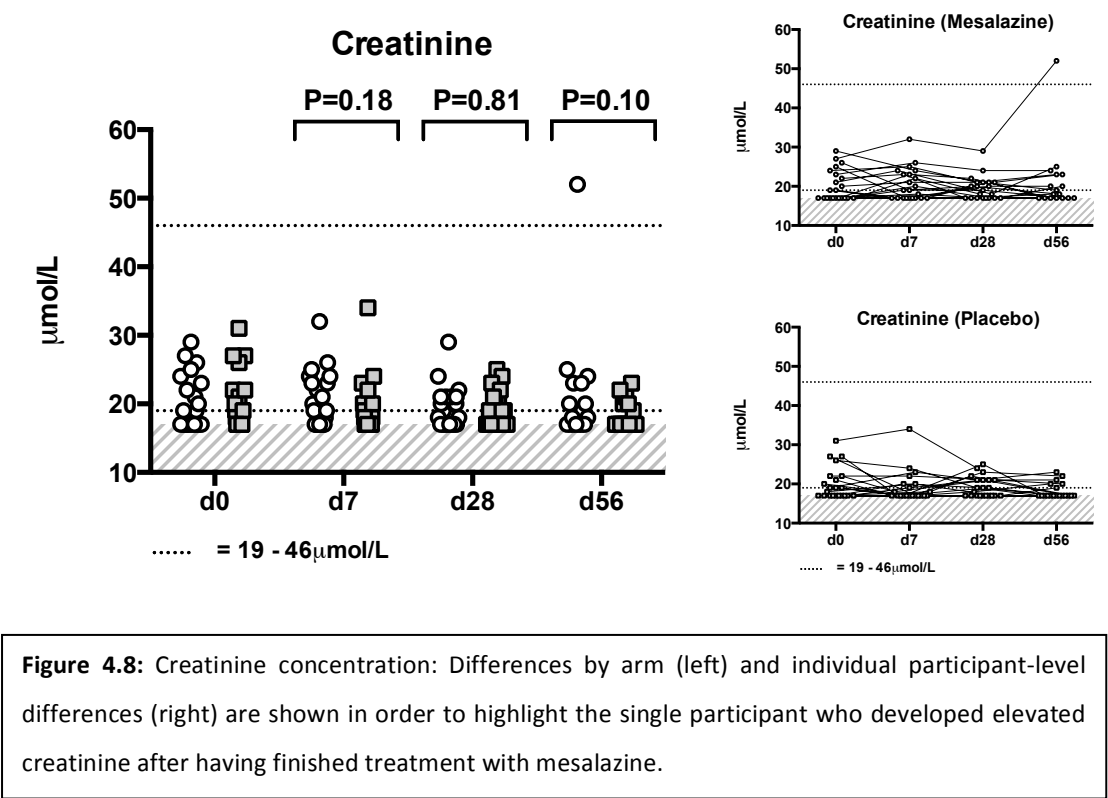


Figure 4.7: Hepatic toxicity: Alanine transaminase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and bilirubin by arm during the trial. Bottom left shows participant-level differences between the liver enzymes at enrolment and day 7, revealing reduced enzyme induction in the mesalazine arm.

Creatinine concentrations across the trial were low. A single elevated concentration occurred at day 56 in a child in the mesalazine arm who had achieved nutritional recovery (Figure 4.8). The child remained well and when tested three months later their creatinine concentration was 27 $\mu\text{mol/l}$.

Figure 4.8: Creatinine



Thirty-four (77%) children were anaemic (Hb < 11.0 g/dL) at enrolment. Rates of resolution were similar in the two arms (Figure 4.9).

Figure 4.9: Haemoglobin

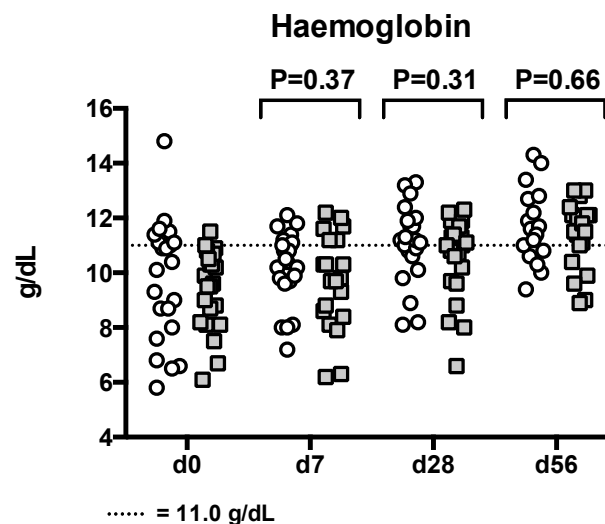


Figure 4.9: Haemoglobin concentration between the arms.

4.2.3 Growth and Recovery

At completion of the 56-day follow-up period, nineteen children (34%) had not recovered, died, or were not remaining in follow-up, nine in the mesalazine arm and ten in the placebo arm. At day 56, there were no differences in RUTF-free days or resolution of oedema between the arms (Table 4.2). There were no differences in linear growth rate or change in HAZ/LAZ (Table 4.2 and Figure 4.10). The rate of increase in MUAC was higher in the placebo than in the mesalazine arm, though this was less pronounced amongst those children who were enrolled without oedema (Figure 4.11).

Figure 4.10: Anthropometry

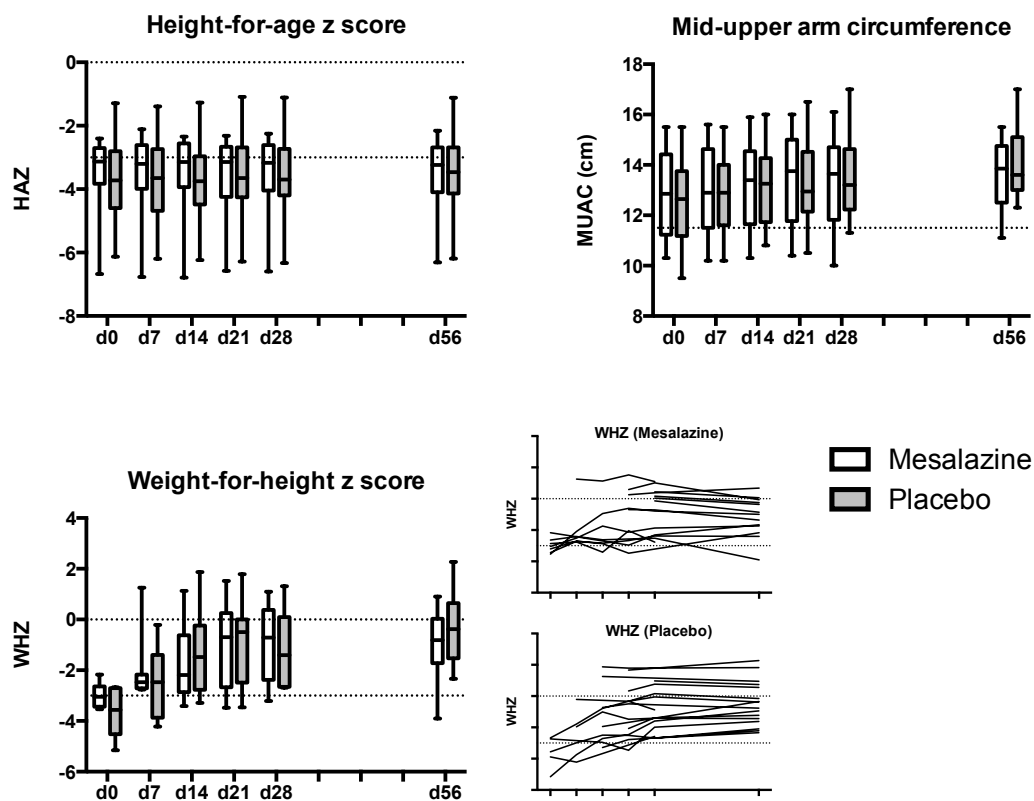


Figure 4.10: Height/length-for-age z-score (HAZ), mid-upper arm circumference (MUAC) and weight-for-height/length z-score (WHZ) by arm. $P > 0.05$ (by Mann Whitney U test) at all timepoints. Boxes enclose inter-quartile range, whiskers enclose range, median is plotted. Dotted lines at 0 and -3 (HAZ – indicating normal and severe stunting, respectively); 11.5 (MUAC – indicating severe acute malnutrition); 0 and -3 (WHZ 0 indicating normal and severe wasting). Although WHZ increases during the course of the study, this is partly due to the fact that children with kwashiorkor, who sometimes do not have (very) low lean mass are included only once their oedema resolves. Separating the WHZ data down to individual participants reveals how few datapoints make up the earlier timepoints (smaller graphs, bottom right).

Figure 4.11: MUAC in kwashiorkor

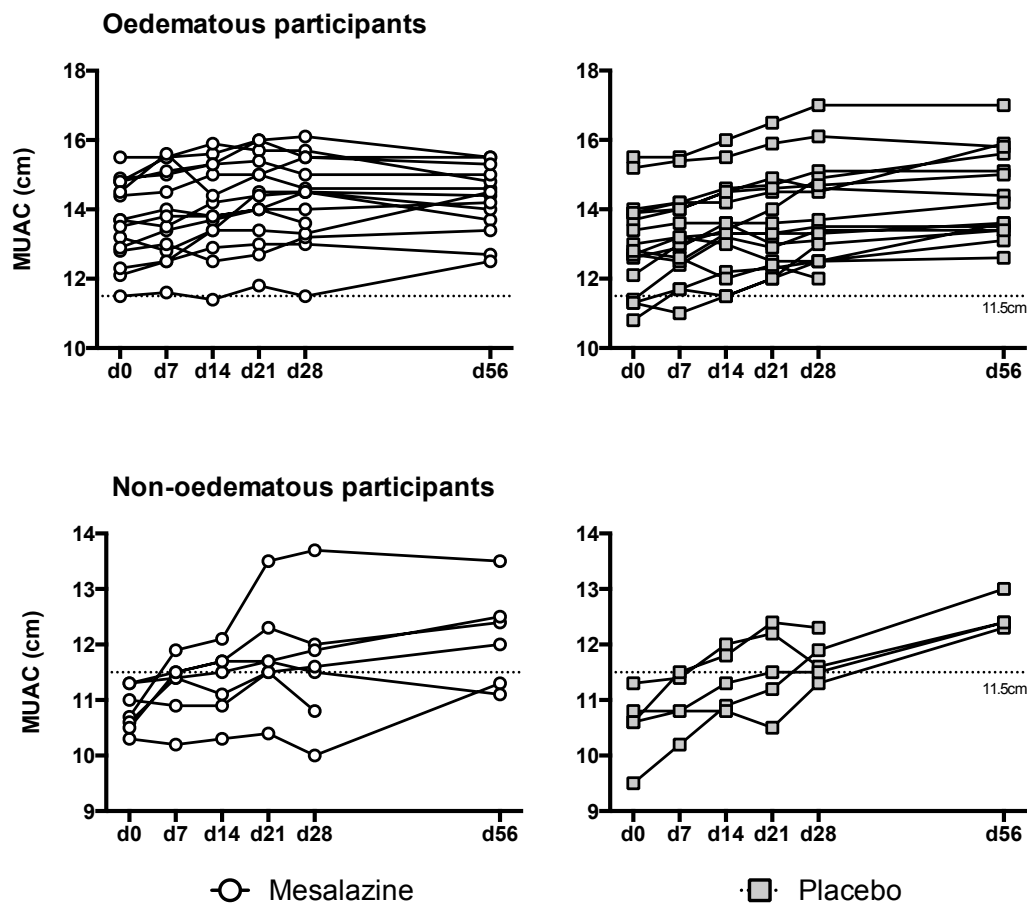


Figure 4.11: MUAC over the course of the study stratified by whether or not the participants were oedematous at enrolment and allocation arm. Amongst those who were not oedematous (i.e. who were enrolled on the basis of low MUAC), most had satisfactory improvements in MUAC by day 28. Individual participant level increases in MUAC in oedematous children were particularly pronounced in the placebo arm, but this is difficult to interpret because the presence of oedema fluid artificially increases MUAC.

IGF-1 increased from enrolment to day 28 in both arms, and was maintained to day 56 (Figure 4.12). IGF-1 concentrations at day 28 correlated with rate of height growth both to day 28 and day 56 (Figure 4.12).

Figure 4.12: Insulin-like growth factor-1

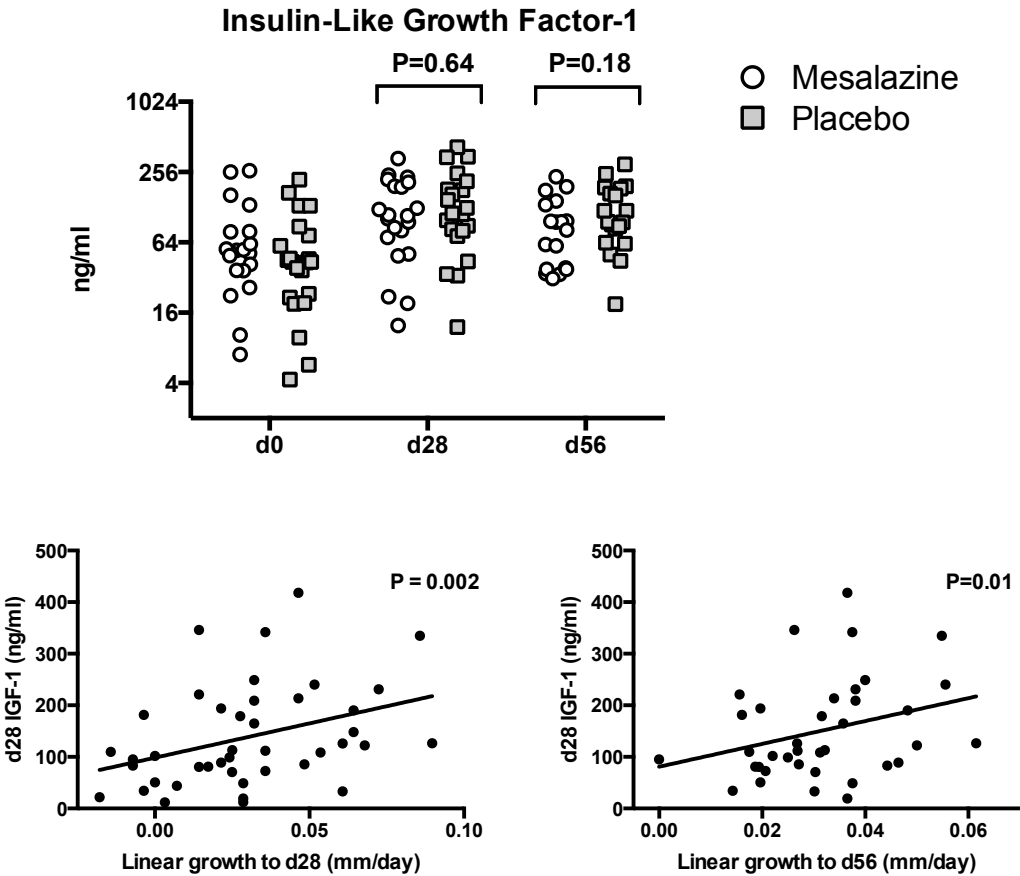


Figure 4.12: Insulin-like growth factor-1 (IGF-1) increased over the course of the study. IGF-1 concentrations at day 28 correlated with linear growth rate from enrolment to day 28 (bottom left), and from enrolment to day 56 (bottom right).

Enrolment IGF-1 concentration had strong inverse correlation with a number of inflammatory mediators (e.g. CRP, $P=0.008$), which was less apparent at the later time points (Figure 4.13).

Figure 4.13: IGF-1 and inflammation

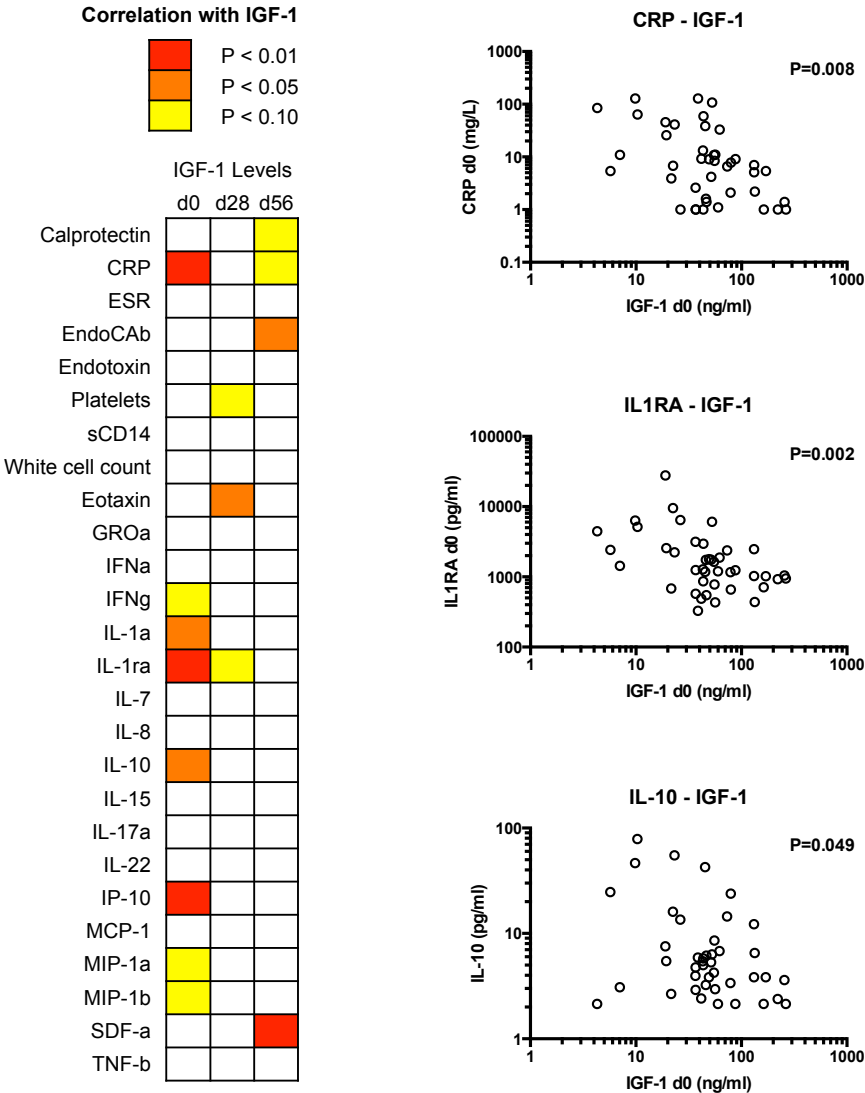


Figure 4.13: Chart shows participant-level correlations between Insulin-like growth factor-1 (IGF-1) and inflammatory/immunologic indices at each of the three timepoints. Three examples are provided (right), showing inverse correlation at enrolment. CRP (C-reactive protein), ESR (erythrocyte sedimentation rate), EndoCAb (IgG anti-endotoxin core antibodies) sCD14 (soluble CD14), GROa (growth regulated oncogene- α), IFN (Interferon), IL (Interleukin), IL-1ra (Interleukin-1 receptor antagonist), IP-10 (interferon- γ induced protein-10), MCP (monocyte chemotactic protein), MIP (macrophage inflammatory protein-1 α), SDF (stromal cell-derived factor), TNF (tumour necrosis factor).

4.2.4 Inflammatory indices

Intestinal inflammation was evident in most children at enrolment, with 43 (97%) having faecal calprotectin higher than 100 µg/g²³⁵. Systemic inflammatory activation was evident, which declined during the course of the trial. Point estimates of most of the clinically important or gut-specific inflammatory markers were lower in the mesalazine arm on completion of treatment at day 28 (Figure 4.14). Differences between the arms were not sustained to day 56. Cytokine concentrations in plasma were similar between the arms at all time points (Figure 4.15).

Figure 4.14: Inflammatory indices

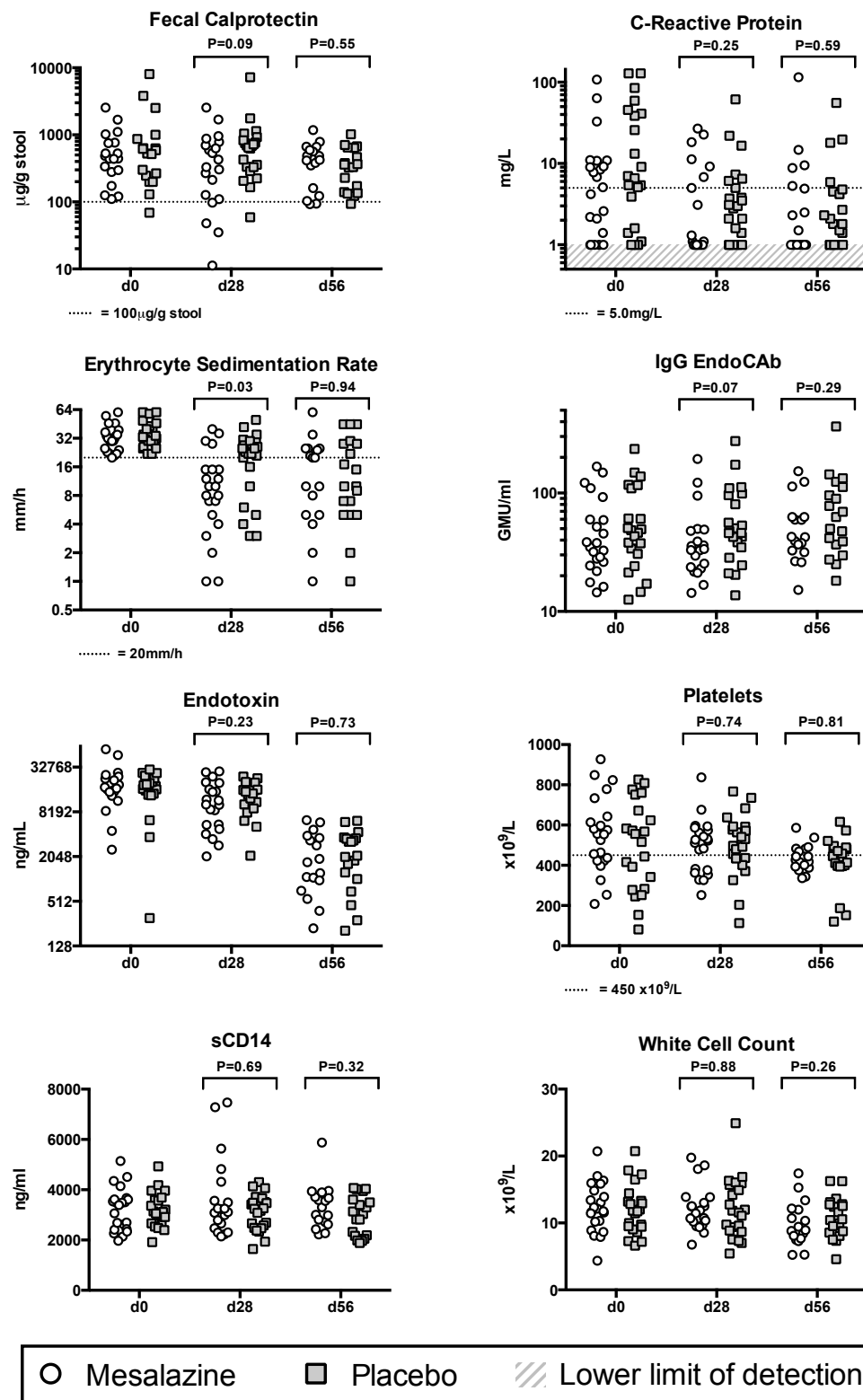


Figure 4.14: Important inflammatory indices by arm. EndoCAb (IgG anti-endotoxin core antibodies) sCD14 (soluble CD14)

Figure 4.15: Cytokines/chemokines

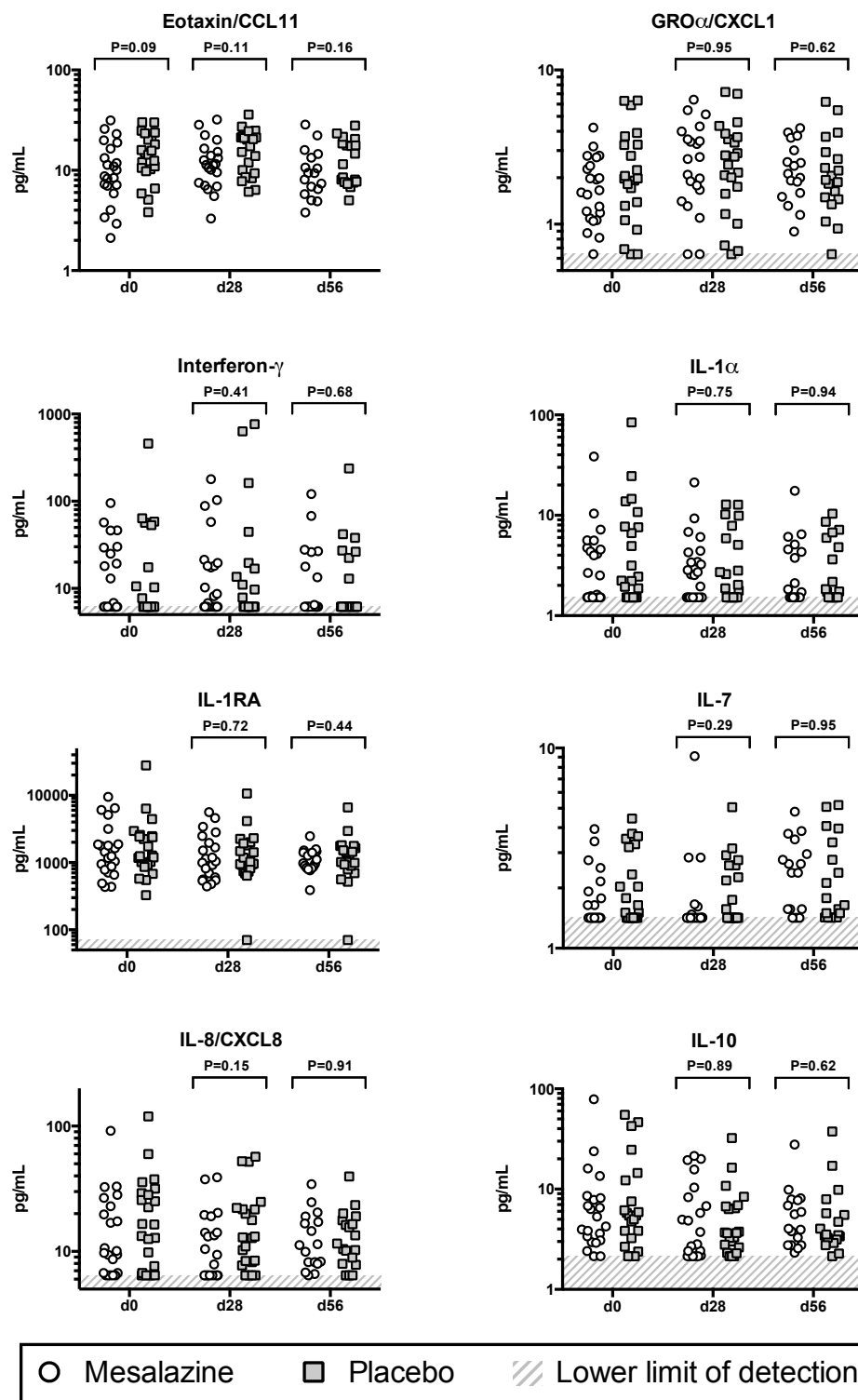


Figure 4.15: Cytokines by arm. CCL (chemokine (C-C motif) ligand), GRO (growth regulated oncogene), CXCL (chemokine (C-X-C motif) ligand), IL (Interleukin), IL-1RA (IL-1 receptor antagonist)

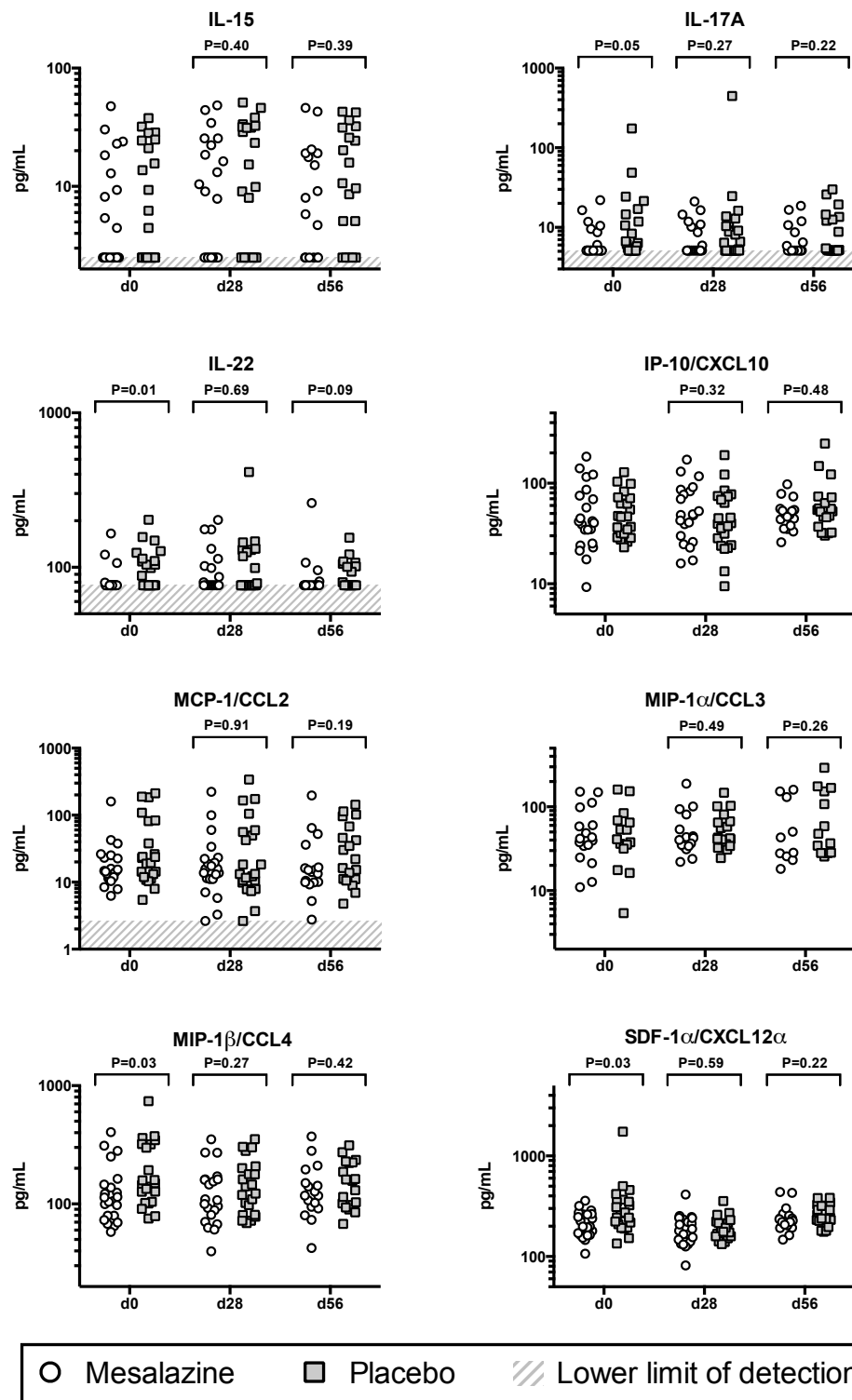


Figure 4.15 (continued): Cytokines by arm. IL (Interleukin), CCL (chemokine (C-C motif) ligand), CXCL (chemokine (C-X-C motif) ligand), MCP (monocyte chemotactic protein), MIP (macrophage inflammatory protein-1 α), SDF (stromal cell-derived factor).

4.3 DISCUSSION

This study represents the first controlled trial of a directly immunomodulatory agent in severe acute malnutrition (SAM). Despite being well enough to be eligible for outpatient management, intestinal and systemic inflammatory activation was very common amongst participants. Levels of faecal calprotectin at enrolment were higher than those reported from healthy populations living in either high-income countries or sub-Saharan Africa. They approached those seen in active inflammatory bowel disease (IBD).^{116, 248} Treatment with mesalazine was demonstrated to be safe. The frequency of acute illness episodes was similar between the interventional arm and placebo, and there was no excess gastrointestinal morbidity or diarrhoea that might suggest a disadvantageous effect on gut mucosal homeostasis. The trial was not designed to have sufficient power to detect an impact of the intervention on resolution of oedema, normalisation of acute malnutrition or linear growth. Individual growth trajectories revealed greater MUAC growth in the placebo arm to day 56. However, because oedema alters MUAC and most of the children in this study were oedematous at enrolment, the clinical significance of this finding is unclear.

In paediatric IBD, linear growth impairment correlates with cytokine-mediated depression of the GH-IGF-1 axis.²⁴⁹⁻²⁵¹ A similar effect was evident amongst participants in this trial, where baseline inflammatory variables were inversely associated with IGF-1. Children in the mesalazine arm experienced modest reductions in several clinically-relevant and gut-specific biomarkers compared to

those receiving placebo. If EED represented an essential level of inflammatory activation and immune-surveillance in the face of high pathogen burden, we would have expected the opposite result: that mesalazine would have been associated with a loss of control at the gut mucosal surface with increased levels of bacterial translocation and systemic immune activation and the development or intensification of gastrointestinal symptoms. Instead, these data have demonstrated that pharmacologically-mediated reduction in enteric inflammation is well tolerated, thereby providing the first clear evidence that EED is likely to be at least partly maladaptive and consequently a potential direct target for therapy. New therapies are urgently required because trials of nutritional support, hygiene-based interventions, probiotics, and empiric treatment for small intestinal bacterial overgrowth or specific pathogens have failed to show clinically significant improvements in linear growth or EED activity in children.^{67, 75, 119, 252} I propose that failure to control host-driven inflammatory activation may have been a barrier to efficacy of these interventions.

An important limitation of this study is that enteric inflammation was inferred on the basis of indirect blood or faecal markers, rather than direct measurement of intestinal structure or function. In 2013, an expert working group concluded that on the basis of myriad ethical and logistical challenges inherent in performing gastrointestinal endoscopy on young children living in poverty, that *“definition of environmental enteric dysfunction will need to rely on biomarkers alone, without biopsy data to connect functional and structural changes”*.⁶¹ The current ‘gold-standard’ for definition and stratification of EED is the lactulose-mannitol dual sugar

absorption test. Because this test takes several hours to complete, I considered that administering it would represent an excessive burden for patients and carers, especially since the schedule of visits was already relatively intensive. Most of the parents of acutely malnourished children in Mathare engage in casual work and the prospect of losing a morning or day's wage may have compromised attendance for follow-up visits. Since the primary outcome of this trial was assessment of safety, the risk of a functional assessment of EED jeopardising timely clinical review was not considered acceptable. Faecal calprotectin, haemoglobin, white cell and platelet counts, C-reactive protein and erythrocyte sedimentation rate were pre-defined as key biomarkers because of their role in IBD activity monitoring. EndoCAB, plasma endotoxin and sCD14 were chosen as markers of bacterial translocation; a key part of EED's pathobiology.

Although the EED has been conceived of primarily as a small intestinal enteropathy, the high levels of faecal calprotectin and systemic inflammatory activation demonstrated in this study are redolent of colonic inflammation.²⁵³⁻²⁵⁵ Biopsy studies of colonic architecture in children at risk of EED have not been reported. In view of the fact that sigmoidoscopy can be achieved more easily than either upper-GI endoscopy or ileocolonoscopy, without the need for either bowel preparation or deep sedation, if colonic inflammation were a component of EED then it might be amenable to serial biopsy-based monitoring in clinical trial settings.

The enrolment of children who were severely acutely was appropriate because children with SAM are at high risk of continued growth disturbance, illness and

death, despite provision of medical and nutritional care, which means that they have the most to gain from any new intervention. However, it may be that ensuring optimal capacity to respond to a new pathogen challenge takes precedence over optimising growth in the context of SAM, where vulnerability to major infection is intense. Targeting those EED in the context of moderate acute malnutrition or in non-acutely malnourished children would be likely to increase the chances of detecting any nascent IGF-1-mediated linear growth benefit. In this regard, a limitation of the study is that the inflammatory enteropathy found in kwashiorkor (which was present in 73% of those enrolled) may be qualitatively different to that present in the context of other forms of acute malnutrition, which may in turn be different to 'EED' where it occurs without acute malnutrition. Results of the current trial should not be considered generalisable either to children with stunting but without acute malnutrition or to populations of moderately or severely malnourished children without high prevalence of kwashiorkor, and future studies in such groups will need to take a similarly cautious and thorough approach as exemplified here.⁷⁷ That said, such studies should consider the use of more intensive treatment schedules because although mesalazine is a reasonable agent for maintenance of remission in IBD, it is less effective in induction. Difficulty in diagnosing tuberculosis in acutely malnourished children may preclude the use of systemic immunosuppressants, but longer courses or higher doses of mesalazine could reasonably be trialled. Furthermore, although EED occurs in all age groups, irreversible stunting mainly occurs in the first two years of life, and may even be apparent at birth.^{24, 79} Treatment prior to the development of clear evidence of growth failure may be optimal, but this requires the development and refinement of

point-of-care biomarkers for EED. Faecal calprotectin may be a suitable marker: point-of-care tests already exist, and evaluation against dual sugar absorption tests should be a research priority. The possibility of targeting short-term medical treatments towards this key early window of vulnerability to linear growth failure, which is also the peak period for development of acute malnutrition and major infectious diseases, means that clinical benefit for individual children might be possible even if modification of environmental determinants was minimal. This reflects social and political realities in many of the settings where EED is endemic.

These results have highlighted that hepatic parenchymal damage is reasonably common in uncomplicated SAM (8% of those screened had elevated liver enzymes in plasma) and that current management strategies may be associated with a transient exacerbation. Fatty infiltration of the liver is a recognised feature of kwashiorkor, and could be exacerbated by a sudden switch onto a high-triglyceride diet.²⁵⁶ That RUTF is safe and effective for use in the community is partly a factor of its high lipid content (which gives low water activity and therefore restricts bacterial growth) and it is likely that any risk associated with a modest short-term exacerbation of liver damage is outweighed by the proven benefits of community-based care. However, there may be a subset of patients in whom this presents a particular problem, especially as RUTF is starting to be used more commonly in the more-unwell group of hospitalised children with complicated SAM.

The participants involved in this trial represented a uniquely vulnerable population. Detection of a severely acutely malnourished child during community screening was

frequently an indicator of multiple social, nutritional and medical problems affecting the whole family. The provision of appropriate ancillary support was an essential component of our trial methodologies, made possible because of the comprehensive integration of medical and social services and long history of community engagement in Mathare by “German Doctors”, which made this setting a particularly appropriate one in which to conduct the trial. Where required, parents and siblings were provided with a daily food ration, free medical care and access to social support and advice. Study visits took place at participants’ home to minimise the opportunity cost of attending the clinic and community health workers performed regular home visits in order to provide support and assess for additional needs. The same benefits were afforded to all children screened for enrolment regardless of their eligibility.

In summary, this is the first controlled trial directly targeting the host inflammatory response in children with SAM. The trial is conceptually novel in considering EED as a maladaptive host response to environmental challenge, and has provided pilot evidence of safety. Larger trials will be needed to assess efficacy. The trend towards reduction in a range of inflammatory markers with mesalazine treatment encourages the design of such studies.

CHAPTER 5

Relationship between n-3 polyunsaturated fatty acid status and anthropometric indices in Kenyan children: a multi-centre case-control observational study

5.1 METHODS

This was a multi-centre case control observational study. The primary objective was to assess for differences in n-3 polyunsaturated fatty acid status between children with SAM and controls. The study recruited children from two settings in three phases. The first phase involved recruitment of children with SAM and hospital inpatient controls from Kilifi District (now County) Hospital (KCH) between May and September 2010. The second phase involved recruitment of children with SAM into the Njugu Plus trial (described in Chapter 6) at KCH between June 2012 and July 2013. Baseline data from the trial have been included in this analysis. The third phase involved recruitment of hospitalised SAM cases and outpatient controls from Mbagathi Hospital (MH) in Nairobi, between March and September 2012.

5.1.1 Study Settings

Kilifi County comprises a mostly rural subsistence-farming community and at least 60% of residents live below the national poverty line.²⁵⁷ KCH is a government referral facility with more than 5000 paediatric admissions per year to either a 54-bed ward or 10-bed high dependency unit where clinical care is supported by staff and funding from the Kenya Medical Research Institute (KEMRI)-Wellcome Trust Research Programme. Children with SAM are cared for in a dedicated bay where integrated

nutritional and medical management is provided. Uncomplicated SAM cases are managed at KCH's on-site outpatient therapeutic feeding program (OTP).

Mbagathi Hospital is a district general-equivalent facility (at the time this work was carried out, the only one) in central Nairobi. It lies near to Kenyatta National Hospital, the country's largest referral centre, but because of cheaper fees it is the main hospital utilised by most of the city's poor. Mbagathi has a 38-bedded paediatric ward that admits 4000 children under 5, annually, 30% of whom have SAM.

5.1.2 Study Participants

Formal inclusion and exclusion criteria were as follows:

Inclusion criteria, CASES:

- Children aged 6 months to 5 years old.
- Provision of informed consent.
- SAM (one or more of MUAC < 11.5 cm, WHZ/WLZ < -3, or bilateral pedal oedema).
- Completed 'stabilisation' phase of nutritional rehabilitation (if required).

Exclusion criteria, CASES:

- Known HIV disease or tuberculosis^{*}. (All participants received provider-initiated HIV testing and referral in the event of a positive test.)
- Requiring high-dependency level of care.
- Children being screened for enrolment to the Njugu Plus study were also ineligible if they had known allergy or hypersensitivity to ingredients found in RUTF or fish.

Inclusion criteria, CONTROLS:

- Children aged 6 months to 5 years old.
- Provision of informed consent.
- No severe acute malnutrition (any of MUAC < 11.5 cm, HHZ/WLZ < -3, or bilateral pedal oedema).
- Attending hospital for routine surgery, outpatient clinic appointment, minor illness or injury.

Exclusion criteria, CONTROLS:

- Known HIV disease or tuberculosis. (All participants received provider-initiated HIV testing and referral in the event of a positive test, as above.)
- Requiring high-dependency level of care.

^{*} Chronic infections, such as HIV and TB, and the drugs used to treat them, may alter lipid metabolism. Children with either of these conditions were excluded in order to limit heterogeneity and maximise statistical power. National guidelines recommend offering provider-initiated testing and counseling for HIV in all cases of SAM.

5.1.3 Screening and enrolment

Cases were identified by routine daily screening of all new admissions (to either KCH or MH) by a member of the study team. Information about the study was given to each eligible child's attending parent or carer and informed consent for participation was sought. Where consent was provided, cases were discussed with the attending clinical team in order to establish a mutually convenient time for venepuncture (in order that clinically required blood tests could be performed at the same time, to minimise discomfort to the participant). Cases who were enrolled in the Njugu Plus trial had venepuncture performed on the first day that they were ready to start for RUTF, as described in Chapter 6.

Controls were identified by opportunistic screening. In Kilifi, children being admitted to the hospital for routine surgery (e.g. hernia repair), injury (e.g. fracture) or illness without any WHO-indicated features of severity were given information about the study by KEMRI-Wellcome Trust Research Programme Clinical Officers as part of their routine admission procedure. They were then approached by a member of the study team in order that informed consent could be sought. In Nairobi, children attending the outpatient child-health clinic were screened for eligibility by Clinical Officers, and referred to the study team who provided information and sought informed consent.

Where consent was provided, participants were enrolled and venepuncture was performed at a convenient time in the next 24 hours (or, for those enrolled to Njugu

Plus, once they were ready to start RUTF). 2 to 4 ml of blood was taken into BD vacutainers using a needle and syringe. Where HIV status had not been previously documented, consent for testing was sought via a provider-initiated testing and counselling strategy endorsed by the WHO and Kenyan Ministry of Health, and the same sample was used for this purpose.

Baseline clinical and demographic features were recorded, and routine anthropometry was performed according to WHO and national guidelines. Comorbidities were defined according to WHO clinical syndromic diagnoses (diarrhoea and pneumonia) or a positive blood film with clinically apparent disease (malaria). A simple dietary recall questionnaire was administered (excluding participants enrolled in Njugu Plus), where carers were asked to estimate on how many days in an ordinary week the family consumed cows' milk, eggs, meat, and fish.

5.1.4 Laboratory Methods

5.1.4.i Separation of blood components for lipidomic analysis

Plasma and leukocyte fractions were extracted from whole blood by separation over a discontinuous density gradient created by layering Histopaque 1077 on top of Histopaque 1119 (Sigma-Aldrich Limited (Gillingham, UK)). After centrifugation at 700 g for 30 minutes, peripheral blood mononuclear cells (PBMCs) were reserved (and processed further in Njugu Plus, see Chapter 6), plasma was stored directly at -

80°C, and the red cell pellet (free of granulocyte contamination) was washed twice in phosphate buffered saline (PBS) and stored at -80°C.

5.1.4.ii Lipidomic analysis

Total lipid was extracted from the stored samples according to the method of Bligh & Dyer (with dichloromethane replacing chloroform), and dried under nitrogen.²⁵⁸ Plasma phosphatidylcholine was isolated using solid phase extraction on aminopropylsilica cartridges (Agilent Technologies (Santa Clara, USA)). Methyl esters were generated by incubation with methanol containing 2% H₂SO₄ and extracted into hexane following neutralisation as described previously.²⁵⁹ Analysis was performed on a Hewlett Packard 6890 gas chromatograph fitted with a BPX-70 column. Fatty acid methyl esters were identified using HPChemStation (Hewlett Packard (Palo Alto, USA)) by retention time compared to standard samples.

5.1.4.iii Other methods

Full blood count was processed by clinical laboratories at the KEMRI-Wellcome Trust Research Programme in Kilifi, or at the University of Nairobi's Kenyatta Hospital Campus in Nairobi.

5.1.5 Statistical Methods

Heterogeneity in clinical and demographic characteristics of the population was assessed by grouping according to recruitment strata (case or control), and location (Kilifi or Nairobi). Mann-Whitney U-tests and ANOVA (where number of groups >2)

were employed for analysis of continuous data, and Fisher's exact test for categorical data.

The primary objective was to assess for differences in n-3 polyunsaturated fatty acid status between children with SAM and controls, with DHA status in the erythrocyte fraction as the nominal primary outcome. Secondary outcomes were differences in other lipids and in both erythrocyte and plasma fractions, and secondary objectives were to assess for relationships between the raw compositional and anthropometric variables (including for HAZ/LAZ). In view of the fact that SAM comprises two distinct clinical syndromes (kwashiorkor and severe wasting) I also assessed for significant differences between them, and between each syndrome and controls.

Differences in fatty acid composition of erythrocyte and plasma phospholipid fractions were assessed at the individual species level using Mann-Whitney U-tests. For linear regression analyses, initial univariate analysis was performed with recruitment location, age, sex, current breastfeeding status (yes or no), haemoglobin concentration, white cell and platelet counts, MUAC, presence of oedema, WHZ/WLZ (only if non-oedematous), and HAZ/LAZ as independent variables and the individual fatty acid species as dependent variables. Where the univariate regression showed a significant relationship with $P < 0.05$, that variable was taken forward to a multivariate model. An exception was where oedema and WHZ/WLZ were both significant, when only WHZ/WLZ was included in the model (in view of the fact that measurement of WHZ/WLZ specifically excludes children with oedema). Z scores for fatty acid compositional indices were calculated for children with oedema where the

reference data (mean and standard deviation for normalisation equation) was calculated from all non-oedematous participants. Statistical significance of differences from the reference was assessed using Mann-Whitney U tests.

Throughout, analysis was performed in Stata (version 12.0) and graphs were drawn with GraphPad Prism (version 6.0). Anthropometric z-scores were calculated using WHO standards (2006).

5.2 RESULTS

5.2.1 Clinical and demographic characteristics

A total of 124 children were recruited to the study, 92 of whom had SAM. 89 were recruited from Kilifi and 35, from Nairobi. Baseline characteristics are summarised in Table 5.1.

Table 5.1: Baseline characteristics of the participants

	Kilifi Case	Kilifi Control	Nairobi Case	Nairobi Control	P (Group)	P (SAM-C)	P (Loc.)
n	77	12	15	20	-	-	-
Age (months)	18 (13 to 26)	23 (10 to 33)	13 (11 to 19)	25 (9 to 36)	0.19	0.26	0.74
Male sex (%)	41	5	7	12	0.75	1.00	0.84
MUAC (mm)	112 (106 to 119)	128 (124 to 138)	108 (100 to 114)	138 (130 to 143)	<0.001	<0.001	-
WHZ/WLZ*	-3.4 (-4.1 to -2.8)	-2.1 (-2.7 to -0.5)	-4.0 (-4.5 to -2.5)	-1.1 (-1.6 to -0.4)	<0.001	<0.001	-
Oedema (%)	13 (17)	0 (0)	5 (33)	0 (0)	-	-	-
HAZ/LAZ	-3.3 (-4.3 to -2.2)	-1.8 (-2.4 to -1.1)	-2.8 (-4.5 to -1.3)	-1.2 (-1.7 to -0.4)	<0.001	<0.001	<0.001
Diarrhoea	25 (37)	6 (50)	4 (27)	4 (20)	0.29	0.83	0.13
Pneumonia	7 (10)	2 (17)	7 (15)	4 (20)	0.01	0.79	0.02
Malaria	2 (12)**	4 (33)	1 (7)	0 (0)	0.02	1.00	0.04
Hb (g/dL)	9.1 (7.5 to 10.4)	8.9 (7.4 to 10.2)	10.0 (9.5 to 10.4)	11.0 (9.6 to 11.8)	0.05	0.18	0.01
WCC (x10 ⁹ /L)	10.8 (9.0 to 14.5)	12.4 (10.1 to 18.7)	9.4 (7.6 to 11.8)	9.7 (7.9 to 18.9)	<0.01	0.36	0.29
PMN (x10 ⁹ /L)	3.3 (2.1 to 4.2)	4.5 (3.2 to 9.8)	2.9 (2.2 to 4.9)	3.7 (2.5 to 5.7)	<0.01	0.03	0.80
Platelets (x10 ⁹ /L)	373 (203 to 513)	322 (197 to 570)	243 (111 to 317)	323 (258 to 669)	0.32	0.61	0.90

Table 5.1: Baseline characteristics of the participants. Statistical significance tests were conducted between all four groups (by ANOVA or Fisher's exact; P(Group)), between children with SAM and controls (by Mann-Whitney U test or Fisher's exact; P(SAM-C)), and by location in Kilifi or Nairobi (by Mann-Whitney U test or Fisher's exact; P(Loc.)). Because the proportion of cases and controls differed by location, significance of differences in indices of acute malnutrition between the locations were not assessed. *Excludes children with oedema; **of 17 children who had a blood film. MUAC (mid-upper arm circumference); WHZ/WLZ (weight-for-height/length z score); HAZ/LAZ (height/length-for-age z score); Hb (haemoglobin); WCC (white cell count); PMN (neutrophils).

The difference in acute nutritional status (MUAC and WHZ/WLZ) between cases and controls was more exaggerated in Nairobi than Kilifi. All of the Kilifi control children had one of diarrhoea, pneumonia or malaria compared to just 40% of Nairobi controls, reflecting recruitment from mainly inpatient versus outpatient populations, respectively. Children from Kilifi were generally more anaemic than those from Nairobi, and oedema was less common.

Despite major differences in acute nutritional status, there were no significant differences in family-level frequency of consumption of cows' milk, eggs, meat or fish between children with SAM and controls (Figure 5.1)

Figure 5.1: Family-level consumption of food items between cases and controls

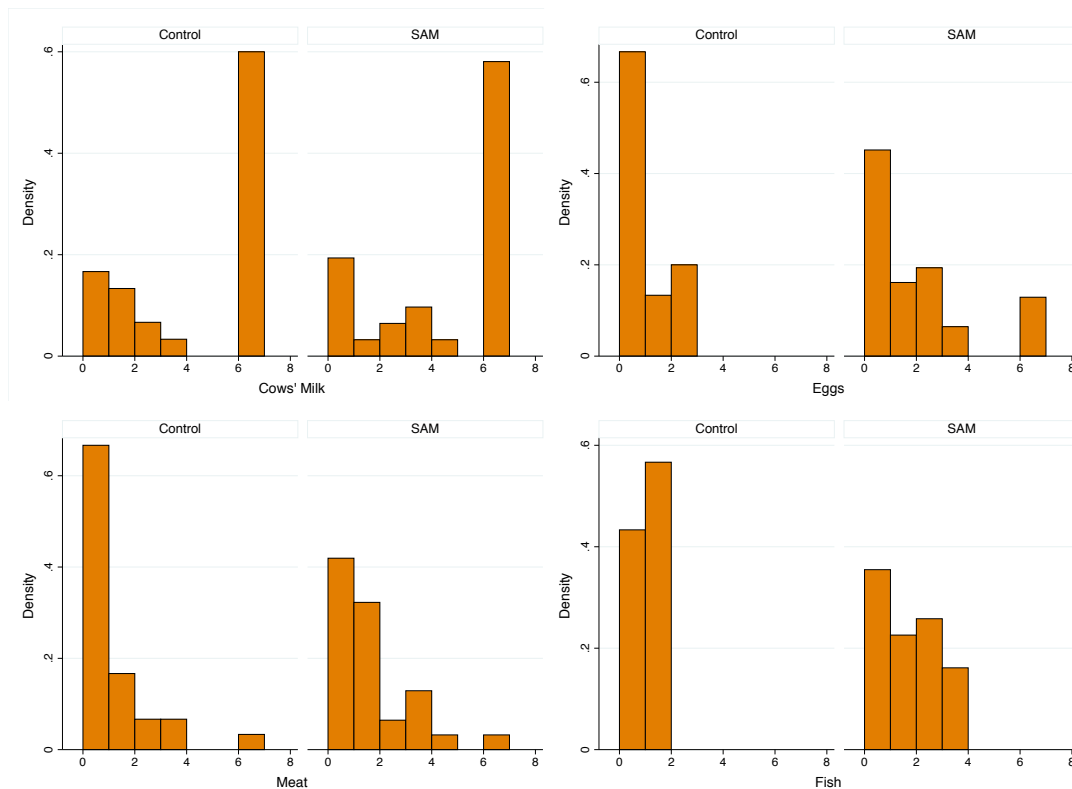


Figure 5.1: Histograms show no significant differences in family-level consumption of cows' milk (top left), eggs (top right), meat (bottom left) and fish (bottom right) between children with SAM and controls ($P > 0.1$ for all foods by Fisher's Exact). X-axes are estimated usual number of days per week on which the foodstuff is consumed by the family. $N=32$ for each group.

5.2.2 Fatty acid composition in SAM

The pattern of abundance of individual fatty acid species in erythrocyte and plasma phospholipid compartments was broadly similar between children with SAM and controls, though a number of significant point differences in abundance were evident (Table 5.2 and Figure 5.2).

Table 5.2: Percentage of fatty acid species in erythrocyte fractions and plasma phospholipid between children with SAM and controls

	Erythrocyte		Significance tests				Plasma		Significance tests			
Species (%)	SAM	Control	SAM-C	SW-K	SW-C	K-C	SAM	Control	SAM-C	SW-K	SW-C	K-C
14:0	0.50 (0.36 to 0.77)	0.53 (0.35 to 1.04)		0.03		0.09	0.38 (0.27 to 0.61)	0.44 (0.24 to 6.39)		<0.001	0.05	
16:0	25.9 (24.2 to 27.3)	26.0 (22.2 to 26.9)		0.02			32.9 (30.0 to 34.6)	31.8 (30.1 to 34.6)		<0.001		<0.01
16:1(n-7)	0.65 (0.42 to 0.96)	0.45 (0.31 to 0.65)	<0.01		<0.01	<0.01	0.57 (0.33 to 0.97)	0.58 (0.39 to 0.86)		<0.01		0.03
18:0	13.1 (12.3 to 14.5)	15.5 (13.7 to 18.0)	<0.001		<0.001	<0.001	13.2 (11.6 to 14.6)	13.9 (12.4 to 15.0)	0.09		0.08	
18:1(n-9)	15.8 (13.9 to 17.3)	15.3 (11.6 to 16.9)	0.08		0.05		14.7 (12.0 to 16.8)	12.2 (10.3 to 13.7)	<0.001		<0.001	0.02
18:1(n-7)	1.35 (1.14 to 1.55)	1.14 (1.01 to 1.44)	0.03		0.02		1.74 (1.32 to 2.16)	1.65 (1.32 to 1.91)		<0.01		
18:2(n-6)	11.6 (9.71 to 12.5)	9.87 (7.90 to 11.4)	<0.001	<0.001	<0.01	<0.001	20.5 (16.5 to 22.3)	15.5 (13.0 to 19.4)	<0.001	0.02	<0.001	
18:3(n-6)	0.10 (0.06 to 0.14)	0.08 (0.06 to 0.11)	0.08		0.04		0.10 (0.07 to 0.19)	0.09 (0.04 to 0.22)		<0.001		<0.01
18:3(n-3)	0.42 (0.23 to 0.69)	0.28 (0.17 to 0.48)	0.06	<0.001	<0.01		0.22 (0.11 to 0.50)	0.18 (0.08 to 0.55)		<0.001		0.02
20:0	0.76 (0.22 to 1.35)	0.38 (0.13 to 1.15)	0.07	0.08	0.02		0.16 (0.10 to 0.27)	0.25 (0.11 to 0.33)		<0.01	0.05	
20:1(n-9)	0.20 (0.16 to 0.25)	0.17 (0.12 to 0.25)				<0.01	0.19 (0.15 to 0.32)	0.17 (0.09 to 0.39)		<0.01		0.07
20:2(n-6)	0.35 (0.27 to 0.42)	0.28 (0.24 to 0.33)	<0.01	<0.01	<0.001		0.30 (0.23 to 0.39)	0.32 (0.22 to 0.38)				
20:3(n-9)	0.08 (0.06 to 0.12) *	0.07 (0.06 to 0.09) *			0.09		0.12 (0.08 to 0.21) *	0.16 (0.09 to 0.21) *				
20:3(n-6)	1.27 (1.06 to 1.48)	1.39 (1.21 to 1.81)	<0.01	0.04	0.03	<0.01	1.58 (1.21 to 2.08)	1.97 (1.32 to 2.61)	0.07	<0.01		<0.01
20:4(n-6)	13.6 (11.9 to 15.2)	14.6 (11.6 to 16.1)		<0.01		0.02	6.83 (5.06 to 8.84)	9.56 (6.93 to 11.8)	<0.001		<0.001	0.01
20:4(n-3)	0.10 (0.06 to 0.16)	0.09 (0.05 to 0.21)		<0.001		0.08	0.09 (0.06 to 0.14)	0.09 (0.07 to 0.13)		<0.01		
20:5(n-3)	0.32 (0.26 to 0.48)	0.36 (0.29 to 0.39)				0.03	0.38 (0.27 to 0.63)	0.45 (0.31 to 0.64)				
22:5(n-3)	1.65 (1.41 to 2.00)	1.61 (1.47 to 1.92)					0.67 (0.46 to 0.89)	0.71 (0.56 to 0.90)		<0.01		<0.01
22:6(n-3)	4.97 (3.65 to 6.66)	4.47 (3.74 to 6.23)					2.41 (1.19 to 3.51)	2.91 (2.29 to 4.05)	0.04			<0.001
24:0	1.04 (0.87 to 1.29)*	1.20 (1.03 to 1.43)*	0.04		0.04		0.25 (0.17 to 0.37)	0.42 (0.24 to 0.49)	<0.01		<0.01	0.08
24:1(n-9)	0.33 (0.27 to 0.40)*	0.31 (0.23 to 0.45)*					0.12 (0.09 to 0.19)	0.13 (0.11 to 0.19)		<0.01	0.07	

Table 5.2: Percentage of individual fatty acid species in erythrocytes and plasma phospholipid extracts between children with SAM and controls. Statistical significance tests were conducted between children with SAM and controls (SAM-C); between children with SAM defined on the basis of severe wasting without oedema and those defined on the basis of oedema (SW-K); between children with SAM defined on the basis of severe wasting without oedema and controls (SW-C); and between children with SAM defined on the basis of oedema and controls (K-C). All testing was via Mann-Whitney U tests, and P values are only displayed if $P < 0.1$. *n=75 (SAM) and 20 (control).

Figure 5.2: Fatty acid composition according to SAM syndrome

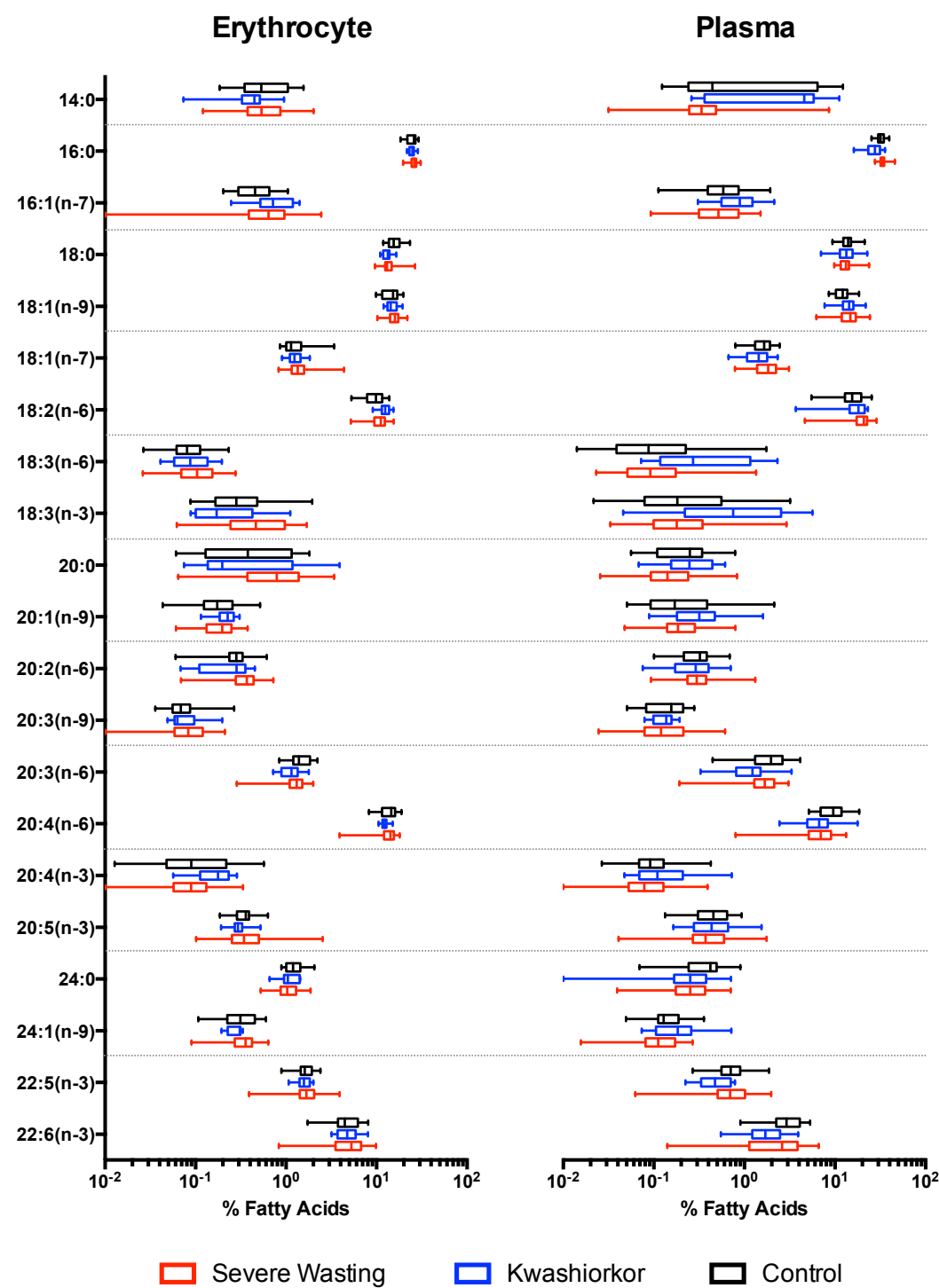


Figure 5.2: Percentage of individual fatty acid species in erythrocytes and plasma phospholipid extracts between children with severe wasting (SAM defined on the basis of severe wasting without oedema), kwashiorkor (SAM defined on the basis of oedema) and controls.

Lower levels some of the saturated fatty acid species were seen in children with SAM (18:0 and 24:0 in erythrocytes, 24:0 in plasma phospholipid), while unsaturated fatty acids tended to be more abundant: In the n-6 series, children with SAM had higher levels of 18:2(n-6) and 20:2(n-6) but lower 20:3(n-6) in erythrocyte fractions, and higher 18:2(n-6) in plasma phospholipid than controls. They had higher 16:1(n-7) and 18:1(n-7) in erythrocytes, and higher 18:1(n-9) in plasma phospholipid. The n-3 species were similar between the groups, though lower 22:6(n-3) was evident in plasma phospholipid from children with SAM.

A large number of statistically significant differences in fatty acid composition were evident between the two different SAM syndromes of severe wasting and kwashiorkor. The biological significance of these differences is unclear because there was inconsistency both within fatty acid series and between the erythrocyte and plasma phospholipid compartments. For example, for children with kwashiorkor, 18:2(n-6) was more relatively abundant in erythrocytes but less relatively abundant in plasma phospholipid compared to children with severe wasting. The opposite relationship existed for 20:3(n-6).

Differences in both the control and SAM populations between Kilifi and Nairobi further complicate the interpretation of these results (Figure 5.3).

Figure 5.3: Fatty acid composition according to SAM/control and location

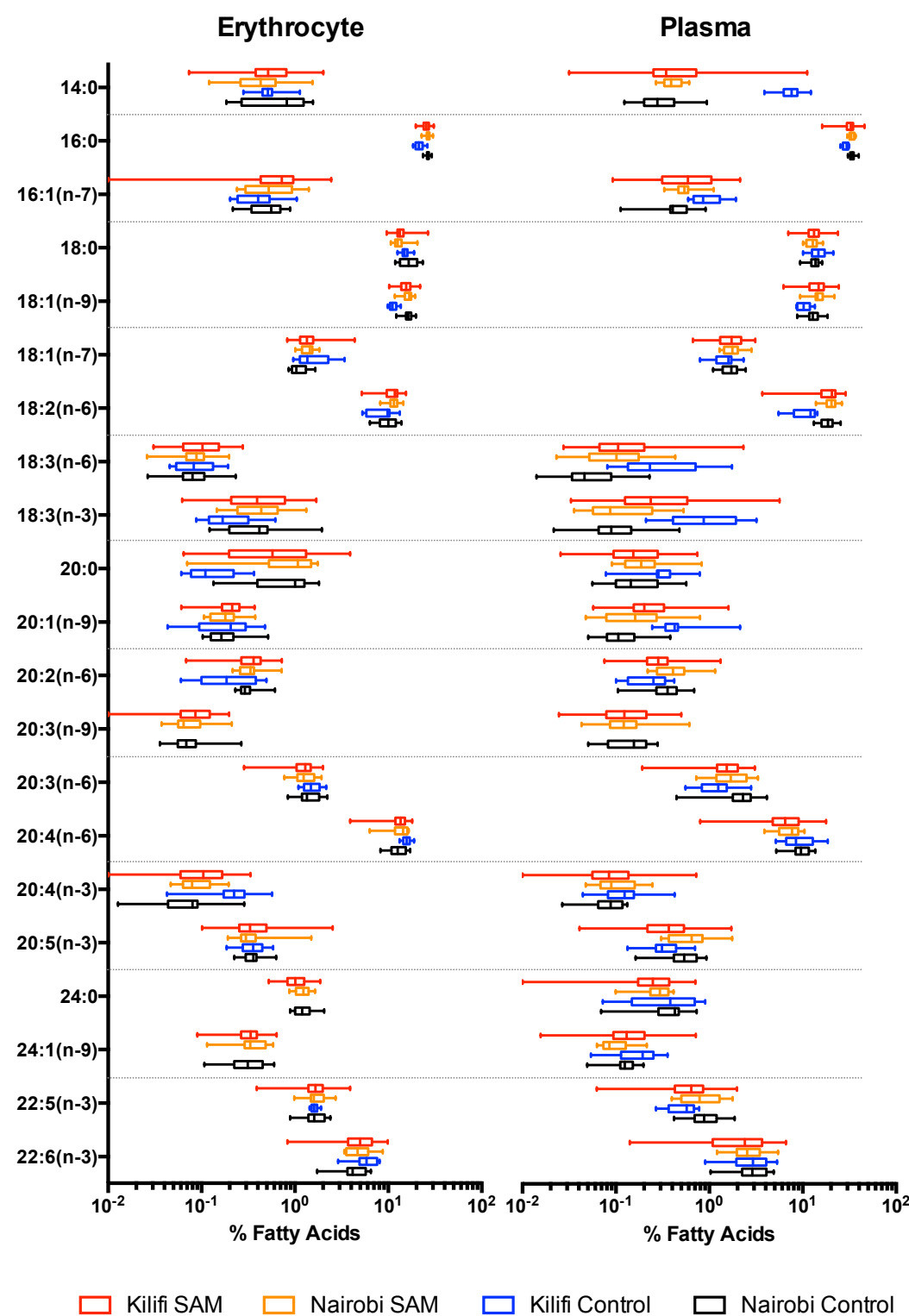


Figure 5.3: Percentage of individual fatty acid species in erythrocytes and plasma phospholipid extracts between children with SAM and controls recruited from Kilifi or Nairobi.

5.2.3 Desaturase and elongase enzyme activity in SAM

Desaturase and elongase enzyme activity was calculated on the basis of product/substrate ratios. Enzyme activity was similar between children with SAM and controls. Children with SAM had increased $\Delta 5$ desaturase activity and decreased elongase activity in the erythrocyte fraction, but there were no such differences evident in plasma phospholipid (Figure 5.4).

Figure 5.4: Desaturase and elongase enzyme activity in SAM

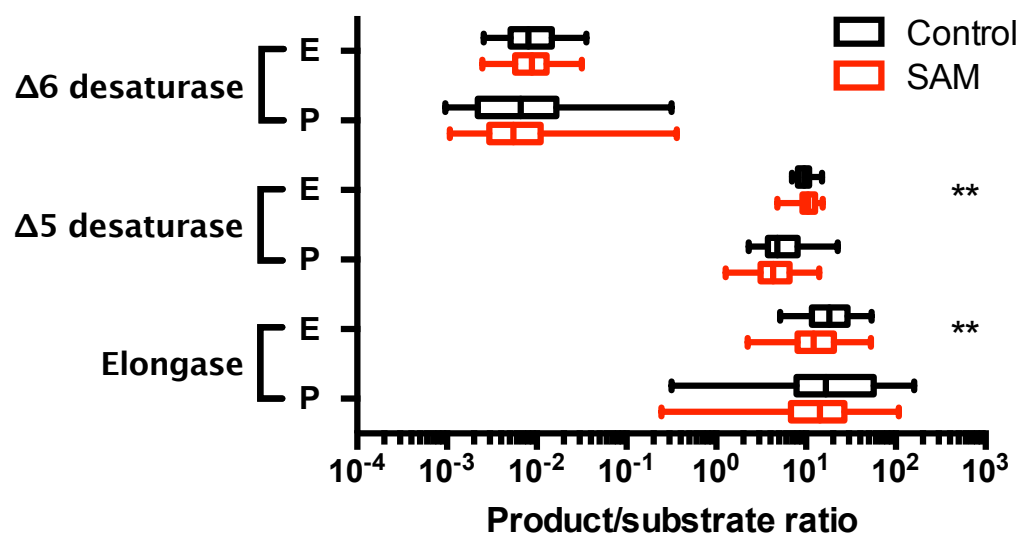


Figure 5.4: $\Delta 6$ desaturase calculated as $18:3(n-6)/18:2(n-6)$; $\Delta 5$ desaturase calculated as $20:4(n-6)/20:3(n-6)$; elongase calculated as $20:3(n-6)/18:3(n-6)$. Data shown for erythrocyte (E) and plasma (P) fractions. ** $P < 0.01$, all others $P > 0.05$

5.2.4 Predictors of fatty acid composition: regression analyses

In order to account for systematic differences between the two locations, and to resolve the potentially confounding impact of markedly different kwashiorkor rates between Kilifi and Nairobi, multiple linear regression analyses were performed to determine the predicting variables of individual fatty acids' relative abundance. The results are summarised in Table 5.3 (erythrocyte) and Table 5.4 (plasma phosphatidylcholine)

Table 5.3: Predictors of individual fatty acids' relative abundance in erythrocytes

Species (Erythrocyte)	Variable	Univariate		Multivariate		
		Coefficient	P	Coefficient	P	aR ²
14:0	Age	-0.000	0.03	-0.000	0.49	7.3
	Breastfeeding	0.190	<0.01	0.140	0.07	
	Oedema	-0.220	0.02	-0.170	0.07	
16:0	Location	-1.590	<0.01	-	-	-
16:1(n-7)	Age	0.003	<0.01	0.000	0.07	31.7
	Breastfeeding	-0.190	0.01	-0.118	0.25	
	Hb	-0.039	0.03	-0.023	0.23	
	Platelets	-0.001	<0.001	-0.000	0.03	
	MUAC	-0.053	0.03	-0.055	0.34	
	WHZ/WLZ	-0.570	0.02	-0.030	0.44	
	HAZ/LAZ	-0.093	<0.001	-0.053	0.11	
	Location	0.165	0.04	0.020	0.89	
18:0	MUAC	0.405	0.02	-0.052	0.85	9.2
	WHZ/WLZ	0.573	<0.01	0.509	0.05	
	Location	-1.350	0.02	-1.040	0.13	
18:1(n-9)	Breastfeeding	-1.000	0.03	-0.713	0.12	12.3
	HAZ/LAZ	-0.250	0.04	-0.359	<0.01	
	Location	-1.490	<0.01	-1.590	<0.01	
18:1(n-7)	Hb	-0.053	0.01	-0.042	0.06	5.1
	HAZ/LAZ	-0.047	0.05	-0.026	0.35	
	Location	0.216	0.02	0.132	0.33	
18:2(n-6)	Age	0.001	0.02	0.001	0.20	23.4
	WCC	-0.101	<0.001	-0.830	<0.01	
	Oedema	2.110	<0.001	1.770	<0.01	
	HAZ/LAZ	-0.256	0.02	-0.170	0.10	
18:3(n-6)	Hb	-0.005	0.03	-0.005	0.10	10.2
	MUAC	-0.009	<0.01	-0.011	0.03	
	HAZ/LAZ	-0.007	0.01	-0.001	0.74	
	Location	0.023	0.04	0.001	0.98	
18:3(n-3)	Age	-0.000	0.04	-0.000	0.25	4.0
	MUAC	-0.055	0.03	-0.042	0.12	
	Oedema	-0.219	0.03	-	-	
20:0	Age	-0.000	0.02	-0.003	0.16	3.9
	MUAC	-0.095	0.04	-0.065	0.21	

20:1(n-9)	WCC	0.003	<0.01	0.003	<0.01	11.1
	Platelets	-0.000	0.03	-0.000	0.01	
20:2(n-6)	MUAC	-0.022	<0.01	-0.022	0.06	8.4
	Oedema	-0.089	<0.01	-	-	
	WHZ/WLZ	-0.021	<0.01	-0.005	0.63	
20:3(n-6)	Oedema	-0.213	0.01	-	-	-
20:4(n-6)	Platelets	0.004	<0.01	0.003	0.02	16.0
	Oedema	-1.340	0.05	-1.381	0.03	
	HAZ/LAZ	0.422	<0.01	0.326	0.02	
20:4(n-3)	Hb	-0.009	0.02	-0.002	0.49	28.8
	WCC	0.005	<0.001	0.005	<0.001	
	Oedema	0.057	0.01	0.069	<0.01	
	Location	0.053	<0.01	0.064	<0.01	
20:5(n-3)	Age	-0.000	<0.01	-0.000	0.08	9.6
	Sex	-0.128	0.01	-0.107	0.07	
	MUAC	-0.453	0.02	-0.027	0.18	
22:6(n-3)	Age	-0.002	<0.001	-0.001	0.02	28.9
	Breastfeeding	1.290	<0.001	0.781	0.04	
	Platelets	0.003	<0.001	0.002	0.04	
	HAZ/LAZ	0.300	<0.01	0.232	0.01	
24:0	Age	0.003	<0.001	0.000	0.03	21.7
	Sex	0.132	0.04	0.075	0.22	
	Breastfeeding	-0.216	<0.01	-0.131	0.06	
	MUAC	0.049	0.02	-0.038	0.14	
	Location	-0.187	<0.01	-0.235	<0.01	
24:1(n-9)	HAZ/LAZ	0.018	0.01	-	-	-

Table 5.3: Predictors of fatty acid abundance in erythrocyte compartment are listed if $P < 0.05$ in univariate analyses. Multivariate analyses were $P < 0.05$ are listed in **bold**. Adjusted R^2 for the multivariate analyses is provided. Note that where oedema and WHZ/WLZ were both predictors in the univariate analyses, oedema was not taken forward to the multivariate model because WHZ/WLZ specifically excluded those participants with oedema. MUAC (mid-upper arm circumference); WHZ/WLZ (weight-for-height/length z score); HAZ/LAZ (height/length-for-age z score); Hb (haemoglobin); WCC (white cell count); PMN (neutrophils).

Table 5.4: Predictors of individual fatty acids' relative abundance in plasma PL

Species (Plasma)	Variable	Univariate		Multivariate		
		Coefficient	P	Coefficient	P	aR ²
14:0	WCC	0.106	<0.01	0.110	<0.01	21.8
	Oedema	2.450	<0.01	2.800	<0.001	
	Location	2.080	<0.001	2.210	<0.01	
16:0	Oedema	-5.790	<0.001	-5.780	<0.001	32.5
	Location	-2.430	<0.01	-2.410	<0.001	
16:1(n-7)	Age	0.000	0.04	0.000	0.12	19.1
	Hb	-0.050	<0.01	-0.039	0.03	
	Platelets	-0.000	0.01	-0.000	0.06	
	Oedema	0.336	<0.01	0.329	<0.01	
	Location	0.220	<0.01	0.159	0.15	
18:0	Age	0.002	0.02	0.001	0.20	8.1
	Platelets	-0.003	0.03	-0.001	0.23	
	WHZ/WLZ	0.366	<0.01	0.334	0.02	
18:1(n-9)	Age	0.002	0.02	0.001	0.41	30.6
	Breastfeeding	-2.420	<0.001	-2.910	<0.01	
	WCC	-0.099	0.03	-0.043	0.31	
	Platelets	-0.004	0.03	-0.002	0.25	
	MUAC	-0.680	<0.01	-1.094	0.03	
	WHZ/WLZ	-0.662	<0.01	-0.090	0.80	
	HAZ/LAZ	-0.655	<0.001	-0.173	0.54	
18:1(n-7)	Oedema	-0.355	<0.01	-	-	-
	WHZ/WLZ	-0.102	<0.01	-	-	
18:2(n-6)	WCC	-0.144	0.03	-0.118	0.06	6.2
	HAZ/LAZ	-0.555	0.02	-0.517	0.06	
18:3(n-6)	Oedema	0.455	<0.001	0.454	<0.001	21.1
	Location	0.176	0.02	0.174	0.01	
18:3(n-3)	Oedema	1.090	<0.001	1.090	<0.001	23.8
	Location	0.550	<0.01	0.550	<0.01	
20:1(n-9)	Oedema	0.149	0.03	0.150	0.03	6.6
	Location	0.124	0.02	0.120	0.02	
20:2(n-6)	Age	-0.000	<0.01	-0.000	<0.01	12.7
	Location	-0.101	<0.01	-0.100	<0.01	
20:3(n-6)	Platelets	0.001	0.02	0.000	0.27	45.3
	MUAC	0.097	0.03	-0.110	0.10	

	Oedema	-0.488	<0.01	-	-	
	WHZ/WLZ	0.113	<0.01	0.082	0.16	
	Location	-0.553	<0.001	-0.452	0.03	
20:4(n-6)	Age	-0.002	0.03	-0.001	0.37	20.5
	WCC	0.116	<0.01	0.084	0.02	
	Platelets	0.004	0.01	0.002	0.25	
	MUAC	0.509	<0.01	0.310	0.28	
	HAZ/LAZ	0.612	<0.001	0.440	0.06	
20:4(n-3)	Oedema	0.091	<0.01	-	-	-
20:5(n-3)	HAZ/LAZ	0.047	<0.01	0.036	0.03	8.7
	Location	-0.188	<0.01	-0.150	0.03	
22:5(n-3)	Platelets	0.000	0.01	0.000	0.08	16.8
	Oedema	-0.277	<0.01	-0.217	0.02	
	HAZ/LAZ	0.055	<0.01	0.020	0.34	
	Location	-0.260	<0.01	-0.290	<0.01	
22:6(n-3)	Age	-0.002	<0.001	-0.001	0.08	38.8
	Breastfeeding	1.190	<0.001	0.761	0.01	
	WCC	0.054	<0.01	0.031	0.05	
	Platelets	0.002	<0.01	0.001	0.20	
	Oedema	-0.867	0.02	-0.430	0.21	
	HAZ/LAZ	0.309	<0.001	0.240	<0.01	
24:0	MUAC	0.038	<0.001	0.005	0.77	14.0
	WHZ/WLZ	0.041	<0.001	0.036	0.01	
	HAZ/LAZ	0.021	0.01	0.008	0.41	
24:1(n-9)	Oedema	0.081	<0.001	0.081	<0.001	13.5
	Location	0.040	0.02	0.040	0.02	

Table 5.4: Predictors of fatty acid abundance in plasma phospholipid (PL) compartment are listed if $P < 0.05$ in univariate analyses. Multivariate analyses were $P < 0.05$ are listed in **bold**. Adjusted R^2 for the multivariate analyses is provided. Note that where oedema and WHZ/WLZ were both predictors in the univariate analyses, oedema was not taken forward to the multivariate model because WHZ/WLZ specifically excluded those participants with oedema. MUAC (mid-upper arm circumference); WHZ/WLZ (weight-for-height/length z score); HAZ/LAZ (height/length-for-age z score); Hb (haemoglobin); WCC (white cell count); PMN (neutrophils).

Overall, presence of oedema and location of recruitment were the variables that were most frequently associated (to statistical significance at $P<0.05$) with fatty acid content in the multivariate analyses. Oedema was associated with decreased levels of the longest and most extensively unsaturated n-6 and n-3 family members with corresponding increases in the other family members, with the exception of 18:3(n-3), which was lower for oedematous children in the erythrocyte fraction (Figure 5.4).

Figure 5.5: Fatty acid composition for oedematous participants compared to others

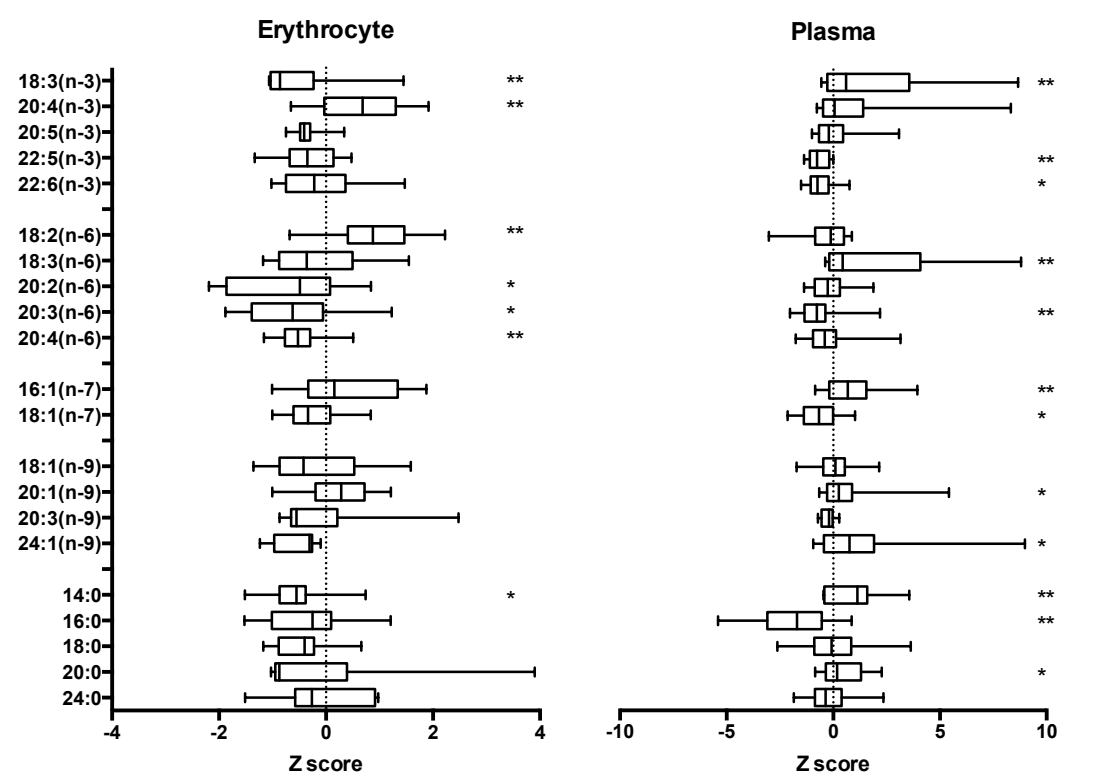


Figure 5.5: Data were normalised to individual species-level mean/standard deviation for non-oedematous participants and are presented as z scores for oedematous children compared to this reference. * $P<0.05$; ** $P<0.01$ by Mann-Whitney U tests

WHZ/WLZ and MUAC, the other indices of acute nutritional status along with presence of oedema, were associated with fatty acid content much less frequently. There were no significant associations on multivariate analysis in the erythrocyte pool. In the plasma phospholipid, more-negative WHZ/WLZ was associated with lower 18:0 and 24:0 saturated fatty acids, and higher 18:1(n-7). Low MUAC was associated with higher 18:1(n-9).

Recruitment of children across a range of HAZ/LAZ allowed me to assess the impact of stunting on fatty acid composition. In both the erythrocyte and plasma compartments, a greater degree of stunting was associated with lower 22:6(n-3) (Figure 5.5). In erythrocytes it was also associated with lower 20:4(n-6), 18:1(n-9) and 24:1(n-9); and in plasma it was also associated with lower 20:5(n-3).

Figure 5.6: Association of HAZ/LAZ with fatty acid content

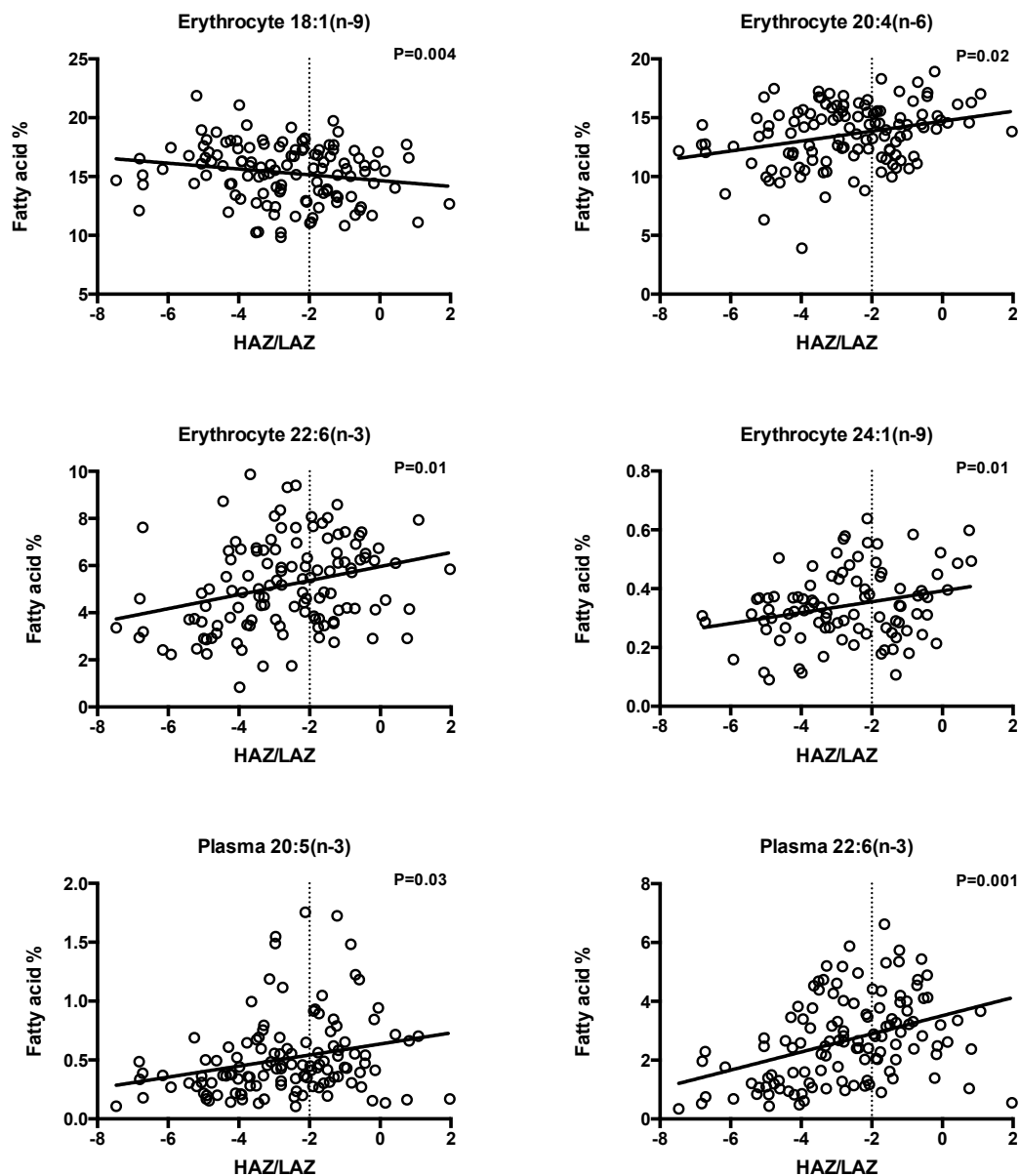


Figure 5.6: Relationship between fatty acid content and HAZ/LAZ. All fatty acids with $P < 0.05$ in multivariate analyses are shown. P values are the output of multivariate analysis but the uncorrected datapoints are plotted.

5.3 Discussion

This study is the largest to date to address fatty acid status in children with SAM, and one of the only studies to measure fatty acids in more than one compartment. The underlying hypothesis that children with SAM were likely to be deficient in long-chain polyunsaturated fatty acids (LC-PUFA) has not been proven. Whilst the key LC-PUFA, 20:4(n-6) (arachidonic acid, AA) and 22:6(n-3) (docosahexaenoic acid, DHA), were lower in plasma phospholipid, they were maintained in the erythrocyte compartment. LC-PUFA precursors, 18:2(n-6) and 18:3(n-3) were increased as a percentage of erythrocyte FA in children with SAM compared to controls. Since the definition of SAM imposes an arbitrarily-defined binary classification on WHZ/WLZ and MUAC, which are continuous variables, regression was used to increase the power to detect linear associations. MUAC was not associated with levels of any FA either in plasma PL or erythrocytes in multivariate analyses, and WHZ/WLZ was only associated with levels of two saturated FA (18:0 and 20:0) in plasma. However, there were several strong and consistent correlations between LC-PUFA status and HAZ/LAZ that suggest an association between LC-PUFA deficiency and stunting that may be of biological and public-health relevance.

A number of previous case-control studies have measured fatty acid status in malnourished children (Table 5.5 and Figure 5.7).^{178-185, 260-263} Results have been heterogeneous, likely reflecting differences in the definition of 'severe malnutrition', in the clinical characteristics of cases and controls, and in local dietary, breastfeeding and weaning practices.

Table 5.5: Fatty acids in SAM: research context & study characteristics

Study	Setting	Participants	Substrate	Notes
Taylor 1972	Nigeria	SM: 29 (all K) C: 30	Serum total FA	No data on setting/recruitment
Naismith 1973	Nigeria Hospital setting	SM: 20 (all K) C: 20 ('healthy')	Plasma total FA	No data on control recruitment
Holman 1981	Argentina Hospital setting	SM: 40 C: 48 (hospital controls)	Plasma PL, CE, TG	Reduced $\Delta 5$ desaturase activity. $\Delta 6$ desaturase and elongase normal (Conventional P/S ratios not reported)
Wolff 1984	Peru Hospital setting	SM: 44 (22 M, 22 K) C: 11 (recovered SM)	Erythrocyte FA, serum total FA	Increased $\Delta 5$ desaturase and $\Delta 6$ desaturase activity. Decreased elongation (Statistical significance testing not reported)
Chen 1985	Honduras Hospital setting	SM: 10 (all M) C: 20 (community controls)	Serum total FA	
Koletzko 1986	Nigeria Hospital setting	SM: 17 (8 M, 9 K) C: 8 (hospital controls)	Plasma CE, TG & PL	No significant differences between Marasmus and Kwashiorkor Large (non-significant) decrease in $\Delta 6$ desaturase activity. $\Delta 5$ desaturase and elongase normal
Vajreswari 1990	India Hospital setting	SM: 10 (4 M, 6 K) C: 17	Erythrocyte FA	Decrease in total desaturation/elongation pathway in SM
Leischenring 1992	Sudan Hospital setting	SM: 18 (10 M, 8 K) C: 20 (community controls)	Plasma CE & PL	Impaired $\Delta 5$ desaturase and $\Delta 6$ desaturase activities
Desci 1995	Romania Orphanage setting	SM: 35 (all M) C: 25 (community controls)	Plasma PL	19 of SM were HIV +ve Impaired $\Delta 5$ desaturase ($\Delta 6$ desaturase and elongase results not provided)
Smit 1997	Pakistan Hospital setting	SM: 21 (20 M, 1 K) C: 26 (hospital controls)	Erythrocyte FA	$\Delta 5$ desaturase, $\Delta 6$ desaturase and elongase activities normal
Franco 1999	Brazil Hospital setting	SM: 15 (5 M, 10 K) C: 8 (hospital controls)	Plasma total FA	
Houssaini 1999	Morocco Hospital setting	SM: 18 (all M) C: 15 (hospital controls)	Total plasma FA	$\Delta 6$ desaturase activity normal. Elongase increased
Jones 2015	Kenya Hospital setting	SM: 92 (74 M, 17 K) C: 32 (hospital controls)	Erythrocyte FA, plasma PL	$\Delta 6$ desaturase normal, $\Delta 5$ desaturase decreased and elongase decreased (erythrocyte); all normal in plasma

Table 5.6: Previous studies on fatty acid status in severely malnourished children: study characteristics. C (control); CE (cholesterol esters); FA (fatty acids); K (kwashiorkor); M (marasmus); PL (phospholipid); SM (severe malnutrition); TG (triglyceride);

Figure 5.7: Fatty acids in SAM: research context & study results

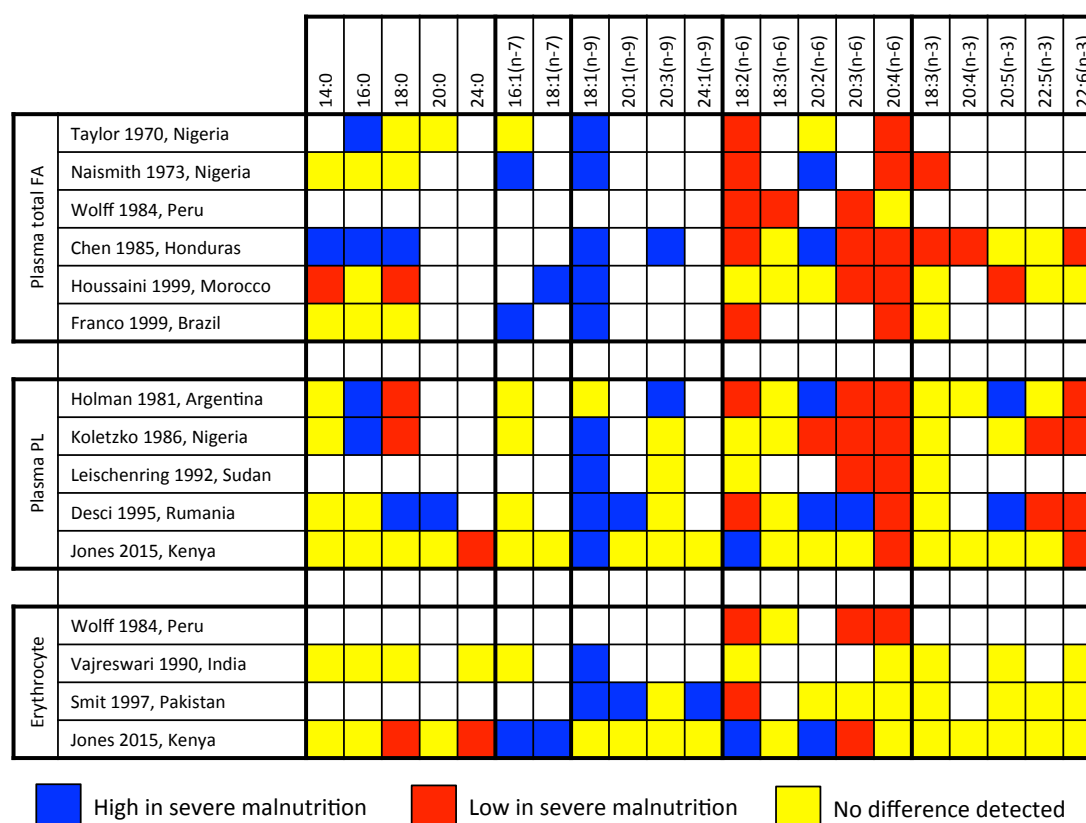


Figure 5.7: Previous studies on fatty acid status in severely malnourished children: study results. Considered 'high' or 'low' in comparison to controls (see Table 5.6) only where $P < 0.05$ (if documented). Blank cells indicate results not measured/reported.

The most commonly reported differences between children with SAM and controls are a relatively increased 18:1(n-9), and decreased 18:2(n-6), 20:3(n-6) and AA. Low DHA was also reported in half of those studies that measured it.

18:1(n-9) (oleic acid) is the most abundant fatty acid in human adipose tissue.²⁶⁴ In the fasted state, fatty acids are released from adipose tissue triglyceride into the circulation as non-esterified fatty acids (NEFA), under the control of hormone-sensitive lipase. NEFA can be metabolised to produce energy or utilised in the generation of structurally integral lipid moieties such as phospholipid. Under conditions of prolonged fasting and starvation, NEFA content of plasma rises as they represent an increasingly important energy source.²⁶⁵⁻²⁶⁷ FA release from adipose tissue TAG into plasma NEFA is selective, favouring shorter chain and more unsaturated FA.²⁶⁸ 18:1(n-9), being relatively long and only monounsaturated, is not preferentially released and in basal conditions the proportional NEFA content is lower than that in adipose tissue TAG. However, during progressive starvation in experimental models, exhaustion of more preferentially-released FA in adipose tissue leads to an increase in 18:1(n-9) content of NEFA, which, in turn, leads to enrichment in other compartments.²⁶⁹ Such effects are also seen in humans, for example in the context of anorexia nervosa, where elevated 18:1(n-9) content of plasma and erythrocyte lipids is well documented.^{270, 271} In the current study, 18:1(n-9) was elevated in plasma phospholipid in the context of SAM, and had a strong inverse correlation with MUAC. MUAC reflects both fat and lean mass, and a potential limitation of the study is the absence of a specific index of fat mass which, according to the rationale above, ought to be the more important determinant of 18:1(n-9) mobilisation and detection in NEFA and phospholipid.²⁷²

The study may have been underpowered to detect its primary outcome. As currently designed it had power of approximately 0.6 to detect a 20% difference in erythrocyte DHA between cases and controls, 138 children with SAM would have

been required to give power of 0.8. However, the clinical or public health significance of such relatively small differences is not questionable, especially since differences in DHA between the groups were inverted in the plasma versus erythrocyte fractions.

Stunting is a major global cause of neurodevelopmental and cognitive delay in young children.^{22, 273} The association between DHA status and HAZ/LAZ seen in this study is provides a potential mechanism for this effect, because sub-optimal DHA status has been associated with similar deficits.²⁷⁴ However, the targeted enrolment of children with SAM means that the anthropometric characteristics of this cohort are very different to that of the wider population. As shown in figure 5.8, while 59.8% of participants were acutely malnourished with WHZ/WLZ < -2 (excluding kwashiorkor), population-based survey data from Coast Province (which includes Kilifi) and Nairobi give prevalences of 4.5% and 2.5%, respectively.²⁷⁵ Therefore the association may not be directly applicable to more representative samples, may be subject to residual confounding, and should properly be considered exploratory at this stage. However, the finding argues for further study in more representative and 'healthy' populations.

Figure 5.8: Anthropometric characteristics of the study cohort

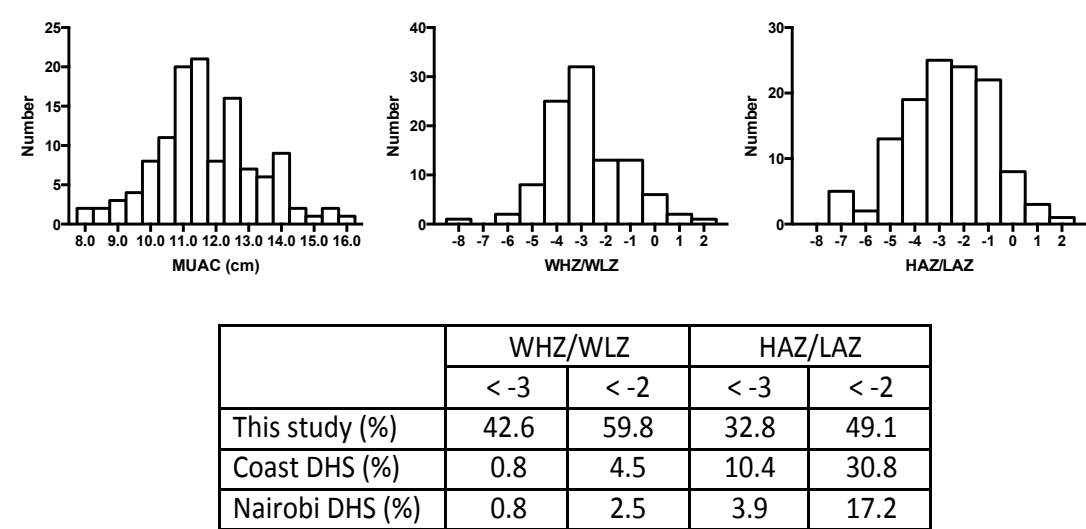


Figure 5.8: Histograms show the distribution of MUAC, WHZ/WLZ, and HAZ/LAZ amongst the 124 study participants. Comparison to population-based demographic and health survey data (2014) from Coast Province, Kenya, and Nairobi.

In summary, this study has failed to demonstrate clinically-relevant abnormalities in PUFA status in the context of SAM. The finding of an association between erythrocyte DHA content and HAZ/LAZ should be a subject to further study, because it may represent a mechanism underlying the association between stunting and impaired neurodevelopmental and cognitive status, and might be a target for therapeutic intervention.

CHAPTER 6

Ready-to-use therapeutic food with elevated n-3 polyunsaturated fatty acid profile, with or without fish oil, to treat severe acute malnutrition: a randomised controlled trial

This chapter contains data that have been reported previously:

Jones KDJ, Ali R, Khasira MA, Odera D, West AL, Koster G, Akomo P, Talbert AWA, Goss VM, Ngari M, Thitiri J, Ndoro S, Garcia MK, Omollo K, Ndungu A, Mulongo MM, Bahwere P, Fegan G, Warner JO, Postle AD, Collins S, Calder PC, Berkley JA. Ready-to-use therapeutic food with elevated n-3 polyunsaturated fatty acid profile, with or without fish oil, to treat severe acute malnutrition: a randomized controlled trial. BMC Medicine (2015) 13: 93

6.1 METHODS

This was a single centre, three-armed randomised controlled trial with balanced randomisation (1:1:1). Children with SAM received nutritional rehabilitation with either a standard RUTF (S-RUTF); flax seed oil-containing RUTF (F-RUTF); or flax seed oil-containing RUTF with additional fish oil capsules (FFO-RUTF). Flax seed oil provides the short-chain n-3 PUFA alpha-linolenic acid (18:3(n-3), ALA) while fish oil capsules provide the long chain n-3 PUFA eicosapentaenoic acid (20:5(n-3), EPA) and DHA (22:6(n-3)). The trial was conducted double-blind between the S-RUTF and F-RUTF arms, and open label with respect to FFO-RUTF.

6.1.1 Participants and setting

The study took place between June 2012 and July 2013 at Kilifi County Hospital (KCH) in Kenya. The study setting was described in Chapter 5. All children admitted to hospital and those presenting to the on-site OTP were screened for potential eligibility. Inclusion and exclusion criteria were as follows:

Inclusion criteria:

- Children aged 6 months to 5 years old.
- Provision of informed consent.
- SAM (one or more of MUAC < 11.5 cm, WHZ/WLZ < -3, or bilateral pedal oedema).
- Completed 'stabilisation' phase of nutritional rehabilitation (if required) and eligible to receive RUTF according to national guidelines.

Exclusion criteria:

- Known HIV disease, tuberculosis or chronic infection*
- Known allergy or hypersensitivity to any of the product ingredients.
- Fails 'appetite test' conducted according to national guidelines.¹⁶
- Requiring high-dependency level of care.

6.1.2 Intervention

I used linear programming analysis (using Microsoft Excel Solver) to design a novel

* Chronic infections, such as HIV and TB, and the drugs used to treat them, may alter lipid metabolism. Children with either of these conditions were excluded in order to limit heterogeneity and maximise statistical power. National guidelines recommend offering provider-initiated testing and counseling for HIV in all cases of SAM.

RUTF with increased short chain n-3 PUFA (ALA) content. The RUTF was based on a standard formulation produced by Valid Nutrition (Lilongwe, Malawi) with the addition of cold-pressed flax seed oil purchased from Seed Oil SA (Somerset West, South Africa). Gas chromatographic analysis of the final recipe (performed as described in Chapter 5) showed that n-3 PUFA, primarily ALA, comprised 3.3% of total energy content, compared to 0.7% in the standard formulation (similar to that found in Plumpy'nut, the most widely available brand of RUTF, see Table 6.1), and n-6 PUFA comprised 7.9% of total energy compared to 8.2% in the standard. Standard and flax seed oil-containing RUTF were packaged in identical 92 g sachets under nitrogen, and stored below 25°C for the duration of the study. The two recipes were organoleptically indistinguishable. Neither recipe contained any preformed n-3 LC-PUFA. Peroxidation of the RUTF was assessed by iodometric endpoint determination (ISO 3960: 2007).

Table 6.1: Measured RUTF fatty acid composition

	S-RUTF	F-RUTF	FFO-RUTF	<i>Plumpy'nut</i>	<i>Fish oil</i>
14:0	0.69	0.00	0.28	<i>0.75</i>	<i>7.94</i>
15:0	0.05	0.04	0.04	<i>0.05</i>	<i>0.00</i>
16:0	30.78	30.54	30.17	<i>28.82</i>	<i>18.58</i>
16:1 (n-7)	0.14	0.14	0.50	<i>0.16</i>	<i>10.23</i>
18:0	7.04	6.93	6.83	<i>3.61</i>	<i>3.64</i>
18:1 (n-9)	43.39	38.55	37.65	<i>48.57</i>	<i>11.30</i>
t18:1 (n-9)	0.00	0.78	0.76	<i>1.03</i>	<i>4.86</i>
18:2 (n-6)	14.89	14.40	13.97	<i>12.62</i>	<i>1.57</i>
18:3 (n-6)	0.08	0.13	0.14	<i>0.06</i>	<i>0.29</i>
18:3 (n-3)	1.32	6.23	6.05	<i>1.11</i>	<i>0.85</i>
20:0	0.47	0.56	0.54	<i>0.55</i>	<i>0.16</i>
20:1 (n-9)	0.34	0.45	0.54	<i>1.03</i>	<i>3.00</i>
20:2 (n-6)	0.03	0.01	0.01	<i>0.03</i>	<i>0.13</i>
20:3 (n-6)	0.00	0.00	0.00	<i>0.00</i>	<i>0.12</i>
20:4 (n-6)	0.50	0.84	0.85	<i>0.98</i>	<i>1.04</i>
20:4 (n-3)	0.00	0.05	0.13	<i>0.09</i>	<i>2.22</i>
20:5 (n-3)	0.00	0.00	0.73	<i>0.04</i>	<i>20.48</i>
24:0	0.29	0.31	0.30	<i>0.45</i>	<i>0.00</i>
24:1 (n-9)	0.00	0.02	0.02	<i>0.06</i>	<i>0.00</i>
22:5 (n-3)	0.00	0.00	0.07	<i>0.00</i>	<i>1.82</i>
22:6 (n-3)	0.00	0.00	0.42	<i>0.00</i>	<i>11.78</i>
n-6 total	15.50	15.38	14.97	13.68	3.14
n-3 total	1.32	6.28	7.40	1.24	37.14
n-6/n-3	11.72	2.45	2.02	11.05	0.08
n-6 %energy	8.21	8.15	7.93	7.25	
n-3 %energy	0.70	3.33	3.92	0.66	

Table 6.1: Relative fatty acid content (% of total) of S-RUTF, F-RUTF and Plumpy'nut (for comparison) measured by gas chromatography. Encapsulated fish oil content was measured and the compositional equivalent of a single sachet of F-RUTF plus 2 fish oil capsules is calculated as FFO-RUTF (likely to be an overestimate since capsules were pierced and the oil squeezed out for consumption rather than being consumed whole). WHO/UNICEF recommendations are for n-6 as 3.0-10.0% total energy and n-3, 0.3-2.5% total energy.

Standard or flax seed oil-containing RUTF was provided to children at a dose determined by weight according to Kenyan national guidelines until MUAC was > 11.5cm, WHZ/WLZ > -3, or oedema had resolved at two consecutive weekly visits (depending on which was the eligibility criteria). Parents were advised that no other foodstuff should be consumed during treatment of SAM apart from breast milk. After recovery from SAM, the RUTF product was provided for use in a supplementary fashion alongside family foods at 50% of the recommended daily therapeutic dose until completion of the study, 84 days after enrolment. RUTF has been safely used in a supplementary fashion before and the recommended compositions of therapeutic and supplementary lipid-based nutritional supplements are similar.^{99, 276} The dose provided during the supplementary phase was often higher than recommended in national guidelines, which stipulate one 92 g sachet per day regardless of body weight. However, provision of markedly different per-kg body weight doses during the study would have introduced additional variation of intake in relation to needs and would have decreased the power of the study.

Children enrolled to the third arm of the trial were provided with two 0.5 ml fish oil capsules, donated for use in the study by Seven Seas (Hull, UK), which provided 214 mg of EPA plus DHA at a ratio of 1.7:1.0 (with 4 International Units of vitamin E), for each 92 g sachet of (flax seed oil-containing) RUTF prescribed. Carers were

instructed to pierce the capsules with a safety pin and squeeze the oil into the child's mouth. This is the same technique used to deliver vitamin A to young children and was familiar and acceptable to participants. I calculated that participants in this arm would effectively receive 3.9% of total energy as n-3 PUFA, 18% of which would be as preformed EPA and DHA; 0.39% and 0.22% total energy, respectively, compared to a DHA recommended intake for infants of 0.10 to 0.18%²⁷⁷. This is likely to be an overestimate since piercing the capsule and squeezing into the participant's mouth was likely to preclude delivery of the entire contents.

6.1.3 Screening, enrolment and randomisation

Information about the study was given to each eligible child's attending parent or carer as soon as possible after presentation, and informed consent for participation was sought. Where consent was provided children were reviewed by a member of the study team daily, until the clinical attending team considered them medically stabilised and ready to start RUTF. At this point they were formally enrolled in the trial, given a study number (see below) and started on blinded standard or flax seed oil-containing RUTF with or without fish oil capsules according to the allocation arm. Participants who required ongoing inpatient care were reviewed by a member of the study team daily until discharge.

Standard and flax seed oil-containing RUTF was produced by Valid Nutrition in Lilongwe, Malawi. Each sachet was stamped with one of 18 indelible four-digit alphanumeric codes. 6 of the codes were designated to the standard recipe, and 12 to the flax seed oil-containing recipe (6 each for the arms with and without fish oil

capsules). Access to the allocation key was restricted to manufacturers and the trial statistician (Greg Fegan). A randomisation list was generated in Stata (version 12.0) with variable block sizes using the following code: *"ralloc blknum blksiz Rx, nsubj(60) ntreat(3)"*²⁴³. The trial statistician prepared 60 opaque envelopes labelled with study numbers, inside each of which was a card identifying a four-digit RUTF code and specifying 'with fish oil' or 'without fish oil'. When a participant was enrolled in the trial they were allocated the next consecutively available study number, which was entered on the allocation log prior to opening the relevant envelope.

Because of difficulties sourcing an appropriate placebo oil capsule, the FFO-RUTF arm was open label with respect to both the provision of fish oil capsules and flax seed oil-containing RUTF.

6.1.4 Monitoring

Scheduled study follow-up took place at days 7, 14, 21, 28, 56 and 84 after enrolment. RUTF and capsules were provided at each visit. Capsules were provided in an amber plastic bottle and were dispensed by weight. Compliance was monitored by interview with the parent or carer, counting full and empty sachets of RUTF and by reweighing the bottles containing fish oil capsules. Percentage compliance was calculated with reference to a 'full ration' taking account of the participant's weight and stage of treatment. During therapeutic feeding, additional RUTF was offered to be used after completion of the full prescribed ration if children were still hungry, in line with national guidelines. Blood samples were taken at enrolment and at days 7, 28 and 84. Monitoring for side effects or adverse events was conducted at all

scheduled and unscheduled visits. Participants' homesteads were mapped and defaulters were traced in the community.

6.1.5 Safety and reporting

All adverse events (AE) occurring in participants during the trial were documented and reported at the end of the trial. An independent data safety monitoring committee was not convened as this was a trial of a food, rather than a pharmaceutical intervention, and the main ingredients were in common use in Kenya. For the same reason, specific toxicities and adverse reactions were not pre-defined.

Safety reporting definitions.

6.1.5.i Adverse Event (AE)

Identical to the definition provided in 4.1.5.i. Briefly, an AE was any untoward medical occurrence, regardless of proven/suspected causal relationship with the investigational product. The definition includes any intercurrent illnesses, injuries or exacerbation of pre-existing conditions.

6.1.5.iii Serious Adverse Event (SAE)

Identical to the definition provided in 4.1.5.ii. Briefly, Any AE (whether or not considered related to the investigational product) that results in any of the following outcomes is defined as an SAE.

- Death
- Life-threatening event
- Persistent or significant disability or incapacity
- Hospitalisation: regardless of length of stay. Hospitalisation (including inpatient or outpatient hospitalisation for an elective procedure) for a pre-existing condition that has not worsened unexpectedly does not constitute an SAE.
- An important medical event that may, based upon appropriate medical judgment, jeopardise the subject and/or require medical or surgical intervention to prevent one of the outcomes listed above.

6.1.6 Outcomes

The primary outcome was erythrocyte n-3 PUFA content (percentage of the major species and n-6:n-3 PUFA content ratio) at day 84 measured by gas chromatography. Main secondary outcomes were safety and acceptability of the intervention, assessed by frequency of adverse events and compliance respectively. The study was not powered or designed to detect differences in rate of recovery or growth, but these data were collected and reported to inform future studies. A large number of other outcomes were measured in order to provide mechanistic insights relevant to future study design. Further analysis of fatty acid abundance in plasma phosphatidylcholine (at enrolment, day 28 and day 84) and T cells (at enrolment and day 84) was performed; inflammatory activation was assessed by measurement of a range of soluble mediators in plasma, Insulin-like growth factor-1 (IGF-1) provided an index of linear growth potential; T cell *in vivo* activation/exhaustion and response to

stimulation with mitogen and recall antigen was measured at enrolment and day 84; and biophysical properties of the erythrocyte membrane were assessed by response to shear stress. These outcomes were considered exploratory.

6.1.7 Laboratory Methods

6.1.7.i Separation of blood components for lipidomic analysis

Whole blood was collected into sodium heparin vacutainers (BD (Franklin Lakes, New Jersey, USA)). Plasma and leukocyte fractions were extracted by separation over a discontinuous density gradient created by layering Histopaque 1077 on top of Histopaque 1119 (Sigma-Aldrich Limited (Gillingham, UK)). After centrifugation at 700 g for 30 minutes, peripheral blood mononuclear cells (PBMCs) were reserved, plasma was stored directly at -80°C, and the red cell pellet (free of granulocyte contamination) was washed twice in PBS and stored at -80°C. CD3+ T cells were isolated from PBMCs by positive selection using CD3 MicroBeads and LS columns (Miltenyi Biotec (Bergisch Gladbach, Germany)) according to the manufacturer's instructions, and also stored at -80°C.

6.1.7.ii Lipidomic analysis

Total lipid was extracted from the stored samples according to the method of Bligh & Dyer with dichloromethane replacing chloroform, and dried under nitrogen.²⁵⁸ Plasma phosphatidylcholine was isolated using solid phase extraction on aminopropylsilica cartridges (Agilent Technologies (Santa Clara, USA)). For fatty acid analysis (erythrocyte and plasma phosphatidylcholine samples), methyl esters were generated by incubation with methanol containing 2% H₂SO₄ and extracted into

hexane following neutralisation as described previously.²⁵⁹ Analysis was performed on a Hewlett Packard 6890 gas chromatograph fitted with a BPX-70 column. Fatty acid methyl esters were identified using HPChemStation (Hewlett Packard (Palo Alto, USA)) by retention time compared to standard samples. For whole lipid analysis (CD3+ cells), samples were reconstituted in a mixture of dichloromethane, methanol, water, and concentrated ammonia (ratio 66:30:3:1), and introduced via direct infusion by nanoflow electrospray ionisation to a triple quadrupole mass spectrometer (xevo-TQ, Waters (Milford, USA)).²⁷⁸ Phosphatidylcholine was analysed in positive ionisation as precursors of 184+, and phosphatidylethanolamine was analysed in the neutral loss of 141+. Individual spectra were checked in MassLynx (Waters (Milford, USA)) for quality and analysed using a custom-designed macro.²⁷⁹

6.1.7.iii T cell activation

T cell activation phenotypes (CD3+ CD4/8+ CD38+ HLA-DR+ lymphocytes with/without PD-1 expression) were evaluated by flow cytometric analysis of fresh whole blood. Blood was collected into sodium heparin vacutainers (BD) and processed within 2 hours of blood draw. Whole blood was stained at room temperature for 20 minutes in the dark. Staining antibodies were anti-CD3 (V450), anti-CD4 (PerCP-Cy5.5), anti-CD8 (APC), anti-PD-1 (PECy7) (all BD), anti-CD38 (PE), anti-HLA-DR (FITC) (both Biolegend), and isotype controls (PE and PECy7 both against IgG1-kappa light chain from ebiosciences and Biolegend, respectively). Blood was lysed by addition of BD FACS Lysing buffer (1X) for 10 minutes, and washed three times in ice-cold PBS. Cells were fixed using BD Cellfix and read on a Beckman Coulter CyAn ADP the same day. BD CompBeads were used for compensation.

Analysis was done in FlowJo (provided free as part of the FlowJo for Africa scheme).

Gating strategy was as shown in Figure 6.1:

Figure 6.1: Gating strategy

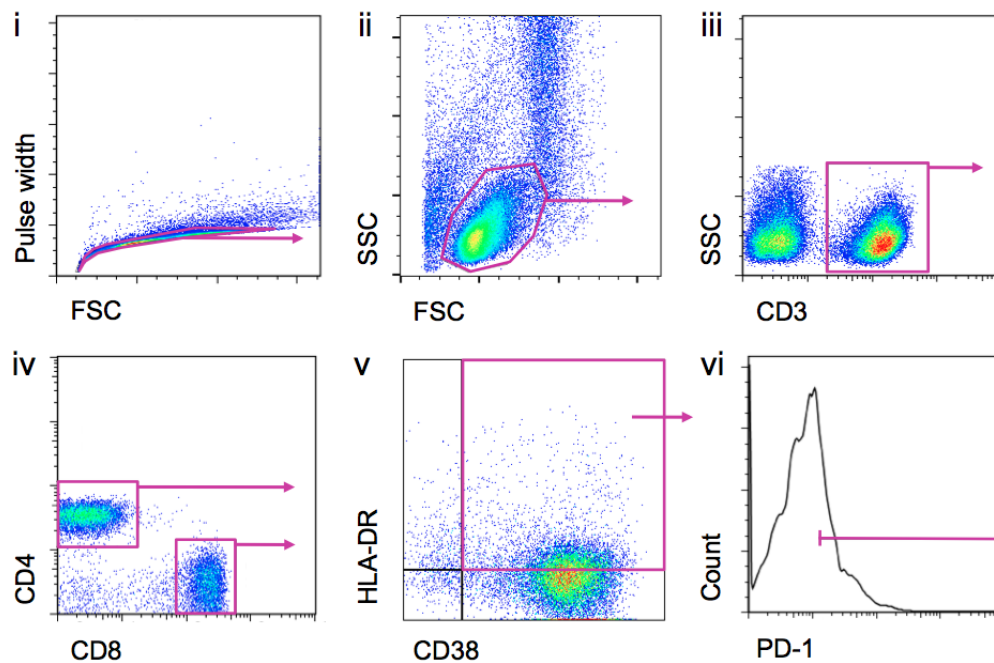


Figure 6.1: Gating strategy for CD3+ CD4/8+ CD38+ HLA-DR+ lymphocytes with/without PD-1 via i) doublet exclusion, ii) lymphocyte gate, iii) CD3+ T cells, iv) CD4+ or CD8+ populations, v) CD38+ and HLA-DR+ co-expression, vi) PD-1+. Gates were placed on the basis of individual-patient isotype controls (for CD38, HLA-DR and PD-1). FSC (forward scatter) SSC (side scatter)

6.1.7.iv T cell function

Whole blood was diluted 1 in 5 with RPMI (supplemented but serum-free) and stimulated in duplicate with phytohaemagglutinin (PHA, 5 µg/ml, Sigma) or tetanus toxoid (TT, 5 Lf/ml, Serum Statens Institut) with or without IL-12 at 50 pg/ml. After 72 hours incubation at 37°C and 5% CO₂, supernatants were removed and interferon

gamma (IFN- γ) concentration was assessed by ELISA (ebioscience) according to manufacturer's instructions.

6.1.7.v Inflammatory mediators in plasma

Plasma IgG EndoCAb (Hycult Biotech) and serum IGF-1 (R&D Systems) were processed according to manufacturers' instructions. Multiplex ELISA (Luminex MAGPIX system) was performed against the following targets in heparinised plasma: Eotaxin (chemokine (C-C motif) ligand (CCL)-11), GRO α (growth regulated oncogene- α , chemokine (C-X-C motif) ligand (CXCL)-1), Interferon- α , Interleukin (IL)-1 α , IL-1 receptor antagonist (IL-1RA), IL-7, IL-8, IL-10, IL-15, IL-17a, IL-22, IL-31, IP-10 (interferon- γ induced protein-10, CXCL10), MCP-1 (monocyte chemotactic protein-1, CCL2), MIP-1 α (macrophage inflammatory protein-1 α , CCL3), MIP-1 β (CCL4), SDF-1 (stromal cell-derived factor-1, CXCL12), tumour necrosis factor- β (TNF β) (Ebioscience). Soluble CD14 (sCD14) was measured using an in-house ELISA (capture clone 55-3, detection clone 3-C39 both from BD, recombinant standard from Sigma).

6.1.7.vi Other methods

Full blood count was processed by the GCLP-compliant clinical laboratories at the KEMRI-Wellcome Trust Research Programme, Kenya. Erythrocyte deformability in response to shear stress was measured on-site using a laser-assisted optical rotation analyser (LORCA) as described previously.²⁸⁰

6.1.8 Statistical Methods

Sample size was calculated with reference to published data on changes over time in erythrocyte membrane fatty acids amongst a group of Thai schoolchildren provided with n-3 LC-PUFA-fortified milk for six months.²⁸¹ DHA composition of total erythrocyte fatty acids increased by 3.6% (standard deviation 1.5). I calculated the sample size based on assessing for an effect size 50% of this magnitude (because the duration of follow-up was only half as long) with 80% power at a significance level of 0.05, which came to 15 in each group. Allowing for up to 25% failure to complete the trial due to mortality or drop-out gave a final size of 20 children per arm, or 60 overall.

All data were double entered and validated with OpenClinica (Isovera (Waltham, USA)). Analysis was performed in Stata (version 12.0) and anthropometric Z-scores were calculated using WHO standards (2006). All analyses were done by intention to treat.

For compositional outcomes (comprising key individual molecular species, calculated total n-6 PUFA and n-3 PUFA content, and n-6:n-3 ratios) ANOVA was used to test differences between the three arms at each time point, and Mann-Whitney U tests to identify within-arm changes from baseline. The total n-6 PUFA and n-3 PUFA content and the n-6:n-3 content ratios were calculated and treated similarly.

Compliance was calculated (see 6.1.4) and compared between arms using ANOVA. For grouped variables (e.g. adverse events), significance was calculated using Fisher's exact test, or Chi-Squared test where the expected number in all cells was > 5.

Changes in anthropometric indices were calculated for individual participants and compared between groups using ANOVA. Where indicated in the text, S-RUTF and F-RUTF arms were considered together as both not containing fish oil, and F-RUTF and FFO-RUTF were considered together as both containing flax seed oil.

I performed a post-hoc analysis to assess for impact of baseline erythrocyte fatty acid composition on changes in erythrocyte membrane composition over the trial using linear regression and assessing for effect modification on the regression coefficient by arm.

'Long-chain' was used to indicate 20-carbon or longer chain fatty acids throughout.

6.1.9 Study Oversight

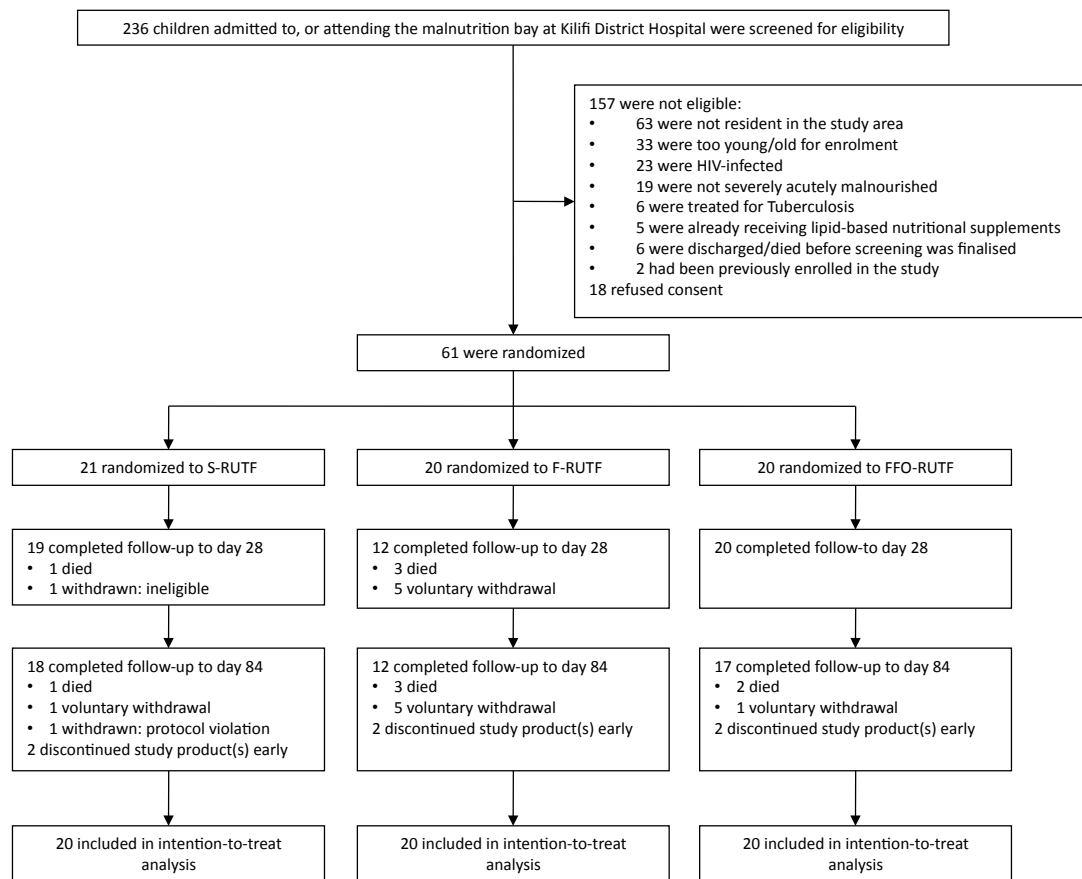
All participants enrolled in the study had individual written informed consent provided by a parent or guardian. The study was approved by the KEMRI Ethical Review Committee and the Oxford Tropical Research Ethics Committee (OxTREC) prior to initiation. The University of Oxford was the sponsor. Clinical trials monitoring was performed by staff from the Clinical Trials Facility at the KEMRI-Wellcome Trust Research Programme. An independent trial steering committee acted as the decision-making body for the study and an independent paediatrician acted as local

safety monitor. Neither the sponsor nor any other party except the named investigators had any role in the design of the study, interpretation of the results, content of manuscripts or decision to publish. The trial was registered at <https://clinicaltrials.gov/ct2/show/NCT01593969>.

6.2 RESULTS

Between 30th May 2012 and 30th April 2013, 236 children admitted to, or attending, the OTP and inpatient malnutrition bay at KCH were assessed for eligibility. 61 children were enrolled in the trial, one of whom was withdrawn as ineligible within 24 hours of enrolment (suspected haematological malignancy) and is not included in any of the analyses (Figure 6.2).

Figure 6.2: Trial flow diagram



6.2.1 Stability of the RUTF

Twelve months after manufacture of the two batches of RUTF (9th May 2012) I initiated a precautionary reanalysis of the lipid composition of the flax seed oil-containing formulation to check for deterioration. Although fatty acid composition (by gas chromatography) and organoleptic qualities were unchanged, on 18th April 2013 the peroxide content of the flax seed oil-containing product was found to be 17.9 mEq/kg, which is higher than the value stipulated by UNICEF as acceptable for newly manufactured batches (< 10 mEq/kg). Because deterioration of RUTF can occur rapidly once peroxidation begins, we began regular checks on palatability and peroxide levels. On the 16th May 2013 the peroxide levels had risen to 33.5 mEq/kg, and investigators considered the flax seed oil-containing product had become less palatable (despite the fact that peroxide levels were 29.7 mEq/kg in the standard formulation, it remained palatable). In discussion with the independent trial steering committee, provision of all study RUTF and capsules was stopped. Children who still required therapeutic or supplementary feeds were switched over to the hospital's standard supply. Six children were affected, 2 in each arm. There were no adverse events considered attributable to the deterioration and these 6 participants were included in intention-to-treat analyses.

6.2.2 Baseline Characteristics:

The arms were comparable at baseline. Compared to the other two arms, children allocated to FFO-RUTF tended to have lower MUAC ($P=0.08$) and were less likely to have diarrhoea at presentation ($P=0.12$) (Table 6.2). There were no major systematic

differences in baseline fatty acid composition of any of the compartments tested, or any of the inflammatory or haematological indices.

Table 6.2: Baseline characteristics of the participants

Baseline Characteristics	S-RUTF	F-RUTF	FFO-RUTF
Participants*	20	20	20
Age (months)	18 (9 to 30)	16 (11 to 25)	14 (11 to 21)
Sex: Male	12 (60)	10 (50)	11 (55)
Nutritional Status			
MUAC (cm)	11.2 (10.9 to 11.4)	11.3 (10.8 to 11.9)	10.9 (10.4 to 11.4)
WHZ.WLZ	-3.11 (-4.43 to -1.89)	-3.31 (-3.88 to -2.90)	-3.65 (-4.12 to -3.19)
Bilateral pedal oedema (kwashiorkor)	1 (5)	1 (5)	0 (0)
HAZ/LAZ	-3.11 (-3.90 to -1.93)	-3.36 (-4.58 to -2.55)	-2.58 (-4.20 to -1.97)
Head circumference-for-age z score	-1.38 (-3.36 to -0.62)	-1.85 (-2.82 to -1.02)	-1.37 (-2.27 to -0.56)
Breastfeeding	8 (40)	8 (40)	12 (60)
Complementary feeds introduced (months of age)	5 (2 to 6)	4 (2 to 6)	4 (3 to 6)
Complications			
Diarrhoea	11 (55)	8 (40)	4 (20)
Pneumonia	3 (15)	3 (15)	1 (5)
Shock	1 (5)	2 (10)	0 (0)
Congenital heart disease	3 (15)	1 (5)	3 (15)
Cerebral palsy	3 (15)	1 (5)	4 (20)
Demographics			
Main carer is not the mother	4 (20)	5 (25)	1 (5)
Main carer no primary education	7 (35)	8 (40)	8 (40)
Lives in mud-walled house	13 (65)	15 (75)	17 (85)
No toilet in house/compound	8 (40)	6 (30)	5 (25)
Fisherman in household	1 (5)	2 (10)	2 (10)

Table 6.2: Data are number (%) or median (inter-quartile range) unless otherwise specified.

*Excluding one child withdrawn due to ineligibility (S-RUTF arm).

6.2.3 Follow up and Compliance:

Seven children were voluntarily withdrawn at parental request: 1 in the S-RUTF arm (at day 84), 5 in the F-RUTF arm (4 before day 7, 1 at day 28) and 1 in the FFO-RUTF arm (at day 42; $P=0.41$ between both elevated n-3 PUFA (F-RUTF & FFO-RUTF) and the standard RUTF arms). Compliance with RUTF feeding and capsules amongst children still in follow-up was high in all three arms (Table 6.3).

Table 6.3: Compliance, Safety and Growth

Compliance	S-RUTF	F-RUTF	FFO-RUTF	P
% RUTF to d28	89 (62 to 102)	95 (79 to 102)	95 (88 to 103)	0.42
% RUTF to d84	90 (80 to 101)	96 (67 to 100)	92 (76 to 100)	0.98
% Capsules to d28	-	-	90 (71 to 99)	-
% Capsules to d84	-	-	88 (79 to 95)	-
Safety				
Deaths	1	3	2	0.86
Other serious adverse events	5	0	4	-
Total death or serious adverse event	6	3	6	0.63
Total illness episodes	50	47	54	0.27
Common illness syndromes:				
Upper respiratory tract infection	7	19	16	0.08
Lower respiratory tract infection	3	2	4	0.90
Rash	10	5	13	0.16
Diarrhoea	7	7	4	0.75
Vomiting	2	6	7	0.20
Growth				
MUAC change				
Enrolment to d28 (mm/day x10 ³)	46 (21 to 72)	52 (24 to 68)	54 (36 to 75)	0.53
Enrolment to d84 (mm/day x10 ³)	21 (17 to 39)	21 (17 to 29)	21 (18 to 29)	0.48
WHZ/WLZ change				
Enrolment to d28 (z score/day x10 ³)	57 (21 to 80)	60 (47 to 84)	58 (34 to 88)	0.23
Enrolment to d84 (z score/day x10 ³)	20 (14 to 35)	21 (14 to 33)	23 (15 to 27)	0.96
HAZ/LAZ change				
Enrolment to d28 (z score/day x10 ³)	-11.9 (-22.9 to -2.5)	-8.0 (-20.9 to -1.0)	-10.4 (-15.7 to 2.9)	0.75
Enrolment to d84 (z score/day x10 ³)	3.0 (-4.5 to 5.6)	4.0 (0.4 to 5.8)	0.2 (-2.3 to 4.6)	0.40
Head circumference-for-age change				
Enrolment to d28 (z score/day x10 ³)	10.1 (1.8 to 17.1)	13.2 (5.8 to 17.0)	6.1 (-6.4 to 20.4)	0.71
Enrolment to d84 (z score/day x10 ³)	8.1 (0.6 to 9.8)	5.8 (3.9 to 8.4)	7.4 (1.2 to 9.3)	0.96

Table 6.3: Data are absolute numbers or median (inter-quartile range) unless otherwise specified. Total illness episodes are compared by Chi-squared test. Individual illness syndromes, deaths, and deaths/serious adverse events are by Fishers Exact test. Compliance and growth by ANOVA.

6.2.4 Safety:

6 children (10%) died during follow-up. 5 of these deaths were associated with severe pneumonia and 1 occurred in the community where it was not possible to ascertain cause of death. 1 death occurred in the S-RUTF arm, 3 in the F-RUTF, and 2 in the FFO-RUTF arms. There were a further 9 severe adverse events (requiring hospitalisation or prolonging hospital stay): 4 were due to lower respiratory tract infection (2 in S-RUTF, 2 in FFO-RUTF), 4 were due to diarrhoea/dehydration (3 in S-RUTF, 1 in FFO-RUTF), and 1 was due to severe malaria (FFO-RUTF). There were no adverse events considered directly attributable to the investigational products.

Non-severe episodes of illness or infection were common during follow-up, as expected in this high-risk population. Total number of illness episodes and the nature of the episodes were similar between the groups (Table 6.3). There was a greater incidence of upper respiratory tract infections and vomiting amongst children receiving the RUTF with elevated n-3 PUFA (F-RUTF and FFO-RUTF arms), but neither of these effects was statistically significant.

6.2.5 Lipidomic analysis:

6.2.5.i Erythrocytes

There were large and highly significant differences in erythrocyte membrane fatty acid composition between the groups at days 28 and 84 (Figure 6.3 and Table 6.4). The percentage of PUFA increased at the expense of saturated fatty acids, but there were wide differences in the behaviour of individual species. In the S-RUTF group there were increases in n-6 PUFA family members dihomo-gamma-linolenic acid (DGLA, 20:3(n-6)) and AA, and also in the n-3 PUFA, EPA. However, DHA declined significantly during follow up. Although breastfeeding was an important determinant of baseline DHA status (Figure 6.4), the decline in DHA occurred in both breastfed and non-breastfed children (Figure 6.5). In the FFO-RUTF group there were marked increases in most of the n-3 LC-PUFA species (except eicosatetraenoic acid (ETA, 20:4(n-3))), while n-6 PUFA family members were unchanged from baseline. Compared to the other two arms, composition in the F-RUTF arm was least affected, with modest increases in EPA and a decline in DHA similar to that seen in S-RUTF.

Figure 6.3: Erythrocyte fatty acid composition

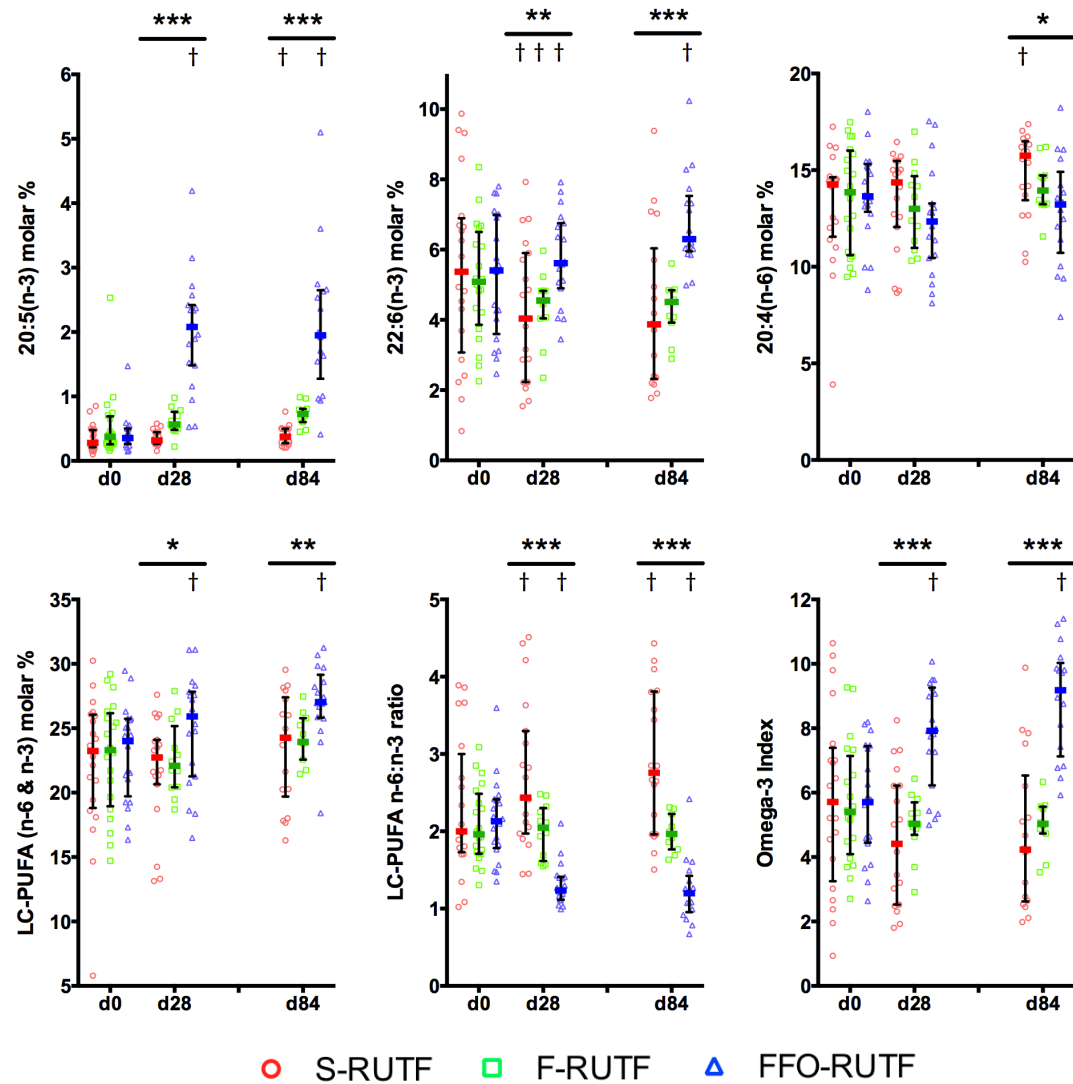


Figure 6.3: Erythrocyte fatty acid composition. Median and interquartile ranges shown for the three arms at baseline, day 28 and day 84. Graphs show (clockwise from top left) DHA, EPA, AA, Omega-3 index (DHA+EPA), n-6:n-3 ratio in LC-PUFA, total LC-PUFA (n-6 and n-3). For between-arm comparisons (ANOVA) at each time point: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. For within-arm comparison (sign test) to baseline values: † $P \leq 0.05$. LC indicates species with carbon chain > 18 .

Table 6.4 Erythrocyte fatty acid composition

	Enrolment			Day 28			Day 84		
	S-RUTF	F-RUTF	FFO-RUTF	S-RUTF	F-RUTF	FFO-RUTF	S-RUTF	F-RUTF	FFO-RUTF
14:0	0.76 (0.47-1.00) *	0.46 (0.36-0.61) *	0.56 (0.33-0.88) *	0.65 (0.26-1.13)	0.36 (0.28-0.57)	0.36 (0.30-0.58)	0.56 (0.39-0.88) *	0.33 (0.25-0.45) *	0.39 (0.23-0.58) * <i>b</i>
16:0	26.4 (25.3-27.5)	26.5 (24.2-27.5)	25.8 (24.7-26.7)	25.1 (24.3-25.7)	25.0 (24.1-26.2)	24.8 (24.2-25.2)	25.0 (24.5-25.7) <i>a</i>	24.8 (23.4-25.5) <i>c</i>	24.7 (24.2-25.1)
16:1(n-7)	0.76 (0.50-1.15)	0.60 (0.43-0.95)	0.77 (0.36-0.96)	0.43 (0.39-0.68) <i>b</i>	0.38 (0.28-0.55) <i>a</i>	0.39 (0.18-0.63) <i>a</i>	0.39 (0.26-0.57) <i>b</i>	0.36 (0.22-0.42)	0.28 (0.20-0.38) <i>c</i>
18:0	14.5 (12.5-16.1) **	13.0 (12.1-13.5) **	12.9 (12.2-13.6) **	14.9 (13.6-18.7)	14.0 (13.6-15.0)	13.4 (13.0-15.1)	15.1 (13.7-17.5) **	14.3 (13.3-14.9) **	13.2 (12.6-13.7) **
18:1(n-9)	15.8 (14.5-17.0)	16.2 (14.4-17.6)	16.7 (14.6-17.6)	15.9 (14.3-17.4)	15.6 (14.8-16.2)	15.0 (14.2-16.3)	16.4 (13.9-16.9)	15.4 (14.6-15.7)	14.7 (13.6-15.9) <i>b</i>
18:1(n-7)	1.35 (1.21-1.72)	1.30 (1.10-1.61)	1.35 (1.17-1.54)	1.18 (1.05-1.37) <i>b</i>	1.11 (1.03-1.18) <i>b</i>	1.16 (0.98-1.28) <i>c</i>	1.12 (1.04-1.20) <i>b</i>	0.95 (0.87-1.21) <i>c</i>	1.13 (0.95-1.21) <i>c</i>
18:2(n-6)	10.3 (8.85-12.0) *	12.0 (11.1-12.8) *	11.6 (9.42-12.4) *	10.4 (9.60-13.2) **	13.0 (12.1-13.9) **	11.5 (11.0-12.1) **	12.2 (10.6-13.2) *	13.5 (13.0-14.3) *	12.8 (11.5-13.6) * <i>a</i>
18:3(n-6)	0.10 (0.06-0.14)	0.12 (0.09-0.17)	0.13 (0.08-0.16)	0.11 (0.09-0.16)	0.10 (0.07-0.13)	0.11 (0.07-0.14)	0.10 (0.06-0.14)	0.11 (0.07-0.13)	0.09 (0.08-0.11)
18:3(n-3)	0.46 (0.26-0.73)	0.58 (0.31-1.07)	0.48 (0.26-1.05)	0.55 (0.33-0.97)	0.52 (0.42-0.64)	0.55 (0.40-0.89)	0.38 (0.22-0.51)	0.55 (0.41-0.88)	0.51 (0.26-0.62)
20:0	0.91 (0.56-1.28)	0.90 (0.35-1.57)	0.98 (0.41-1.57)	1.09 (0.65-1.84)	1.01 (0.60-1.24)	1.09 (0.46-1.65)	0.86 (0.24-1.03)	0.73 (0.44-1.07)	0.74 (0.52-0.98)
20:1(n-9)	0.18 (0.13-0.22)	0.19 (0.14-0.29)	0.40 (0.36-0.53)	0.26 (0.19-0.36) <i>b</i>	0.25 (0.18-0.40)	0.30 (0.19-0.34)	0.25 (0.16-0.40)	0.29 (0.23-0.40)	0.22 (0.16-0.27)
20:2(n-6)	0.34 (0.25-0.41) **	0.40 (0.30-0.44) **	0.40 (0.36-0.53) **	0.39 (0.35-0.43)	0.51 (0.41-0.51) <i>a</i>	0.43 (0.38-0.49)	0.38 (0.36-0.45)	0.45 (0.39-0.46)	0.38 (0.35-0.44)
20:3(n-9)	0.08 (0.06-0.11)	0.10 (0.06-0.13)	0.09 (0.07-0.12)	0.10 (0.07-0.12)	0.09 (0.07-0.11)	0.07 (0.04-0.10)	0.09 (0.07-0.10)	0.07 (0.07-0.11)	0.08 (0.06-0.09)
20:3(n-6)	1.29 (0.95-1.47)	1.24 (1.02-1.47)	1.34 (1.10-1.44)	1.58 (1.25-1.86) * <i>c</i>	1.48 (1.27-1.84) *	1.28 (1.09-1.47) *	1.61 (1.40-1.85) <i>b</i>	1.57 (1.36-1.76)	1.36 (1.15-1.60)
20:4(n-6)	14.3 (11.7-14.6)	13.9 (10.7-16.0)	13.6 (12.9-15.3)	14.4 (12.1-15.5)	13.0 (11.1-14.5)	12.3 (10.5-13.3)	15.8 (13.7-16.4) * <i>a</i>	13.9 (13.2-14.7) *	13.2 (11.4-14.2) *
20:4(n-3)	0.09 (0.06-0.11)	0.07 (0.05-0.11)	0.10 (0.05-0.16)	0.08 (0.06-0.14)	0.14 (0.11-0.17) <i>c</i>	0.13 (0.07-0.15)	0.08 (0.05-0.11) *	0.12 (0.06-0.16) *	0.09 (0.08-0.11) *
20:5(n-3)	0.28 (0.22-0.48)	0.37 (0.26-0.68)	0.35 (0.28-0.50)	0.32 (0.26-0.44) ***	0.56 (0.49-0.74) ***	2.08 (1.48-2.42) *** <i>c</i>	0.37 (0.28-0.50) *** <i>a</i>	0.73 (0.60-0.81) ***	1.95 (1.54-2.63) *** <i>c</i>
24:0	1.05 (0.80-1.31)	1.01 (0.87-1.26)	0.99 (0.87-1.13)	1.03 (0.96-1.28) **	0.99 (0.76-1.11) ** <i>a</i>	0.82 (0.65-0.94) ** <i>b</i>	1.04 (0.88-1.14) ***	0.70 (0.53-1.06) *** <i>b</i>	0.60 (0.46-0.69) *** <i>c</i>
24:1(n-9)	0.32 (0.24-0.37) *	0.30 (0.26-0.37) *	0.37 (0.32-0.43) *	0.33 (0.30-0.39)	0.39 (0.22-0.45)	0.36 (0.28-0.44)	0.39 (0.33-0.45) <i>a</i>	0.35 (0.31-0.39)	0.33 (0.27-0.44)
22:5(n-3)	1.54 (1.24-1.90)	1.86 (1.43-2.21)	1.59 (1.42-2.01)	1.70 (1.40-1.82) ***	2.29 (1.99-2.80) ***	3.07 (2.32-3.90) *** <i>c</i>	1.66 (1.57-1.88) ***	3.11 (2.53-3.44) *** <i>c</i>	3.42 (3.03-3.91) *** <i>c</i>
22:6(n-3)	5.37 (3.28-6.83)	5.08 (3.98-6.39)	5.41 (3.75-6.96)	4.04 (2.23-5.90) ** <i>a</i>	4.55 (4.05-4.83) ** <i>a</i>	5.61 (4.90-6.75) ** <i>b</i>	3.88 (2.36-5.70) ***	4.51 (3.92-4.85) ***	6.30 (6.02-7.33) *** <i>c</i>
Total n-6 + n-3	34.9 (30.9-36.6)	35.5 (31.1-38.1)	35.3 (31.8-38.1)	34.6 (30.3-38.3)	37.0 (34.3-38.5)	39.0 (32.7-40.5)	38.0 (31.2-39.9) ** <i>a</i>	38.8 (37.4-40.1) **	40.9 (40.1-41.7) ** <i>b</i>
Total LCn-6 + LCn-3	23.2 (19.0-26.0)	23.3 (19.2-26.0)	24.0 (20.2-25.7)	22.7 (20.7-24.1) *	22.1 (20.4-24.6) *	25.9 (21.3-27.8) * <i>a</i>	24.3 (20.3-27.2) **	23.9 (22.6-25.8) **	27.0 (25.9-28.6) ** <i>b</i>
n-6:n-3	3.24 (2.64-4.31)	3.46 (2.70-3.92)	3.21 (2.88-3.83)	3.85 (2.90-5.21) *** <i>a</i>	3.58 (3.06-3.90) ***	2.15 (1.93-2.31) *** <i>c</i>	4.48 (3.22-6.12) *** <i>b</i>	3.23 (3.06-3.77) ***	2.03 (1.90-2.23) *** <i>c</i>
LCn-6:LCn-3	2.00 (1.75-2.90)	1.96 (1.72-2.45)	2.13 (1.80-2.41)	2.44 (1.97-3.30) *** <i>b</i>	2.05 (1.64-2.28) ***	1.24 (1.11-1.41) *** <i>c</i>	2.76 (1.96-3.80) *** <i>b</i>	1.97 (1.77-2.23) ***	1.20 (0.99-1.36) *** <i>c</i>
Omega-3 Index	5.71 (3.48-7.27)	5.41 (4.22-6.94)	5.70 (4.47-7.42)	4.41 (2.52-6.22) ***	5.03 (4.76-5.59) ***	7.92 (6.23-9.26) *** <i>c</i>	4.23 (2.65-6.20) ***	5.03 (4.73-5.56) ***	9.18 (7.43-9.86) *** <i>c</i>

Table 6.4: Erythrocyte fatty acid composition. Median (IQR). For between-arm comparisons (ANOVA) at each time point: * P≤0.05; ** P≤0.01; *** P≤0.001. For within-arm comparison (sign test) to baseline values: *a* P≤0.05; *b* P≤0.01; *c* P≤0.001

Figure 6.4: Enrolment LC-PUFA by breastfeeding status

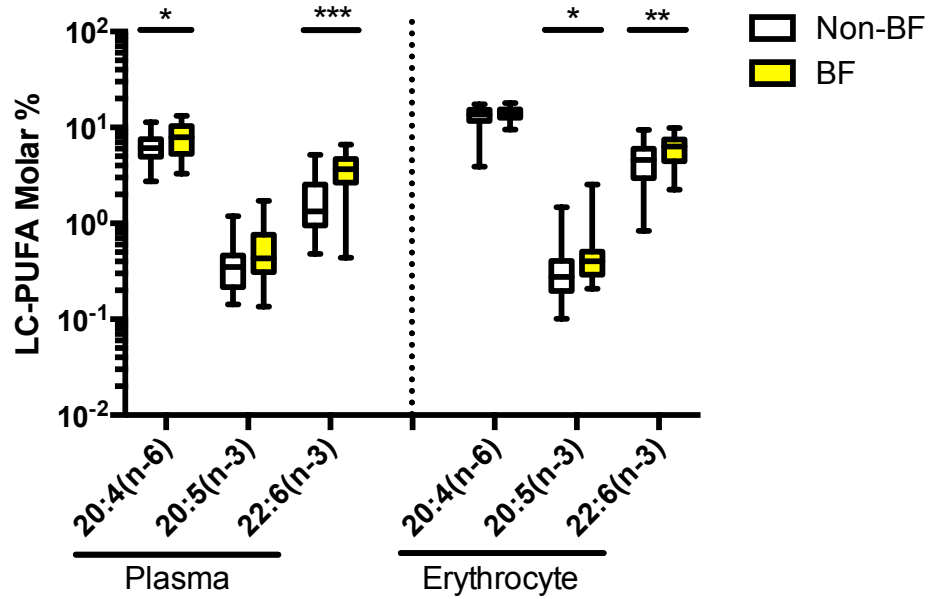


Figure 6.4: Enrolment 20:4(n-6), 20:5(n-3), and 22:6(n-3) in plasma PC and erythrocyte compartments according to whether the participant was being breastfed (at all) at enrolment. Breastfeeding was associated with increases in both n-3 and n-6 LC-PUFA family members. BF (breastfeeding). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ (by Mann Whitney U test).

Figure 6.5: Erythrocyte DHA by breastfeeding status

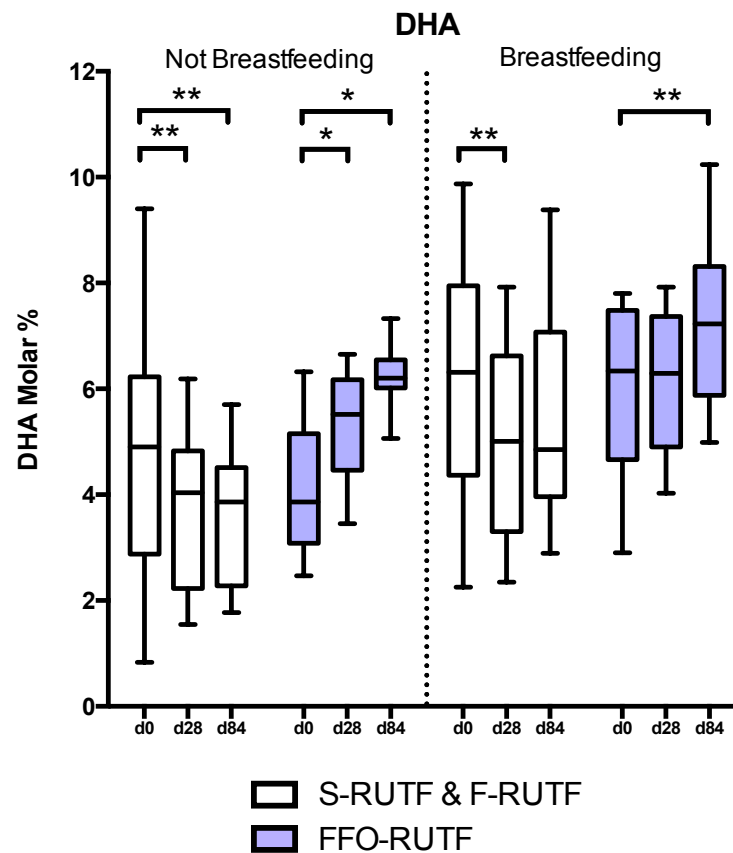


Figure 6.5: Erythrocyte DHA content during the trial is presented according to whether or not the participant was being breastfed at all on admission to the trial. Data are for the arm receiving fish oil (FFO-RUTF) or the other arms combined. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ (by sign test).

Alteration in the LC-PUFA species lay behind significant changes in the overall erythrocyte n-6:n-3 PUFA ratio, but despite the provision of diets with dramatically different linoleic acid (LA, 18:2(n-6)) and ALA content there was no difference in the LA:ALA ratio between the groups at any timepoint, and no change from baseline.

Ratios of 20:4(n-6)/20:3(n-6), 18:3(n-6)/18:2(n-6) and 20:3(n-6)/18:3(n-6) were calculated as indices of $\Delta 5$ -desaturase, $\Delta 6$ -desaturase and elongase activity,

respectively. There were no differences between the arms at any timepoint, and no evidence suggesting longitudinal change in enzyme activity during nutritional rehabilitation (Figure 6.6).

Figure 6.6: Desaturase and elongase activity

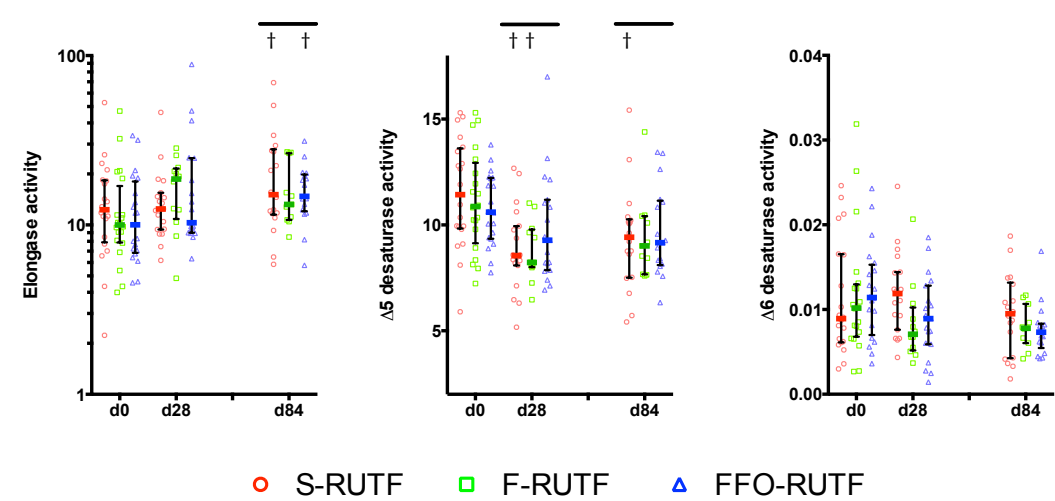


Figure 6.6: Calculated in erythrocyte lipids as n-6 product/substrate ratios as described in the text. For between-arm comparisons (ANOVA) at each time point, $P > 0.05$ throughout. For within-arm comparison (sign test) to baseline values: † $P \leq 0.05$.

Baseline composition of LC-PUFA was an important modulator of the compositional response to PUFA provision. In the FFO-RUTF arm, children with low baseline values of DHA had large increases, whilst amongst those with relatively higher baseline DHA, enrichment was much less marked (Figure 6.7). For those in both of the non-fish oil arms, relatively low baseline DHA remained constant, however there was a marked decrease in DHA amongst children who had higher levels at baseline. The regression coefficients for the change in DHA by baseline composition differed

between the trial arms ($P=0.045$ overall, and $P=0.025$ between the S-RUTF and F-RUTF arms) (Figure 6.8).

Figure 6.7: Erythrocyte DHA/EPA stratified by baseline status

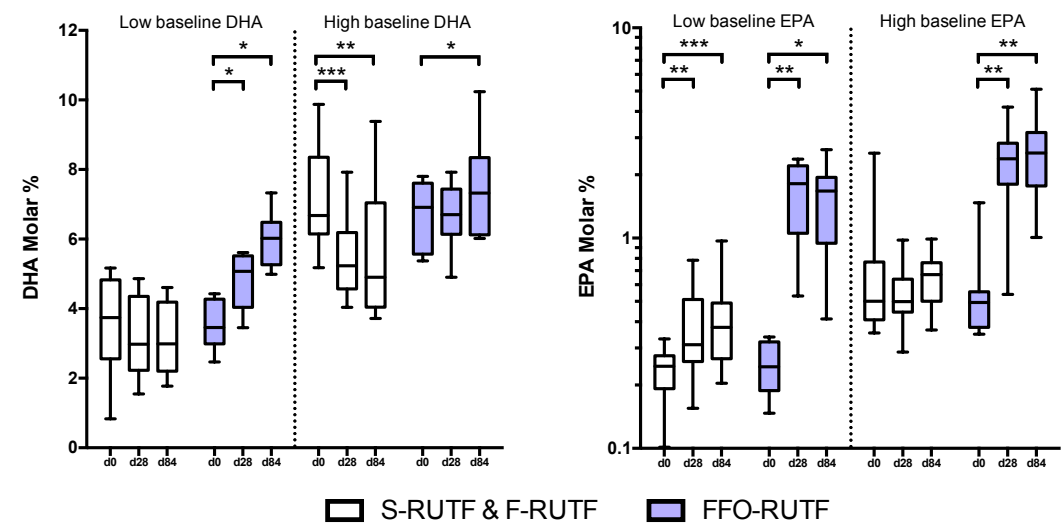


Figure 6.7: Erythrocyte DHA and EPA between both arms without fish oil (S-RUTF and F-RUTF) compared to FFO-RUTF. Participants are stratified on the basis of their baseline DHA or EPA values. Low indicates equal to or below the median, and high indicates above the median. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ (by sign test)

Figure 6.8: Impact of baseline PUFA status on response

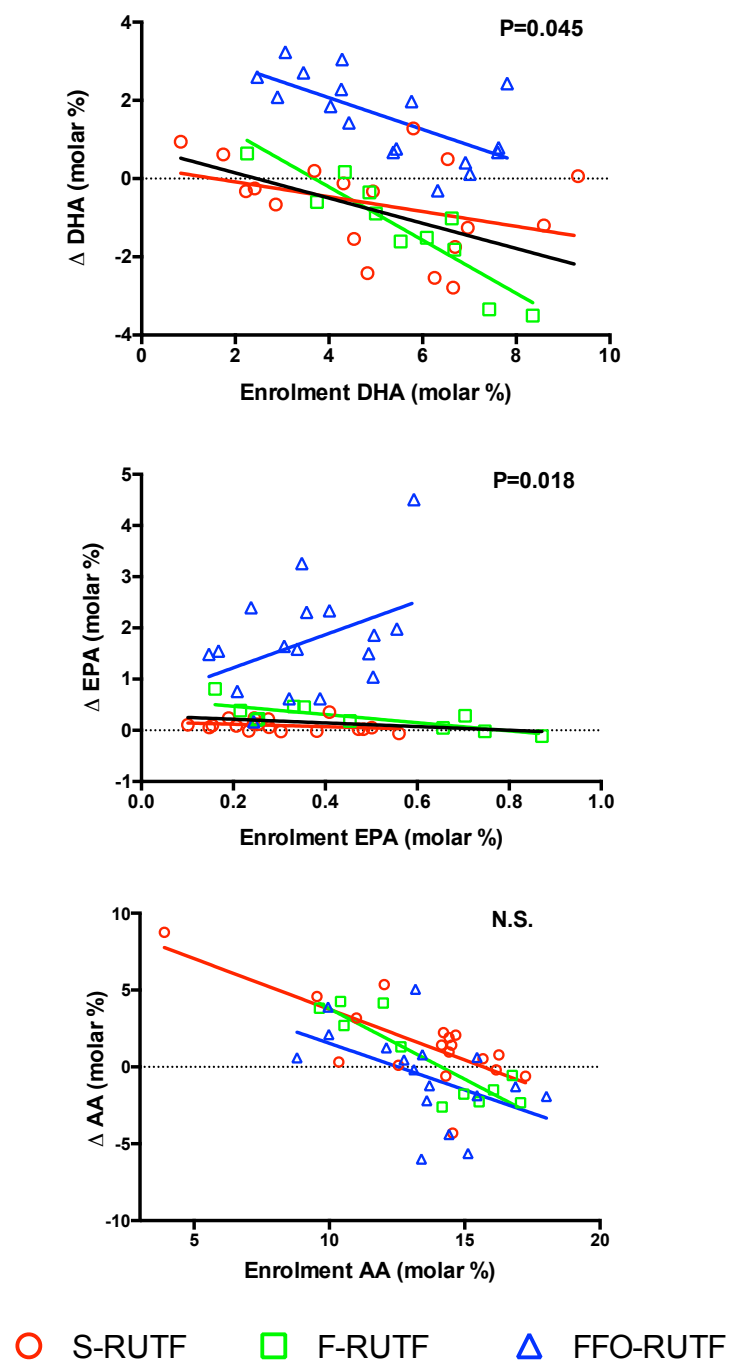


Figure 6.8: Change in erythrocyte DHA, EPA or AA from baseline to day 84 plotted against baseline values. Regression lines are coloured the same as corresponding group symbols, black regression line is for S-RUTF & F-RUTF combined. P value is for effect modification across the three arms as described in the text. N.S. ($P > 0.05$)

For EPA the situation was different. Fish oil supplementation was associated with large increases in EPA content regardless of baseline status, and for those in the two non-fish oil arms, only those with relatively low baseline EPA levels had any appreciable relative increase during the study. The regression coefficients for the change in EPA by baseline composition were significantly different between arms ($P=0.018$ overall, and $P=0.032$ between FFO-RUTF and F-RUTF arms). The fact that increases in EPA composition were largest in those children in the fish oil group who had highest levels at baseline, implies that provision of pre-formed EPA bypassed regulatory mechanisms (Figure 6.8).

6.2.5.ii Plasma phosphatidylcholine:

Average changes in percentage composition of plasma phosphatidylcholine (PC) PUFA were very similar to those seen in the erythrocyte fraction, although plasma PC was less enriched in PUFA overall and the observed effect sizes were smaller (Table 6.5). However, the relationships between participant-level erythrocyte and plasma PC data were highly variable (Figure 6.9 and Table 6.6).

Table 6.5 Plasma phosphatidylcholine fatty acid composition

	Enrolment			Day 28			Day 84		
	S-RUTF	F-RUTF	FFO-RUTF	S-RUTF	F-RUTF	FFO-RUTF	S-RUTF	F-RUTF	FFO-RUTF
14:0	0.29 (0.20-0.40)	0.30 (0.21-0.39)	0.32 (0.23-0.46)	0.22 (0.16-0.33)	0.20 (0.16-0.28)	0.30 (0.17-0.38)	0.22 (0.12-0.32) <i>a</i>	0.25 (0.17-0.39)	0.24 (0.17-0.38) <i>a</i>
16:0	33.7 (32.0-35.1)	33.9 (31.9-35.2)	33.4 (32.3-34.3)	31.1 (29.7-31.9) <i>a</i>	31.5 (30.4-32.9) <i>a</i>	31.6 (30.6-32.9)	30.8 (29.8-31.8) <i>b</i>	31.9 (30.1-33.5)	31.8 (30.7-32.3) <i>b</i>
16:1(n-7)	0.38 (0.25-0.75)	0.55 (0.33-0.90)	0.59 (0.28-0.91)	0.19 (0.17-0.32) <i>b</i>	0.23 (0.17-0.31)	0.21 (0.17-0.38) <i>c</i>	0.26 (0.20-0.35)	0.28 (0.16-0.45)	0.21 (0.14-0.40) <i>c</i>
18:0	12.8 (11.5-15.0)	13.3 (11.6-14.0)	13.4 (12.4-13.8)	15.5 (14.0-16.0) <i>a</i>	15.6 (13.7-16.4) <i>b</i>	15.5 (14.6-16.5) <i>c</i>	15.8 (14.1-16.4) <i>a</i>	14.7 (13.4-17.3)	15.4 (14.9-15.8) <i>b</i>
18:1(n-9)	15.1 (11.9-18.5)	15.7 (12.9-17.3)	15.0 (11.8-18.8)	11.7 (9.13-13.7) <i>c</i>	11.0 (10.2-13.2)	10.8 (9.76-12.3) <i>c</i>	11.4 (9.10-11.7) * <i>b</i>	11.5 (10.8-14.2) *	10.7 (9.49-11.4) * <i>c</i>
18:1(n-7)	1.92 (1.54-2.51)	2.10 (1.68-2.20)	1.68 (1.30-2.23)	1.45 (1.24-1.71)	1.39 (1.18-1.45) <i>a</i>	1.45 (1.27-1.67)	1.51 (1.38-1.69) <i>a</i>	1.34 (1.13-1.83)	1.47 (1.32-1.70) <i>a</i>
18:2(n-6)	20.3 (17.7-22.4)	21.8 (20.1-22.7)	19.3 (21.4-24.4)	24.7 (21.5-26.3) *	25.8 (23.4-28.1) * <i>c</i>	22.3 (21.4-25.2) *	22.6 (19.8-25.2)	23.0 (20.4-27.9)	23.2 (21.0-24.1)
18:3(n-6)	0.09 (0.05-0.15)	0.09 (0.05-0.15)	0.09 (0.07-0.16)	0.10 (0.06-0.23)	0.09 (0.06-0.13)	0.08 (0.05-0.17)	0.08 (0.04-0.10)	0.06 (0.05-0.12)	0.11 (0.05-0.14)
18:3(n-3)	0.16 (0.09-0.26)	0.17 (0.12-0.24)	0.23 (0.11-0.36)	0.20 (0.14-0.27) ***	0.43 (0.30-0.57) ***	0.57 (0.29-0.67) *** <i>b</i>	0.11 (0.09-0.17) ***	0.33 (0.24-0.62) ***	0.23 (0.19-0.50) ***
20:0	0.11 (0.08-0.14)	0.11 (0.10-0.20)	0.17 (0.07-0.29)	0.18 (0.11-0.28) <i>a</i>	0.15 (0.10-0.19)	0.18 (0.12-0.26)	0.12 (0.09-0.15)	0.14 (0.11-0.29)	0.14 (0.11-0.27)
20:1(n-9)	0.19 (0.16-0.27)	0.17 (0.12-0.21)	0.18 (0.13-0.23)	0.23 (0.18-0.30)	0.23 (0.18-0.25)	0.20 (0.16-0.27)	0.19 (0.13-0.25) *	0.24 (0.20-0.27) *	0.20 (0.16-0.24) *
20:2(n-6)	0.33 (0.24-0.39)	0.29 (0.23-0.34)	0.28 (0.22-0.35)	0.32 (0.27-0.38)	0.26 (0.21-0.35)	0.31 (0.22-0.36)	0.39 (0.30-0.45)	0.30 (0.24-0.34)	0.36 (0.28-0.45) <i>a</i>
20:3(n-9)	0.13 (0.09-0.22)	0.12 (0.07-0.21)	0.11 (0.05-0.23)	0.15 (0.07-0.17)	0.08 (0.05-0.15)	0.08 (0.05-0.16)	0.11 (0.09-0.20)	0.09 (0.05-0.20)	0.09 (0.06-0.15)
20:3(n-6)	1.72 (1.35-2.07)	1.48 (1.25-1.96)	1.73 (1.37-2.11)	2.71 (2.00-3.17) *** <i>b</i>	1.88 (1.64-2.32) **	1.65 (1.11-2.12) **	2.41 (2.06-3.32) *** <i>b</i>	1.71 (1.46-2.02) ***	1.90 (1.43-2.01) ***
20:4(n-6)	6.34 (5.53-8.36)	6.87 (5.31-8.01)	6.23 (4.77-9.04)	7.71 (6.17-8.94) *	5.10 (4.42-7.71) *	5.70 (4.25-8.33) *	9.73 (8.52-11.2) *** <i>a</i>	6.87 (5.75-9.38) **	7.82 (6.10-9.84) **
20:4(n-3)	0.09 (0.06-0.13)	0.07 (0.04-0.12)	0.08 (0.04-0.14)	0.09 (0.06-0.14)	0.12 (0.09-0.18) <i>b</i>	0.12 (0.08-0.16)	0.07 (0.05-0.09) *	0.08 (0.07-0.13) * <i>b</i>	0.12 (0.07-0.13) * <i>a</i>
20:5(n-3)	0.37 (0.22-0.60)	0.44 (0.29-0.61)	0.37 (0.29-0.49)	0.44 (0.35-0.57) ***	0.69 (0.39-0.84) ***	1.74 (0.71-2.40) *** <i>c</i>	0.44 (0.32-0.52) ***	0.88 (0.51-0.94) ***	1.01 (0.68-1.96) *** <i>c</i>
24:0	0.27 (0.18-0.40)	0.23 (0.17-0.29)	0.25 (0.16-0.34)	0.33 (0.21-0.44) ***	0.21 (0.14-0.30) ***	0.14 (0.11-0.22) *** <i>a</i>	0.40 (0.36-0.52) ***	0.25 (0.19-0.33) ***	0.18 (0.15-0.22) *** <i>a</i>
24:1(n-9)	0.12 (0.07-0.16)	0.13 (0.08-0.17)	0.12 (0.09-0.18)	0.10 (0.08-0.13)	0.09 (0.08-0.17)	0.10 (0.07-0.15)	0.11 (0.87-0.14)	0.13 (0.10-0.17)	0.13 (0.09-0.17)
22:5(n-3)	0.69 (0.39-1.29)	0.71 (0.53-0.90)	0.71 (0.51-0.82)	0.76 (0.53-1.00) ***	1.02 (0.69-1.19) *** <i>a</i>	1.64 (0.94-1.92) *** <i>b</i>	0.91 (0.66-1.11) *	1.05 (0.83-1.33) *	1.32 (1.09-1.49) * <i>b</i>
22:6(n-3)	2.51 (1.00-4.63)	2.63 (1.11-3.66)	2.35 (1.15-3.69)	2.23 (1.02-3.35) ***	2.08 (1.38-2.97) ***	4.26 (3.27-4.98) *** <i>c</i>	2.36 (1.52-4.43) **	2.70 (2.05-2.96) **	4.15 (3.57-5.36) **
Total n-6+n-3	33.6 (30.3-37.1)	34.7 (31.3-36.2)	35.3 (31.4-38.2)	39.1 (37.1-41.9) <i>c</i>	39.9 (36.7-41.2) <i>a</i>	38.9 (38.0-40.4) <i>b</i>	40.5 (38.9-41.6) <i>c</i>	39.6 (36.2-41.3)	40.4 (39.3-42.9) <i>b</i>
Total LCn-6+LCn-3	12.3 (9.45-15.6)	13.3 (9.35-14.8)	11.7 (9.77-16.5)	13.5 (12.5-16.3) *	11.6 (9.40-15.3) *	16.2 (13.2-17.9) * <i>a</i>	16.9 (15.2-19.9) <i>a</i>	13.3 (12.9-15.6)	16.3 (15.2-20.6) <i>b</i>
n-6:n-3	8.04 (4.17-13.3)	7.72 (5.27-11.0)	7.93 (6.67-11.1)	8.24 (7.15-14.7) ***	7.82 (6.69-8.98) ***	3.60 (3.29-4.86) *** <i>c</i>	9.20 (5.52-12.4) ***	6.63 (6.17-7.57) ***	4.40 (3.86-5.64) *** <i>b</i>
LCn-6:LCn-3	2.73 (1.67-3.72)	2.59 (1.71-3.48)	2.41 (2.15-3.08)	2.93 (2.08-4.64) ***	1.94 (1.74-2.32) ***	1.01 (0.73-1.43) *** <i>c</i>	3.41 (2.48-4.68) ***	2.25 (1.72-2.29) ***	1.50 (1.05-1.75) *** <i>b</i>

Table 6.5: Plasma phosphatidylcholine fatty acid composition. Median (IQR). For between-arm comparisons (ANOVA) at each time point: * P≤0.05; ** P≤0.01; *** P≤0.001. For within-arm comparison (sign test) to baseline values: *a* P≤0.05; *b* P≤0.01; *c* P≤0.001

Figure 6.9 Correlation between plasma phosphatidylcholine and erythrocyte fatty acids for key species

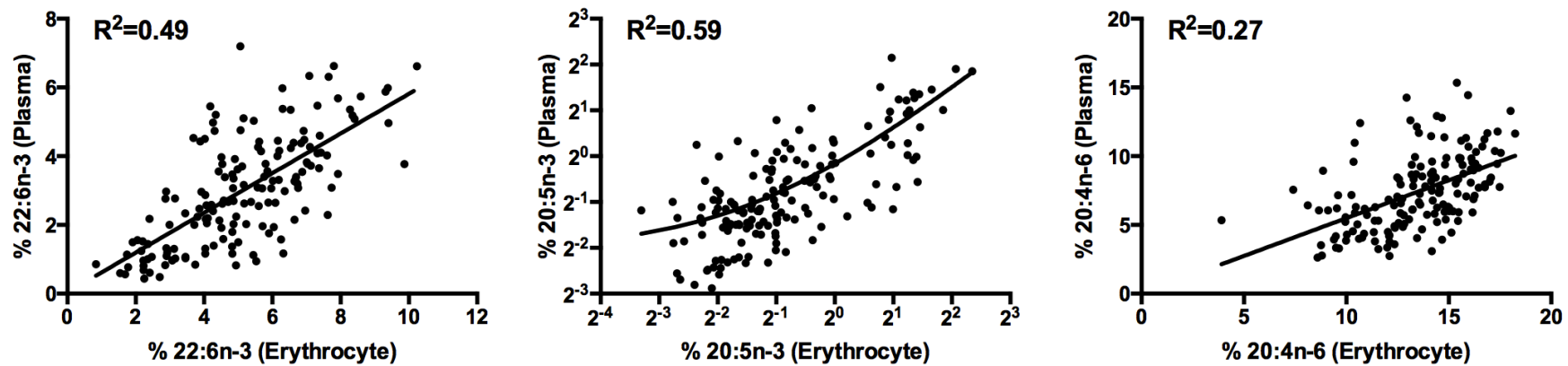


Table 6.6: Plasma phosphatidylcholine/erythrocyte fatty acid correlation.

	Enrolment	Day 28	Day 84	Total
14:0	0.07	0.10	0.08	0.09
16:0	0.00	0.01	0.00	0.04
16:1(n-7)	0.21	0.03	0.12	0.26
18:0	0.13	0.01	0.08	0.07
18:1(n-9)	0.39	0.35	0.27	0.32
18:1(n-7)	0.07	0.22	0.29	0.22
18:2(n-6)	0.21	0.09	0.27	0.19
18:3(n-6)	0.01	0.00	0.01	0.00
18:3(n-3)	0.00	0.00	0.13	0.01
20:0	0.03	0.01	0.02	0.01
20:1(n-9)	0.05	0.06	0.07	0.01
20:2(n-6)	0.10	0.09	0.10	0.08
20:3(n-9)	0.00	0.02	0.01	0.00
20:3(n-6)	0.25	0.21	0.47	0.34
20:4(n-6)	0.22	0.28	0.29	0.27
20:4(n-3)	0.01	0.25	0.00	0.05
20:5(n-3)	0.08	0.71	0.54	0.59
24:0	0.10	0.17	0.45	0.16
24:1(n-9)	0.00	0.01	0.00	0.00
22:5(n-3)	0.02	0.26	0.29	0.25
22:6(n-3)	0.52	0.46	0.55	0.49
Total n-6 + n-3	0.12	0.03	0.02	0.11
Total LCn-6 + LCn-3	0.29	0.19	0.12	0.25
n-6:n-3	0.50	0.86	0.71	0.62
LCn-6:LCn-3	0.56	0.87	0.83	0.74

Table 6.6: Data are R-squared values from linear regression analyses between erythrocyte GC data and plasma phosphatidylcholine GC data for each molecular species at the three time points and (total) for all the values at the three timepoints combined.

6.2.5.iii T-cells

Differences in the T-cell compartment were less marked. Modest relative increases in AA in the S-RUTF arm and in EPA in the FFO-RUTF arm were evident in PC species, but DHA was unaffected. There was no effect of the intervention on phosphatidylethanolamine species (where EPA was undetectable) (Table 6.7 and 6.8).

Table 6.7 CD3+ (T cell) phosphatidylcholine fatty acid composition.

	Enrolment			Day 84		
	S-RUTF	F-RUTF	FFO-RUTF	S-RUTF	F-RUTF	FFO-RUTF
16:0 & 14:0	0.50 (0.34-0.75)	0.55 (0.44-0.69)	0.61 (0.45-0.93)	0.67 (0.56-1.10)	0.51 (0.39-0.74)	0.67 (0.45-1.03)
16:0a & 16:0	0.70 (0.65-0.86)	0.84 (0.56-1.08)	0.75 (0.59-1.04)	0.84 (0.65-1.07)	0.84 (0.68-0.96)	0.78 (0.61-0.87)
16:0 & 16:1	1.10 (0.90-1.27)	1.17 (1.00-1.44)	1.10 (0.93-1.32)	1.10 (0.90-1.46)	0.93 (0.82-1.15) <i>a</i>	0.93 (0.79-1.12)
16:0 & 16:0	5.39 (5.36-8.99)	8.95 (6.06-9.44)	7.09 (6.43-7.54)	8.82 (6.24-9.95)	7.55 (6.32-9.98)	8.61 (6.68-10.1)
16:0a & 18:1	1.50 (1.27-1.72)	1.62 (1.19-1.78)	1.31 (1.17-1.72)	1.51 (1.20-1.62)	1.57 (1.13-1.68)	1.32 (1.19-1.59)
16:0 & 18:2	7.46 (6.75-8.67)	7.45 (6.29-8.94)	7.71 (6.76-8.60)	8.25 (6.08-9.24)	8.34 (7.87-9.41)	8.48 (7.10-9.82)
16:0 & 18:1	14.8 (14.2-16.9)	16.4 (14.5-17.1)	15.5 (13.2-17.2)	12.7 (11.7-14.5)	14.3 (13.7-15.2)	13.6 (12.3-14.9)
18:0 & 16:0	0.64 (0.51-0.84)	0.86 (0.58-1.04)	0.76 (0.46-1.02)	0.78 (0.71-0.93)	0.78 (0.63-0.91)	0.88 (0.80-1.05)
16:0a & 20:4	0.86 (0.79-1.16)	0.83 (0.72-0.91)	0.85 (0.75-1.15)	1.05 (0.86-1.12) **	0.76 (0.65-0.86) **	0.63 (0.60-0.97) **
16:0 & 20:5	0.21 (0.17-0.31)	0.26 (0.21-0.36)	0.24 (0.18-0.30)	0.29 (0.25-0.32) **	0.39 (0.27-0.42) **	0.44 (0.33-1.00) ** <i>b</i>
16:0 & 20:4	8.03 (7.24-9.45)	7.49 (6.27-10.1)	6.81 (5.71-7.92)	9.77 (8.75-10.4) **	7.84 (6.81-8.74) **	7.11 (6.13-8.53) **
18:1 & 18:2	5.93 (5.44-6.67)	5.63 (4.97-6.74)	5.14 (3.61-6.25)	4.99 (3.82-6.11)	6.34 (5.16-7.89)	5.79 (4.97-6.44)
18:0 & 18:2	7.69 (6.80-8.52)	8.09 (7.57-8.54)	6.90 (5.99-8.06)	8.03 (6.08-8.97)	8.77 (7.74-10.2)	7.77 (7.74-9.52) <i>b</i>
18:0 & 18:1	2.47 (1.86-2.95)	3.01 (2.25-3.22)	2.93 (1.94-3.72)	2.23 (1.94-2.53)	2.67 (2.45-2.90)	2.41 (1.95-3.13)
18:1 & 20:4	1.17 (1.00-1.47)	1.08 (0.87-1.21)	1.10 (0.99-1.25)	1.27 (1.13-1.39) **	1.26 (1.06-1.45) **	0.97 (0.97-1.20) **
16:0 & 22:6	1.11 (0.89-1.36)	1.09 (0.89-1.42)	1.14 (1.00-1.42)	1.25 (1.08-1.58) *	1.09 (1.00-1.19) *	1.44 (1.34-1.64) *
18:1 & 20:4	4.25 (3.79-5.19)	3.74 (3.54-4.23)	3.59 (3.10-4.64)	3.96 (3.70-4.62)	3.64 (3.2504.61)	3.67 (3.33-4.01)
18:0 & 20:4	7.37 (6.79-8.26) *	6.65 (5.37-7.61) *	6.06 (5.15-7.25) *	8.54 (6.89-10.2) *	6.99 (6.13-8.66) *	6.28 (4.93-7.68) *
18:0 & 20:3	1.73 (1.34-1.98)	1.54 (1.22-1.70)	1.67 (1.26-1.95)	1.60 (1.47-1.86)	1.74 (1.67-2.09)	1.80 (1.70-2.08)
18:0 & 22:6	0.77 (0.64-0.97)	0.72 (0.58-0.85)	0.76 (0.70-0.89)	0.79 (0.65-1.05)	0.69 (0.62-0.80)	0.76 (0.70-0.96)
18:0 & 22:5	0.78 (0.70-1.10)	0.76 (0.65-0.81)	0.83 (0.72-0.98)	0.80 (0.63-0.83)	0.81 (0.65-0.96)	0.70 (0.66-0.78)
20:4(n-6)	10.8 (9.76-12.8) *	9.50 (8.71-11.4) *	9.54 (8.17-11.0) *	12.4 (11.1-13.5) **	10.4 (9.51-11.3) **	9.64 (8.03-10.5) **
20:5(n-3)	0.11 (0.09-0.16)	0.13 (0.10-0.18)	0.12 (0.09-0.15)	0.15 (0.12-0.16) **	0.19 (0.13-0.21) **	0.22 (0.16-0.50) ** <i>b</i>
22:6(n-3)	0.87 (0.84-1.24)	0.90 (0.79-1.08)	0.94 (0.86-1.15)	1.02 (0.89-1.30)	0.91 (0.81-0.98)	1.12 (1.02-1.30)

Table 6.7: Median (IQR). For between-arm comparisons (ANOVA) at each time point: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. For within-arm comparison (sign test) to baseline values: *a* $P \leq 0.05$; *b* $P \leq 0.01$; *c* $P \leq 0.001$. Output data are mass spectra corresponding to PC (or PE, below) diacylglycerol components. Predicted mass identity is listed and total percentage content of AA, EPA and DHA is listed.

Table 6.8 CD3+ (T cell) phosphatidylethanolamine fatty acid composition.

	Enrolment			Day 28		
	S-RUTF	F-RUTF	FFO-RUTF	S-RUTF	F-RUTF	FFO-RUTF
16:1 & 16:1	0.32 (0.15-0.37)	0.22 (0.12-0.36)	0.29 (0.23-0.42)	0.24 (0.17-0.50)	0.43 (0.21-0.72)	0.23 (0.11-0.34)
16:0 & 16:1	0.13 (0.10-0.20)	0.19 (0.15-0.26)	0.24 (0.16-0.37)	0.14 (0.12-0.43) <i>a</i>	0.20 (0.10-0.62)	0.12 (0.10-0.17)
16:0 & 16:0	1.31 (0.85-1.82)	0.85 (0.38-1.06)	1.39 (0.88-2.31)	1.35 (1.00-2.29)	0.86 (0.72-1.90)	1.97 (1.11-3.21)
16:1 & 18:2	0.16 (0.10-0.22)	0.14 (0.11-0.20)	0.21 (0.18-0.25)	0.16 (0.09-0.37)	0.18 (0.13-0.47)	0.18 (0.10-0.24)
16:0 & 18:2	1.66 (1.46-2.20)	2.09 (1.69-2.45)	1.80 (1.53-2.24)	1.68 (1.28-2.41)	1.94 (1.47-2.96)	1.80 (1.53-2.01)
16:0 & 18:1	2.47 (2.01-2.90)	2.65 (2.29-3.19)	2.61 (2.10-3.10)	2.36 (1.71-2.80)	2.75 (2.01-3.69)	2.14 (1.92-2.48)
16:0 & 20:4	4.51 (3.54-4.93)	4.97 (4.53-5.60)	4.86 (4.01-5.34)	4.25 (3.34-4.70)	3.98 (3.34-4.78)	4.03 (3.68-4.67)
18:1 & 18:2	2.33 (1.76-2.84)	2.51 (1.74-3.80)	2.59 (1.99-3.14)	2.38 (1.49-2.86)	2.93 (2.20-4.19)	2.67 (2.44-2.84)
18:1 & 18:1	6.42 (5.48-7.55)	7.95 (6.05-9.11)	6.85 (6.76-7.47)	7.27 (5.96-7.81)	8.87 (6.33-10.3)	8.97 (6.77-9.39)
18:0 & 18:1	2.86 (2.39-3.43)	3.27 (3.06-3.62)	3.29 (3.05-3.67)	2.77 (1.90-3.03)	2.95 (2.46-3.78)	3.10 (2.27-3.91)
18:0p & 20:4	1.05 (0.97-1.08)	0.95 (0.81-1.08)	0.92 (0.83-1.18)	0.85 (0.71-1.24)	0.87 (0.83-1.00)	0.91 (0.78-1.08)
18:0p & 20:3	0.88 (0.74-1.01)	0.80 (0.68-0.98)	0.93 (0.87-1.13)	0.81 (0.66-0.93)	0.72 (0.62-0.87)	0.88 (0.60-0.96)
16:0 & 22:6	1.93 (1.31-2.20)	2.24 (1.78-2.75)	2.11 (1.67-2.73)	2.05 (1.82-2.62)	1.77 (1.43-2.11)	2.28 (1.98-2.99)
18:1 & 20:4	5.12 (4.21-5.73)	5.15 (4.42-5.53)	4.96 (4.50-5.23)	4.27 (3.05-4.80)	4.08 (3.38-4.93)	4.86 (3.92-5.70)
18:0 & 20:4	27.5 (22.2-32.6)	29.1 (27.2-29.5)	26.0 (22.3-28.4)	26.4 (21.2-33.4)	24.9 (21.7-29.6)	25.5 (20.9-28.5)
18:0 & 20:3	3.12 (2.76-3.90)	3.50 (2.97-3.94)	3.23 (2.57-3.83)	3.01 (2.48-3.38)	3.16 (2.45-3.40)	3.11 (2.68-3.62)
18:1 & 22:6	2.26 (1.26-2.60)	2.11 (1.47-2.64)	1.88 (1.67-2.78)	1.95 (1.68-2.60)	2.02 (1.64-2.14)	2.00 (1.63-2.36)
18:0 & 22:6	3.30 (2.46-4.06)	3.37 (2.87-4.62)	3.35 (2.64-4.20)	3.59 (3.05-4.48)	2.70 (2.42-2.90)	3.59 (2.71-4.97)
18:0 & 22:5	2.75 (2.39-3.18)	3.12 (2.45-3.29)	2.35 (2.11-3.04)	2.74 (2.04-3.01)	2.50 (2.45-3.27)	3.04 (2.64-3.79)
18:0 & 22:4	2.57 (2.20-3.36) *	2.46 (2.20-2.66) *	2.01 (1.66-2.47) *	2.65 (1.78-2.95)	2.19 (1.64-2.34)	1.65 (1.52-1.98)
20:4(n-6)	19.3 (16.0-22.1)	20.4 (20.0-21.1)	18.8 (16.1-19.4)	18.2 (13.8-21.3)	17.5 (14.5-20.3)	17.4 (15.0-19.3)
22:6(n-3)	3.64 (2.51-4.52)	3.71 (3.30-5.06)	3.46 (3.23-4.57)	3.81 (3.31-4.90)	3.12 (2.81-3.43)	3.75 (3.17-5.21)

Table 6.8: Median (IQR). For between-arm comparisons (ANOVA) at each time point: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. For within-arm comparison (sign test) to baseline values: *a* $P \leq 0.05$; *b* $P \leq 0.01$; *c* $P \leq 0.001$. Output data are mass spectra corresponding to PE (or PC, above) diacylglycerol components. Predicted mass identity is listed and total percentage content of AA and DHA is listed.

6.2.6 Growth:

There were no detectable differences between the arms in any of the growth indices measured (Table 6.3). There was no difference in IGF-1 between the arms at any timepoint but all arms had a highly significant increase in IGF-1 by day 28 compared to baseline, which was sustained to day 84 (Table 6.9).

Table 6.9 Inflammatory indices.

	Enrolment			Day 7			Day 28			Day 84		
	S-RUTF	F-RUTF	FFO-RUTF	S-RUTF	F-RUTF	FFO-RUTF	S-RUTF	F-RUTF	FFO-RUTF	S-RUTF	F-RUTF	FFO-RUTF
IGF-1	44.5 (30.7-58.2)	50.3 (30.2-69.8)	44.7 (15.5-62.7)				140 (63.1-169) <i>c</i>	143 (77.9-275) <i>c</i>	114 (45.3-143) <i>b</i>	103 (65.9-180) <i>b</i>	94.4 (74.2-138)	68.8 (47.9-114) <i>a</i>
Platelets	315 (210-480)	437 (210-513)	394 (287-541)	421 (304-759)	391 (297-608)	522 (405-772) <i>a</i>	452 (321-622)	470 (323-583)	462 (291-621)	447 (323-582)	410 (359-490)	494 (344-548)
WCC	10.6 (8.50-14.8)	10.7 (8.65-14.0)	10.7 (9.40-15.9)	13.2 (8.80-15.9)	11.9 (9.90-13.5)	12.7 (10.6-14.5)	12.0 (5.90-16.3)	13.3 (8.85-15.5)	11.5 (8.90-14.0)	11.9 (9.90-14.1)	12.5 (9.10-14.5)	11.1 (8.20-13.8)
Neutrophils	3.25 (1.78-4.30)	3.24 (1.64-4.18)	3.71 (2.94-4.28)	4.85 (2.73-7.60)	3.62 (2.71-4.93)	4.47 (4.00-5.10)	3.20 (2.45-5.61)	3.97 (3.63-5.55)	3.87 (3.08-4.61)	3.22 (2.39-4.41)	2.94 (2.66-5.35)	3.45 (2.36-4.37)
Lymphocytes	5.93 (4.24-6.91)	5.74 (5.36-8.35)	5.84 (4.84-7.66)	4.80 (3.34-7.91)	5.60 (5.24-8.53)	5.92 (5.05-8.36)	5.39 (3.53-8.54)	6.82 (4.55-9.23)	6.47 (3.86-7.29)	6.81 (4.23-8.30)	8.01 (5.17-8.36)	6.48 (4.85-7.70)
Hb	9.10 (7.00-10.3)	8.45 (7.65-9.75)	9.20 (7.35-11.4)	7.90 (7.50-10.1)	9.20 (8.70-10.2)	9.20 (7.60-10.0)	10.0 (9.00-10.3) <i>b</i>	10.1 (9.60-11.0) <i>a</i>	9.70 (8.70-11.0)	10.5 (10.1-11.5) <i>b</i>	11.0 (10.9-11.6) <i>a</i>	10.5 (9.60-12.0) <i>a</i>
EndoCAB	39.7 (21.3-85.2)	52.3 (33.4-138)	60.2 (38.2-103)				51.0 (27.6-80.0)	70.7 (29.4-121)	61.8 (37.6-123)	39.9 (17.0-78.5)	60.3 (44.3-141)	70.1 (29.0-115)
sCD14	2369 (1862-2784)	2444 (2240-3399)	2298 (1965-2897)				2870 (2532-3827) <i>b</i>	3113 (2583-3351)	2551 (2246-3053)	2861 (2499-3188) <i>a</i>	2625 (2154-3277)	2567 (2036-3206)
MCP-1	16.9 (12.3-20.1)	25.4 (12.3-63.8)	18.3 (9.25-23.9)				10.7 (6.23-19.0)	17.1 (9.16-30.7) <i>c</i>	10.7 (9.22-20.0)	13.2 (9.58-21.2)	15.6 (6.96-24.8)	10.2 (7.35-17.8)
SDF-1	265 (199-314)	245 (193-295)	237 (200-307)				230 (192-258)	223 (137-265)	213 (165-253)	108 (151-257) <i>a</i>	222 (136-268)	203 (174-238) <i>b</i>
CXCL10	68.0 (36.7-93.5)	45.4 (25.9-98.8)	32.7 (19.4-58.3)				42.1 (25.6-77.9)	44.0 (39.1-57.1)	28.6 (14.3-42.3)	39.1 (24.5-57.8) <i>b</i>	31.7 (18.6-41.3) <i>a</i>	38.5 (31.4-54.3)
IL-7	2.47 (1.65-3.26)	2.52 (1.59-4.65)	2.62 (1.51-3.34)				3.89 (3.05-5.21) <i>a</i>	4.30 (2.67-4.89)	3.04 (2.04-5.39)	1.42 (1.42-1.42)	1.42 (1.42-1.42)	1.42 (1.42-1.42)
IL-8	16.7 (9.07-26.9)	15.2 (11.4-25.6)	12.5 (8.64-15.7)				9.91 (6.60-14.1)	7.18 (6.44-11.5)	6.44 (9.10-15.2)	6.44 (6.44-6.74) <i>b</i>	6.44 (6.44-6.77)	6.44 (6.44-11.5)
IL-10	4.31 (2.43-8.18)	4.65 (2.64-5.85)	3.09 (2.14-7.27)				2.48 (2.14-6.91)	2.83 (2.14-5.44)	3.22 (2.14-5.66)	2.14 (2.14-3.54) <i>b</i>	2.23 (2.14-4.19) <i>a</i>	2.14 (2.14-2.20) <i>a</i>
Eotaxin	10.2 (6.93-14.2)	9.67 (8.51-11.8)	10.6 (8.19-15.5)				9.38 (6.01-12.2)	8.45 (6.34-10.0)	7.90 (6.35-10.9)	10.8 (7.47-13.5)	8.38 (7.49-12.9)	10.2 (8.56-12.4)
IL-17a	5.12 (5.12-5.12)	5.12 (5.12-5.12)	5.12 (5.12-5.12)				5.12 (5.12-6.14)	5.12 (5.12-5.12)	5.12 (5.12-7.06)	5.12 (5.12-5.12)	5.12 (5.12-5.12)	5.12 (5.12-5.12)
IL-31	34.1 (34.1-34.1)	34.1 (34.1-34.1)	34.1 (34.1-34.1)				34.1 (34.1-34.1)	34.1 (34.1-34.1)	34.1 (34.1-34.1)	34.1 (34.1-34.1)	34.1 (34.1-34.1)	34.1 (34.1-34.1)
IL-1RA	1054 (2549-6182)	2322 (972-4289)	1228 (674-1882)				1708 (864-4446) <i>*</i>	1307 (997-1989) <i>*</i>	1036 (511-1665) <i>*</i>	1600 (743-2584)	1246 (622-2150)	1086 (572-1946)
MIP-1b	195 (94.3-287)	169 (93.6-300)	116 (94.9-209)				119 (78.8-215)	112 (81.5-166)	120 (94.5-205)	140 (111-181)	101 (60.1-168)	120 (96.9-147)
IFN- α	1.42 (1.42-1.42)	1.42 (1.42-1.42)	1.42 (1.42-1.42)				1.42 (1.42-1.42)	1.42 (1.42-1.42)	1.42 (1.42-1.42)	1.42 (1.42-1.42)	1.42 (1.42-1.42)	1.42 (1.42-1.42)
MIP-1a	76.2 (55.0-127)	64.5 (56.4-92.7)	77.1 (175-59.7)				88.7 (53.6-116)	111 (62.1-215)	64.0 (56.0-118)	80.6 (43.3-99.7)	63.5 (47.7-99.0)	57.4 (48.3-84.5)
TNF- β	10.0 (10.0-10.0)	10.0 (10.0-10.0)	10.0 (10.0-10.0)				10.0 (10.0-10.0)	10.0 (10.0-10.0)	10.0 (10.0-10.0)	10.0 (10.0-10.0)	10.0 (10.0-10.0)	10.0 (10.0-10.0)
GRO α	3.46 (2.45-4.56)	3.16 (2.07-4.25)	2.50 (2.24-3.94)				2.48 (1.43-3.70)	2.35 (2.10-3.32)	2.53 (1.43-3.51)	2.13 (1.43-3.54) <i>a</i>	1.90 (1.09-3.44)	1.66 (0.92-4.72)
IL-1 α	3.79 (1.52-12.7)	1.63 (1.52-4.42)	3.94 (1.52-5.92)				1.52 (1.52-3.04)	1.52 (1.52-1.85)	1.80 (1.52-5.84)	5.35 (1.52-7.87)	1.94 1.52-3.41)	3.02 (1.52-4.16)
IL-15	2.67 (2.50-5.90)	3.67 (2.50-7.54)	2.50 (2.50-7.01)				2.50 (2.50-9.62)	2.92 (2.50-7.24)	2.93 (2.50-8.73)	3.07 (2.50-6.76)	2.75 (2.50-5.79)	2.50 (2.50-3.47)
IL-22	216 (86.7-339)	238 (151-285)	319 (241-379)				205 (76.6-442)	293 (252-350)	323 (180-451)	76.6 (76.6-124)	76.6 (76.6-190)	93.7 (76.6-155)

Table 6.9: Median (IQR). For between-arm comparisons (ANOVA) at each time point: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. For within-arm comparison (sign test) to baseline values: *a* $P \leq 0.05$; *b* $P \leq 0.01$; *c* $P \leq 0.001$.

6.2.7 Haematologic indices:

Haemoglobin increased over the course of the trial in all arms, and there were no differences between the arms in any of the haematologic indices measured (including red cell deformability) (Figure 6.10). Marked thrombocytosis developed over the early part of the study in some participants. There was no clinical evidence of coagulopathy in any participant at any point in the trial.

Figure 6.10: A: Red cell deformability measured by LORCA for the three arms at day 84, compared to the mean baseline values for all participants. † indicates $P \leq 0.05$ for the S-RUTF arm compared to baseline (sign test) and ‡ indicates $P \leq 0.05$ for the FFO-RUTF arm compared to baseline (sign test). All other tests (including between group by ANOVA) $P > 0.05$. **B:** Haemoglobin and platelets by arm: For within-arm comparison (sign test) to baseline values: † $P \leq 0.05$.

6.2.8 Inflammatory indices:

There were no major differences in soluble inflammatory markers in plasma between the arms at any time point (Table 6.9). There was a general reduction in levels of both pro- (e.g. IL-8, CXCL10) and anti-inflammatory (e.g. IL-10) cytokines during the course of the study. There were no differences in the proportion of activated (CD38, HLA-DR co-expressing with/without PD-1 expression) CD4 or CD8 T cells between groups at day 84, though the proportion of PD-1 co-expressing CD8 T cells had fallen in the S-RUTF and F-RUTF arms (Table 6.10). There was no significant difference between the arms in IFN- γ release upon stimulation with PHA with or without IL-12, or TT with IL-12, at enrolment or day 84. Modest increases in IFN- γ release in both of the PHA stimulation conditions were most pronounced in the F-RUTF and FFO-RUTF arms. IFN- γ release induced by TT without IL-12 was undetectable in most participants (Table 6.10). There were no significant associations between any of the T cell functional parameters and corresponding T cell LC-PUFA compositional indices (Figure 6.11).

Table 6.10 T cell functional indices.

	Enrolment			Day 84		
	S-RUTF	F-RUTF	FFO-RUTF	S-RUTF	F-RUTF	FFO-RUTF
CD4: CD38+ HLA-DR+	11.9 (9.44-17.6)	9.25 (7.00-16.0)	11.4 (8.49-14.2)	12.3 (11.2-14.2)	14.5 (10.3-18.6)	17.3 (8.07-20.5)
CD4: CD38+ HLA-DR+ PD-1+	5.49 (3.77-7.82)	4.64 (3.22-6.93)	5.67 (3.33-6.90)	6.02 (4.33-7.49)	6.36 (4.60-8.26)	6.70 (3.36-10.8)
CD8: CD38+ HLA-DR+	46.6 (38.6-54.9)	48.9 (34.5-60.0)	49.2 (24.6-65.0)	47.4 (29.9-70.0)	51.6 (40.7-62.0)	51.6 (37.1-62.3)
CD8: CD38+ HLA-DR+ PD-1+	23.5 (15.6-28.9)	24.6 (11.3-36.1)	18.6 (9.35-32.3)	17.5 (11.1-28.7)	18.2 (14.5-22.8)	20.1 (16.6-22.7)
IFNg release: PHA	1017 (434-2086)	722 (135-3090)	273 (8-600)	827 (325-3927)	1127 (224-1526)	970 (572-1504)
IFNg release: PHA+IL-12	4510 (2802-7579)	3338 (1724-11681)	3569 (2132-5766)	4525 (2050-12519)	5207 (3156-9138)	5109 (3520-8841)
IFNg release: TT+IL-12	303 (69-765)	233 (86-1225)	203 (35-328)	184 (48-489)	83 (56-204)	214 (69-965)

Table 6.10: Percentage co-expression of CD38 and HLA-DR; and CD38, HLA-DR and PD-1 on CD4+ or CD8+ T lymphocytes. Interferon-gamma release (with background subtraction) upon stimulation with phytohemagglutinin (PHA) +/- IL-12 or tetanus toxoid (TT) + IL-12 in pg/ml. Median (IQR). For between-arm comparisons (ANOVA) at each time point: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. For within-arm comparison (sign test) to baseline values: *a* $P \leq 0.05$; *b* $P \leq 0.01$; *c* $P \leq 0.001$

Figure 6.11 T cell compositional/functional correlations

	CD4+ (CD38+ HLADR+)	Δ CD4+ (CD38+ HLADR+)	CD8+ (CD38+ HLADR+)	Δ CD8+ (CD38+ HLADR+)	CD4+ (CD38+ HLADR+ PD1+)	Δ CD4+ (CD38+ HLADR+ PD1+)	CD8+ (CD38+ HLADR+ PD1+)	Δ CD8+ (CD38+ HLADR+ PD1+)	PHA (IFNg release)	Δ PHA (IFNg release)	PHA+IL12 (IFNg release)	Δ PHA+IL12 (IFNg release)	DHA	Δ DHA	EPA	Δ EPA	AA	Δ AA
CD4+ (CD38+ HLADR+)																		
Δ CD4+ (CD38+ HLADR+)	+																	
CD8+ (CD38+ HLADR+)	+																	
Δ CD8+ (CD38+ HLADR+)	+	+	+															
CD4+ (CD38+ HLADR+ PD1+)	+	+	+	+														
Δ CD4+ (CD38+ HLADR+ PD1+)		+			+	+												
CD8+ (CD38+ HLADR+ PD1+)	+		+		+													
Δ CD8+ (CD38+ HLADR+ PD1+)		+		+	+	+												
PHA (IFNg release)	+																	
Δ PHA (IFNg release)	+	+							+									
PHA+IL12 (IFNg release)		-																
Δ PHA+IL12 (IFNg release)		+							+	+	+							
DHA																		
Δ DHA																		
EPA						+		+					+					
Δ EPA		+				+		+						+	+			
AA					-	-												
Δ AA					-		-							+			+	

Figure 6.11: Selected key T cell phenotypic and functional characteristics alongside CD3+ PC content for DHA, EPA and AA. Values are Day 84 readouts, or differences between day 84 and baseline (Δ). Cells show direction of relationship by Spearman's rank correlation with statistical significance level indicated by colour: Red (P<0.001), Orange (P<0.01), Yellow (P<0.05), White (P<0.10).

6.3 DISCUSSION

This study has shown that treatment of SAM with conventional RUTF is associated with a decline in DHA status. The production and administration of RUTF with elevated n-3 PUFA (as ALA), with or without additional fish oil as a supplement, to children with complicated SAM is technically feasible, acceptable to patients and their carers, and safe: the 10% mortality rate was in line with our and others' experience treating this extremely vulnerable population.^{107, 110-112} Provision of RUTF with elevated ALA had minimal impact on n-6 and n-3 PUFA status, but addition of fish oil was associated with marked increases in n-3 LC-PUFA across multiple compartments. Importantly, in both arms without fish oil, percentage composition of DHA in erythrocytes declined suggesting an absolute requirement for pre-formed DHA in the nutritional management of SAM.

An essential aim in the design of RUTF is to provide optimal nutrient intake for growth and development of somatic and neural tissues. RUTF compositional specifications were based on infant formula guidelines, but in the period since they were first developed a new consensus has emerged that pre-formed LC-PUFA should be added to infant formula in view of the fact that formula-fed infants' erythrocyte DHA declines without such exogenous provision.²⁸² Dietary supply of DHA is considered conditionally essential for infants and young children.²⁷⁷ Our data have shown that similar declines occur during nutritional rehabilitation of SAM using RUTF according to current compositional guidelines. The impact of small relative decreases in erythrocyte DHA content in this context remains unclear. However, DHA is a major component of neural lipid, and deficiency during early childhood has been linked to

a range of neurodevelopmental abnormalities.²⁸³ Children with SAM are at risk of long-term cognitive and behavioural deficits,²⁸⁴ and it is plausible that inadequate DHA provision during nutritional rehabilitation could be an exacerbating factor. Because routinely providing pre-formed n-3 LC-PUFA to children with SAM may have substantial resource and practical implications, policy on formulation should be based on a clear assessment of clinical (especially neurodevelopmental) utility, and trials evaluating these outcomes should be prioritised. In 2011 RUTF was provided to 1.96 million children, fewer than 10% of the many millions who needed it.¹⁰¹ Concerns around its composition should not detract from the clear current imperative to support and expand coverage.

ALA can theoretically act as a substrate for biosynthesis of all the longer chain n-3 PUFA family members but participants in the F-RUTF arm, who received far more ALA than those in the S-RUTF arm, saw relatively modest changes in n-3 LC-PUFA after three months' treatment. Point estimates for percentage content of EPA and docosapentaenoic acid (DPA, 22:5(n-3)) were increased, but the failure to impact positively on DHA was notable. The results bear comparison to previous clinical studies, which have suggested that while provision of ALA can be shown to drive increases in EPA and DPA content, metabolic conversion to DHA is inefficient, and a dietary supply of preformed DHA is conditionally essential for its tissue enrichment.²⁸⁵⁻²⁸⁷ Recent experimental work suggests that provision of large quantities of dietary ALA might inadvertently further limit its already slow metabolism to DHA. By systematically varying LA and ALA intake in rats, Gibson *et al.* found that increasing ALA above an optimal concentration was independently

associated with a decrease in tissue DHA accumulation due to competitive inhibition of a part of the conversion step from DPA to DHA catalysed by $\Delta 6$ desaturase.²⁸⁸ Similar inhibition may occur by competition for elongase enzyme activity.²⁸⁹ It is therefore possible that although by providing elevated ALA content we increased the available substrate for DHA biosynthesis, ALA may have inhibited those same metabolic pathways to which it is subject, rendering the supplementation futile as regards DHA accretion. Although it is reassuring that children in the arm receiving elevated ALA without fish oil did not see a decrease in DHA levels beyond that seen with the standard RUTF formulation, this theoretical concern means that we do not believe that RUTF formulations with high ALA content should be taken forward to further clinical trials. Furthermore, although desaturase and elongase activity was similar between the arms, the fact that AA differed after three months' follow-up is difficult to explain by any means other than an ALA-dependent decrease in LA conversion to AA. This alone might have had important consequences, since AA status has been related to growth.²⁹⁰

An alternative strategy to improve DHA accretion during nutritional rehabilitation may be to reduce the n-6 PUFA content of RUTF, because it is well recognised that n-6 PUFA can interfere with desaturation and elongation of ALA.²⁹¹ An attractive feature of such an approach is the possible avoidance of problems associated with stability during storage for products with increased PUFA content. The fact that the flax seed oil-containing RUTF became unusable after just a year despite packaging under nitrogen and storage below 25°C presents a major practical impediment to its use in countries or areas with limited resources. There are precedents for such an

approach, although the effectiveness in terms of DHA accumulation in clinical studies has been modest to date.²⁹²⁻²⁹⁴ An important consideration will be to ensure that n-6 LC-PUFA status itself is not compromised, since this may have adverse consequences for growth, as noted previously.²⁹⁰ Notwithstanding these concerns, a major part of the reason that RUTF has been successful is that it is straightforward to administer, and the development of a formulation that could address DHA accretion without resorting to provision of encapsulated fish oil should be the eventual aim. In pilot work (unpublished) I attempted to synthesise an RUTF containing fish oil as part of the RUTF mix, but it quickly became rancid. There is considerable interest in the production of n-3 LC-PUFA-based products that are resistant to environmental oxidation, but achieving durable stability as part of a mix with highly oxidising micronutrients such as iron will be a major challenge. On the other hand, a recently published paper describing the results of a clinical trial of using RUTF with reduced LA and elevated ALA in the treatment of SAM in Malawi, has suggested that even higher doses of ALA than employed in Njugu Plus might have benefit.²⁹⁵ In this study, treatment with standard RUTF was associated with a decrease in DHA content of plasma phospholipids after four weeks, but the modified RUTF protected against this decline. There was no evidence of a detrimental impact on growth or recovery from SAM, in fact children in the modified RUTF arm experienced a greater improvement in WHZ/WLZ than those in the standard arm, although the high prevalence of kwashiorkor at baseline means that this result is difficult to interpret. However although the modified formulation contained less LA than their standard (in which LA comprised 21.3% of total fatty acids), its LA content was similar to Plumpy'nut, the most widely used RUTF product, and the major difference was the extremely

elevated ALA level – at 13.1% of total fatty acids, more than twice that in our flax seed oil-enriched formulation. Our experience suggests that achieving long-term stability of a preparation with such high ALA content may be difficult, but if stability could be achieved then this formulation might minimise DHA depletion whilst maintaining the practicality of an exclusively RUTF-based regime.

Although there is no precise cut-off for defining DHA deficiency, Luxwolda *et al.* demonstrated that transplacental materno-fetal transfer of DHA is actively regulated towards providing infants with 5.9% DHA composition in erythrocytes at birth, and it is hypothesised that DHA composition of around 7% is optimal for prevention of some non-communicable diseases in adulthood.²⁹⁶⁻²⁹⁸ By comparison, children enrolled in this study had marginally low baseline DHA (median (IQR): 5.2% (3.7-6.7)), but regulated their erythrocyte composition at around 7% in the presence of preformed DHA in the diet. Breastfeeding was partially protective, since breastfed children had a median baseline DHA of 6.3% (IQR 4.5-7.4).

While DHA content appeared to be regulated, EPA did not – the greatest increases in erythrocyte EPA with fish oil provision being among those participants with the highest baseline values. Percentage EPA composition reached levels equivalent to those seen in populations that consume very large amounts of marine fish.²⁹⁹ It is plausible that providing preformed EPA (but not, apparently, DHA) bypasses a physiologically beneficial regulatory checkpoint, and detailed assessment of safety outcomes should form an important component of future studies if oils containing high levels of EPA, as used here, are employed. I deliberately elected to use fish oil

with high EPA content because I hypothesised that its anti-inflammatory properties might be beneficial in the context of SAM. The lack of any measurable impact on inflammation in this study further discourages the use of such oils in future work. Understanding the kinetics of EPA accumulation would be helped by fatty acid desaturase gene cluster profiling, given that common polymorphisms could have a substantial impact on PUFA metabolic responses to supplementation.³⁰⁰ This should be considered as a component of further research.

This study builds on previous work, which has been described in Chapter 5. Of particular relevance, Smit *et al.* randomised 17 Pakistani children with low weight-for-age z-score to 500 mg/day of fish oil alongside standard care (n=10), which consisted of multivitamin provision and parental nutritional education, or standard care alone for up to 12 weeks.¹⁹⁰ They demonstrated a marked increase in erythrocyte composition of all n-3 LC-PUFA in the intervention arm but no change from baseline in the controls. Koletzko *et al.* demonstrated an increase in n-3 LC-PUFA in plasma phospholipids from baseline in a cohort of 8 Nigerian children with SAM after two weeks of follow-up, when provided a rehabilitation diet that contained fish.¹⁷⁹ In a recent controlled trial of fish oil supplementation for young infants without SAM in the Gambia, van der Merwe *et al.* effected increases in plasma n-3 LC-PUFA and in MUAC in the intervention group.¹¹⁶ There was no recorded differences in intestinal health, frequency of illness or neurocognitive development, but breastfeeding rates were high in the trial and the mothers' breast milk was rich in DHA, meaning that the participants were much more likely to be n-3 LC-PUFA replete than those in Njugu Plus.

N-6 and n-3 LC-PUFA and their metabolites are potent immunomodulatory agents, with n-6 PUFA family members being broadly pro-inflammatory and n-3, anti-inflammatory.¹⁷⁵ Children with SAM have a chronic inflammatory T-cell associated enteropathy (environmental enteric dysfunction, EED), which may be partly maladaptive and disrupt beneficial effects of nutritional rehabilitation.³⁰¹ Provision of RUTF with a high n-6 PUFA content could exacerbate this inflammatory activation, thereby further disturbing mucosal homeostasis and contributing to worsening of gastrointestinal symptoms, increase in microbial translocation, and persistent growth failure. However, although Njugu Plus demonstrated major differences in n-3 PUFA composition between the three arms across multiple compartments, there was no evidence of consistent or major impact on any of the numerous immunologic and inflammatory indices measured in exploratory analyses. Hospitalised children with SAM are an extremely heterogeneous group. Some have or are recovering from a major infection, others have chronic illnesses and some present early without major medical or metabolic complications, but require a short period of hospital care because of inadequate facilities for care at home. A wide range of inflammatory and immunologic status would therefore be expected at baseline and the impossibility of controlling or correcting for this in a small trial render the likelihood of a Type 2 error, high. Additionally, while understanding of the importance of PUFA in immune function is informed by a wealth of data from model and experimental settings encompassing a wide range of methodological approaches, our cellular and molecular understanding of the functionally immunocompromised state associated with SAM is extremely limited.^{35, 175} Choice of immune/inflammatory assays was

hypothesis-based but evidence underlying these hypotheses is weak. Further careful observational work to determine the relationship between enteric inflammation, mucosal and systemic immune function, and nutritional status is a research priority, and the utilisation of exploratory and systems biological approaches are likely to be useful in challenging established doctrine.

Although this trial was well powered to assess for its primary outcome, it was not designed or powered to assess for clinically important secondary outcomes such as growth and frequency of infectious episodes. The safety and acceptability of the approach employed provides a sound foundation for future trials targeting such outcomes on a much larger scale. Although I believe that the high drop-out rate in the F-RUTF arm is likely to be stochastic (it did not occur in FFO-RUTF arm, who also received the flax seed oil-containing product), the fact that this arm did not enrich for DHA and quickly became unusable, suggests that simply increasing the stipulated ALA content of RUTF is not a sufficient adaptation to current standards, and such a formulation is not a rational one to take forward for larger-scale trials. Low n-6 PUFA content formulations are potential candidates, but for trials designed to show proof of concept that raising n-3 LC-PUFA has clinical and growth benefits, providing the pre-formed molecules themselves may provide the clearest, quickest and most unambiguous answer.

CHAPTER 7

Future research directions

The work presented in this thesis has provided evidence that:

1. SAM is not associated with abnormalities in systemic cytokine response to innate immune challenge in an *ex vivo* model compared to control children (Chapter 3).
2. Both more severe intestinal inflammation, and kwashiorkor, may be associated with reduced systemic cytokine response to innate immune challenge in an *ex vivo* model (Chapter 3).
3. Children with SAM and stunting have clear evidence of enteric inflammation (Chapter 4).
4. Children with SAM and stunting have evidence of cytokine-mediated inhibition of the GH-IGF-1 axis, which may play a role in linear growth failure (Chapter 4).
5. Treatment of children with SAM and stunting with mesalazine appeared to be safe, and there was limited evidence of an impact on enteric inflammation (Chapter 4).
6. Stunting is associated with LC-PUFA deficiency, but acute malnutrition is not (Chapter 5).
7. Production and administration of an RUTF with elevated n-3 PUFA was technically feasible, but was not associated with positive outcomes in growth, recovery or immune function compared to standard treatment (Chapter 6).
8. Provision of pre-formed DHA in RUTF may be essential to preserve optimal tissue levels (Chapter 6).

These findings have revealed or highlighted a number of areas that could be the focus of further research.

7.1 *Understanding gut function/inflammation in SAM*

Our understanding of the pathogenesis of EED owes much to pioneering studies conducted using small intestinal biopsy techniques.^{76, 156} In those with children, SAM is disproportionately prevalent, meaning that separating out the independent (or cumulative) effects of nutritional and environmental exposures on gut health has proven difficult.^{76, 77} At least one study is currently recruiting children across a range of nutritional status for small intestinal biopsy (led by Asad Ali, Aga Khan University, Pakistan). Such studies are essential, for understanding the immune and inflammatory pathways important in generation of the observed impairments in epithelial function should be used to guide trials of therapeutic interventions. The compartmentalisation of the enteric immune system means that tissue is required for robust analysis. It is possible that the intestinal lesion is different between SAM and (non-SAM) EED, particularly in the case of kwashiorkor, and may respond differently to different interventions. The fact that mesalazine appears to be safe to take forward to larger-scale studies and that other gut-specific immunomodulatory agents are becoming available provides a clear incentive for improving our understanding of the enteric immunopathogenesis both of EED and SAM-enteropathy.^{302, 303} Such studies also provide an opportunity to test and refine better biomarkers of enteric inflammation and microbial translocation, since the performance of some widely utilised tests (e.g. EndoCAb) has been called into question.⁶⁶

Work with animal models has the potential to provide important insights into nutritional and environmental modulators of enteric inflammation. Murine models of weanling undernutrition have revealed potentiation of infection and tissue damage by common intestinal pathogens.^{92, 304} A model of EED that incorporates modulation of nutritional intake and intestinal microbiome has been shown to drive accumulation of intraepithelial lymphocytes in the duodenum with enhanced cytokine release.³⁰⁵ Combining clinical observation with experimental pre-clinical work has led to the development of highly efficacious therapies for IBD.³⁰⁶ The SAM/EED field can learn from this success.

7.2 Further trials of immunomodulation in EED

Provision of mesalazine to children with SAM, stunting, and evidence of EED appeared to be safe and acceptable. The mesalazine trial reported herein was very small, and while this was an integral part of its design, the low absolute number of severe adverse events means that assessing the extent to which it was adequately powered for safety is not possible. However, and notwithstanding the need to direct immunomodulation against targets defined by understanding of these conditions' immunopathology, larger-scale safety and efficacy trials can be countenanced. I believe the most logical way forward is to proceed to a Phase 2-style study powered to demonstrate reduction in faecal calprotectin as a biomarker for intestinal inflammation. An effect size equivalent to that seen in the pilot trial reported herein but extrapolated to three months (provided by longer duration of treatment), would equate to an approximate 40% reduction in calprotectin in the intervention arm. A

study with 90% power to detect such a reduction at a significance level of 0.05 would require 85 children in each arm (not allowing for withdrawal or loss to follow-up) ^{*}.

Although the pilot study enrolled children with SAM and stunting, establishing efficacy may be easier in less acutely malnourished children, based on the assumption that gut structure and function is closer to normal. This might mean that the requirement for an induction (of remission) agent is less pronounced and an agent, like mesalazine, that is more effective in maintenance of remission (in IBD, at least) would be more appropriate. I believe that recruitment of children with MAM ($11.5 \text{ cm} \leq \text{MUAC} < 12.5 \text{ cm}$) and stunting ($\text{WHZ}/\text{WLZ} < -2$) aged between 6 and 18 months would strike the right balance between offering the intervention to a group with the most to gain, but who also have a good chance of achieving normalisation. Treatment for three months would provide an adequate opportunity to assess efficacy. Nutritional and WASH support would need to be provided alongside the interventional product. Different dosing strategies could be employed if more than active/placebo arms were achievable, potentially including a short quasi-induction phase of high-dose treatment. Blood tests would be needed for monitoring toxicity, and could be used to assess systemic inflammatory activation, microbial translocation, with IGF-1 and IGF-binding protein 3 as indices of growth potential. Further studies would be required to assess for impact on clinically important or growth outcomes, for which I suspect sample sizes would run into the hundreds.

^{*} Sample size calculation uses log-transformed calprotectin data. Mean log(calpro) was 6.5 (S.D. 1.0).
Sample size calculation in Stata: *"sampsi 6.5 6.0, sd1(1.0) sd2(1.0) power(.90)"*

Agents with therapeutic potential other than mesalazine exist, including:

- Exclusive enteral nutrition (EEN) is the standard of care for induction of remission for most cases of paediatric Crohn's disease. The mechanism of action remains unclear, but may involve modulation of the intestinal microbiome. A trial of short-course EEN in the management of complicated SAM is underway in Malawi (ClinicalTrials.gov NCT02639416). Whether or not such an approach would be achievable outside a clinical trial setting is unclear, since EEN involves milk based feeds, which cannot generally be safely implemented outside a hospital environment. The generation of an EEN-appropriate RUTF formulation may be possible, and should be a focus of research.
- Oral budesonide is moderately efficacious in induction of remission for Crohn's disease and is associated with few systemic side effects due to first-pass metabolism.³⁰⁷ There are anecdotal reports of efficacy in a range of conditions associated with small intestinal villous atrophy.³⁰⁸⁻³¹⁰
- Larazotide acetate is a first-in-class intestinal epithelial tight junction stabiliser that has shown some promise in the treatment of adults with coeliac disease who remain symptomatic despite gluten-free diet.³¹¹

7.3 Further trials of LC-PUFA in SAM

The Njugu Plus trial provided a mechanism by which significant alterations could be made to erythrocyte and plasma LC-PUFA status. In particular, the use of preformed DHA in fish oil facilitated far greater enrichment than the only similar study.²⁹⁵ However, such an approach would be expensive to provide at scale given current

overheads and likely supply chain. Therefore, in my opinion the logical next step is to conduct a large-scale efficacy trial of conventional-recipe RUTF +/- fish oil capsules with clinically relevant endpoints. Such a study would recruit most efficiently if targeting uncomplicated SAM in a setting with high throughput.

A suitable primary outcome might be a composite of death, hospitalisation or failure to recover from SAM at 12 weeks post-enrolment. A sample size of 1722 children would give 80% power to detect a hazard ratio of 0.7 (assuming an conservative overall failure rate (including defaulters) of 15% - note that Sphere criteria for acceptable cure rate is >75%). In Njugu Plus, large differences in LC-PUFA were achieved by day 28, so the fish oil capsules could be given for a set period (28 days) rather than needing to continue throughout nutritional rehabilitation, meaning a single administration could be performed, which would simplify logistics. Because the study would rely on hard clinical endpoints, I believe it could be run open-label. This is important, because in Njugu Plus it became apparent that fish oil gave participants' stools a distinctive odour that would be difficult to control for or eliminate.

Secondary outcomes would be growth and frequency of minor infections or illnesses. Because DHA may be important in neurodevelopment, it would be beneficial to assess for a longer-term impact on this. Several tools exist that have been optimised for use in resource-limited and African settings.^{312, 313}

While a key objective of RUTF design is to keep the formula affordable in order to expand coverage, this should not stifle innovation. If fish oil were associated with significant improvements in clinically relevant outcomes, then the job of trying to make such a strategy cost-minimal would be critical. For now, seeing if there is a worthwhile effect to work towards is key.

References

1. Black RE, Allen LH, Bhutta ZA, Caulfield LE, de Onis M, Ezzati M, et al. Maternal and child undernutrition: global and regional exposures and health consequences. *Lancet*. 2008; 371(9608): 243-60.
2. Ashworth A, Jackson A, Khanum S, Schofield C. Ten steps to recovery. *Child health dialogue*. 1996; (3-4): 10-2.
3. UNICEF, WHO, World Bank. Levels and trends in child malnutrition. Joint child malnutrition estimates. 2012. Available at:
http://www.who.int/nutgrowthdb/jme_unicef_who_wb.pdf
4. Black RE, Victora CG, Walker SP, Bhutta ZA, Christian P, de Onis M, et al. Maternal and child undernutrition and overweight in low-income and middle-income countries. *Lancet*. 2013; 382(9890): 427-51.
5. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajani-refah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*. 2014; 345(6204): 1251086.
6. Hall JA, Grainger JR, Spencer SP, Belkaid Y. The role of retinoic acid in tolerance and immunity. *Immunity*. 2011; 35(1): 13-22.
7. Stock A, Booth S, Cerundolo V. Prostaglandin E2 suppresses the differentiation of retinoic acid-producing dendritic cells in mice and humans. *The Journal of experimental medicine*. 2011; 208(4): 761-73.
8. Ericksen P TP, Notenbaert A, Cramer L, Jones P, Herrero M. Mapping hotspots of climate change and food insecurity in the global tropics. CGIAR Research Program on Climate Change, Agriculture and Food Security (CCAFS). Copenhagen, Denmark; 2011. Available at: www.ccafs.cgiar.org
9. Wang JQ, Jeelall YS, Ferguson LL, Horikawa K. Toll-Like Receptors and Cancer: MYD88 Mutation and Inflammation. *Frontiers in immunology*. 2014; 5: 367.
10. WHO. Pocket book of hospital care for children. 2005. Available at:
www.who.int/maternal_child_adolescent/documents/9241546700/en/index.html
11. WHO. Updates on the management of severe acute malnutrition in infants and children: Guideline. 2013. Available at:
www.who.int/nutrition/publications/guidelines/updates_management_SAM_infant_andchildren/en/index.html

12. Briend A. Use of MUAC for Severe Acute Malnutrition. 2012 Available at: [http://www.cmamforum.org/Pool/Resources/FAQ-1-Use-of-MUAC-Briend-Eng-June-2012\(1\).pdf](http://www.cmamforum.org/Pool/Resources/FAQ-1-Use-of-MUAC-Briend-Eng-June-2012(1).pdf)
13. Briend A, Myatt M, Dent, N, Brown, R. Putting kwashiorkor on the map. CMAM Forum. 2013. Available at: www.cmamforum.org/Pool/Resources/Putting-kwashiorkor-on-the-map-CMAM-Forum-2013.pdf
14. Kerac M, Blencowe H, Grijalva-Eternod C, McGrath M, Shoham J, Cole TJ, et al. Prevalence of wasting among under 6-month-old infants in developing countries and implications of new case definitions using WHO growth standards: a secondary data analysis. Archives of disease in childhood. 2011; 96(11): 1008-13.
15. Briend A, Collins S, Golden M, Manary M, Myatt M. Maternal and child nutrition. Lancet. 2013; 382(9904): 1549.
16. Isanaka S, Grais RF, Briend A, Checchi F. Estimates of the duration of untreated acute malnutrition in children from Niger. American journal of epidemiology. 2011; 173(8): 932-40.
17. Vollmer S, Harttgen K, Subramanyam MA, Finlay J, Klasen S, Subramanian SV. Association between economic growth and early childhood undernutrition: evidence from 121 Demographic and Health Surveys from 36 low-income and middle-income countries. The Lancet Global health. 2014; 2(4): e225-34.
18. World Bank. Africa's Pulse. 2013. Available at: http://www.worldbank.org/content/dam/Worldbank/document/Africa/Report/Africa-Pulse-brochure_Vol8.pdf
19. Pelletier DL, Frongillo EA, Jr., Schroeder DG, Habicht JP. The effects of malnutrition on child mortality in developing countries. Bulletin of the World Health Organization. 1995; 73(4): 443-8.
20. Bejon P, Mohammed S, Mwangi I, Atkinson SH, Osier F, Peshu N, et al. Fraction of all hospital admissions and deaths attributable to malnutrition among children in rural Kenya. The American journal of clinical nutrition. 2008; 88(6): 1626-31.
21. Coly AN, Milet J, Diallo A, Ndiaye T, Benefice E, Simondon F, et al. Preschool stunting, adolescent migration, catch-up growth, and adult height in young senegalese men and women of rural origin. The Journal of nutrition. 2006; 136(9): 2412-20.
22. Victora CG, Adair L, Fall C, Hallal PC, Martorell R, Richter L, et al. Maternal and child undernutrition: consequences for adult health and human capital. Lancet. 2008; 371(9609): 340-57.

23. Adair LS, Fall CH, Osmond C, Stein AD, Martorell R, Ramirez-Zea M, et al. Associations of linear growth and relative weight gain during early life with adult health and human capital in countries of low and middle income: findings from five birth cohort studies. *Lancet*. 2013; 382(9891): 525-34.
24. Victora CG, de Onis M, Hallal PC, Blossner M, Shrimpton R. Worldwide timing of growth faltering: revisiting implications for interventions. *Pediatrics*. 2010; 125(3): e473-80.
25. Hoddinott J, Behrman JR, Maluccio JA, Melgar P, Quisumbing AR, Ramirez-Zea M, et al. Adult consequences of growth failure in early childhood. *The American journal of clinical nutrition*. 2013; 98(5): 1170-8.
26. Martorell R, Horta BL, Adair LS, Stein AD, Richter L, Fall CH, et al. Weight gain in the first two years of life is an important predictor of schooling outcomes in pooled analyses from five birth cohorts from low- and middle-income countries. *The Journal of nutrition*. 2010; 140(2): 348-54.
27. Case AP, C. Stature and Status: Height, Ability, and Labor Market Outcomes. *Journal of Political Economy*. 2008; 116(3): 499-532.
28. Fall CH. Fetal malnutrition and long-term outcomes. *Nestle Nutrition Institute workshop series*. 2013; 74: 11-25.
29. Sebayang SK, Dibley MJ, Kelly PJ, Shankar AV, Shankar AH. Determinants of low birthweight, small-for-gestational-age and preterm birth in Lombok, Indonesia: analyses of the birthweight cohort of the SUMMIT trial. *Tropical medicine & international health : TM & IH*. 2012; 17(8): 938-50.
30. Addo OY, Stein AD, Fall CH, Gigante DP, Guntupalli AM, Horta BL, et al. Parental childhood growth and offspring birthweight: Pooled analyses from four birth cohorts in low and middle income countries. *American journal of human biology : the official journal of the Human Biology Council*. 2014. DOI 10.1002/ajhb.22614
31. Scrimshaw NS, Taylor CE, Gordon JE. Interactions of nutrition and infection. *Monograph series World Health Organization*. 1968; 57: 3-329.
32. Scrimshaw NS, Taylor CE, Gordon JE. Interactions of nutrition and infection. *The American journal of the medical sciences*. 1959; 237(3): 367-403.
33. Richard SA, Black RE, Gilman RH, Guerrant RL, Kang G, Lanata CF, et al. Diarrhea in early childhood: short-term association with weight and long-term association with length. *American journal of epidemiology*. 2013; 178(7): 1129-38.

34. Liberato SC, Singh G, Mulholland K. Zinc supplementation in young children: A review of the literature focusing on diarrhoea prevention and treatment. *Clin Nutr*. 2014. DOI 10.1016/j.clnu.2014.08.002
35. Rytter MJ, Kolte L, Briend A, Friis H, Christensen VB. The immune system in children with malnutrition--a systematic review. *PloS one*. 2014; 9(8): e105017.
36. Smith R. Investigating the previous studies of a fraudulent author. *BMJ*. 2005; 331(7511): 288-91.
37. Boussiotis VA, Tsai EY, Yunis EJ, Thim S, Delgado JC, Dascher CC, et al. IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. *The Journal of clinical investigation*. 2000; 105(9): 1317-25.
38. Harland PS. Tuberculin reactions in malnourished children. *Lancet*. 1965; 2(7415): 719-21.
39. Geefhuysen J, Rosen EU, Katz J, Ipp T, Metz J. Impaired cellular immunity in kwashiorkor with improvement after therapy. *British medical journal*. 1971; 4(5786): 527-9.
40. Schlesinger L, Stekel A. Impaired cellular immunity in marasmic infants. *The American journal of clinical nutrition*. 1974; 27(6): 615-20.
41. Smythe PM, Brereton-Stiles GG, Grace HJ, Mafoyan A, Schonland M, Coovadia HM, et al. Thymolymphatic deficiency and depression of cell-mediated immunity in protein-calorie malnutrition. *Lancet*. 1971; 2(7731): 939-43.
42. McMurray DN, Loomis SA, Casazza LJ, Rey H, Miranda R. Development of impaired cell-mediated immunity in mild and moderate malnutrition. *The American journal of clinical nutrition*. 1981; 34(1): 68-77.
43. Moore DL, Heyworth B, Brown J. PHA-induced lymphocyte transformations in leucocyte cultures from malarious, malnourished and control Gambian children. *Clinical and experimental immunology*. 1974; 17(4): 647-56.
44. Vasquez-Garibay E, Campollo-Rivas O, Romero-Velarde E, Mendez-Estrada C, Garcia-Iglesias T, Alvizo-Mora JG, et al. Effect of renutrition on natural and cell-mediated immune response in infants with severe malnutrition. *Journal of pediatric gastroenterology and nutrition*. 2002; 34(3): 296-301.
45. McMurray DN, Watson RR, Reyes MA. Effect of renutrition on humoral and cell-mediated immunity in severely malnourished children. *The American journal of clinical nutrition*. 1981; 34(10): 2117-26.

46. Hughes SM, Amadi B, Mwiya M, Nkamba H, Tomkins A, Goldblatt D. Dendritic cell anergy results from endotoxemia in severe malnutrition. *J Immunol.* 2009; 183(4): 2818-26.
47. de Vries E. Patient-centred screening for primary immunodeficiency: a multi-stage diagnostic protocol designed for non-immunologists. *Clinical and experimental immunology.* 2006; 145(2): 204-14.
48. Liston A, Enders A, Siggs OM. Unravelling the association of partial T-cell immunodeficiency and immune dysregulation. *Nature reviews Immunology.* 2008; 8(7): 545-58.
49. Savy M, Edmond K, Fine PE, Hall A, Hennig BJ, Moore SE, et al. Landscape analysis of interactions between nutrition and vaccine responses in children. *The Journal of nutrition.* 2009; 139(11): 2154S-218S.
50. Malave I, Vethencourt MA, Pirela M, Cordero R. Serum levels of thyroxine-binding prealbumin, C-reactive protein and interleukin-6 in protein-energy undernourished children and normal controls without or with associated clinical infections. *Journal of tropical pediatrics.* 1998; 44(5): 256-62.
51. Rikimaru T, Taniguchi K, Yartey JE, Kennedy DO, Nkrumah FK. Humoral and cell-mediated immunity in malnourished children in Ghana. *European journal of clinical nutrition.* 1998; 52(5): 344-50.
52. Ekanem EE, Umotong AB, Raykundalia C, Catty D. Serum C-reactive protein and C3 complement protein levels in severely malnourished Nigerian children with and without bacterial infections. *Acta Paediatr.* 1997; 86(12): 1317-20.
53. Akenami FO, Koskiniemi M, Siimes MA, Ekanem EE, Bolarin DM, Vaheri A. Assessment of plasma fibronectin in malnourished Nigerian children. *Journal of pediatric gastroenterology and nutrition.* 1997; 24(2): 183-8.
54. Sauerwein RW, Mulder JA, Mulder L, Lowe B, Peshu N, Demacker PN, et al. Inflammatory mediators in children with protein-energy malnutrition. *The American journal of clinical nutrition.* 1997; 65(5): 1534-9.
55. Doherty JF, Golden MH, Raynes JG, Griffin GE, McAdam KP. Acute-phase protein response is impaired in severely malnourished children. *Clin Sci (Lond).* 1993; 84(2): 169-75.
56. Paiardini M, Muller-Trutwin M. HIV-associated chronic immune activation. *Immunological reviews.* 2013; 254(1): 78-101.
57. Brandacher G, Hoeller E, Fuchs D, Weiss HG. Chronic immune activation underlies morbid obesity: isIDO a key player? *Current drug metabolism.* 2007; 8(3): 289-95.

58. Mariani F, Sena P, Roncucci L. Inflammatory pathways in the early steps of colorectal cancer development. *World journal of gastroenterology : WJG*. 2014; 20(29): 9716-31.
59. Heilskov S, Rytter MJ, Vestergaard C, Briend A, Babirekere E, Deleuran MS. Dermatoses in children with oedematous malnutrition (Kwashiorkor): a review of the literature. *Journal of the European Academy of Dermatology and Venereology : JEADV*. 2014; 28(8): 995-1001.
60. Prendergast A, Kelly P. Enteropathies in the developing world: neglected effects on global health. *The American journal of tropical medicine and hygiene*. 2012; 86(5): 756-63.
61. Keusch GT, Rosenberg IH, Denno DM, Duggan C, Guerrant RL, Lavery JV, et al. Implications of acquired environmental enteric dysfunction for growth and stunting in infants and children living in low- and middle-income countries. *Food and nutrition bulletin*. 2013; 34(3): 357-64.
62. McKay S, Gaudier E, Campbell DI, Prentice AM, Albers R. Environmental enteropathy: new targets for nutritional interventions. *International health*. 2010; 2(3): 172-80.
63. Lindenbaum J, Gerson CD, Kent TH. Recovery of small-intestinal structure and function after residence in the tropics. I. Studies in Peace Corps volunteers. *Annals of internal medicine*. 1971; 74(2): 218-22.
64. Ramakrishna BS. Tropical sprue: a riddle wrapped in a mystery inside an enigma. *The Indian journal of medical research*. 2013; 137(1): 12-4.
65. Denno DM, VanBuskirk K, Nelson ZC, Musser CA, Hay Burgess DC, Tarr PI. Use of the lactulose to mannitol ratio to evaluate childhood environmental enteric dysfunction: a systematic review. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2014; 59 Suppl 4: S213-9.
66. Kosek M, Guerrant RL, Kang G, Bhutta Z, Yori PP, Gratz J, et al. Assessment of environmental enteropathy in the MAL-ED cohort study: theoretical and analytic framework. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2014; 59 Suppl 4: S239-47.
67. Trehan I, Shulman RJ, Ou CN, Maleta K, Manary MJ. A randomized, double-blind, placebo-controlled trial of rifaximin, a nonabsorbable antibiotic, in the treatment of tropical enteropathy. *The American journal of gastroenterology*. 2009; 104(9): 2326-33.

68. Mondal D, Minak J, Alam M, Liu Y, Dai J, Korpe P, et al. Contribution of enteric infection, altered intestinal barrier function, and maternal malnutrition to infant malnutrition in Bangladesh. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2012; 54(2): 185-92.
69. Menzies IS, Zuckerman MJ, Nukajam WS, Somasundaram SG, Murphy B, Jenkins AP, et al. Geography of intestinal permeability and absorption. *Gut*. 1999; 44(4): 483-9.
70. Weisz AJ, Manary MJ, Stephenson K, Agapova S, Manary FG, Thakwalakwa C, et al. Abnormal gut integrity is associated with reduced linear growth in rural malawian children. *Journal of pediatric gastroenterology and nutrition*. 2012; 55(6): 747-50.
71. Campbell DI, Elia M, Lunn PG. Growth faltering in rural Gambian infants is associated with impaired small intestinal barrier function, leading to endotoxemia and systemic inflammation. *The Journal of nutrition*. 2003; 133(5): 1332-8.
72. Lin A, Arnold BF, Afreen S, Goto R, Huda TM, Haque R, et al. Household environmental conditions are associated with enteropathy and impaired growth in rural Bangladesh. *The American journal of tropical medicine and hygiene*. 2013; 89(1): 130-7.
73. Kosek M, Haque R, Lima A, Babji S, Shrestha S, Qureshi S, et al. Fecal markers of intestinal inflammation and permeability associated with the subsequent acquisition of linear growth deficits in infants. *The American journal of tropical medicine and hygiene*. 2013; 88(2): 390-6.
74. Dewey KG, Adu-Afarwuah S. Systematic review of the efficacy and effectiveness of complementary feeding interventions in developing countries. *Maternal & child nutrition*. 2008; 4 Suppl 1: 24-85.
75. Humphrey JH. Child undernutrition, tropical enteropathy, toilets, and handwashing. *Lancet*. 2009; 374(9694): 1032-5.
76. Campbell DI, Murch SH, Elia M, Sullivan PB, Sanyang MS, Jobarteh B, et al. Chronic T cell-mediated enteropathy in rural west African children: relationship with nutritional status and small bowel function. *Pediatric research*. 2003; 54(3): 306-11.
77. Amadi B, Fagbemi AO, Kelly P, Mwiya M, Torrente F, Salvestrini C, et al. Reduced production of sulfated glycosaminoglycans occurs in Zambian children with kwashiorkor but not marasmus. *The American journal of clinical nutrition*. 2009; 89(2): 592-600.
78. Campbell DI, McPhail G, Lunn PG, Elia M, Jeffries DJ. Intestinal inflammation measured by fecal neopterin in Gambian children with enteropathy: association with

- growth failure, *Giardia lamblia*, and intestinal permeability. *Journal of pediatric gastroenterology and nutrition*. 2004; 39(2): 153-7.
79. Prendergast AJ, Rukobo S, Chasekwa B, Mutasa K, Ntozini R, Mbuya MN, et al. Stunting is characterized by chronic inflammation in zimbabwean infants. *PloS one*. 2014; 9(2): e86928.
 80. Gaspari S, Marcovecchio ML, Breda L, Chiarelli F. Growth in juvenile idiopathic arthritis: the role of inflammation. *Clinical and experimental rheumatology*. 2011; 29(1): 104-10.
 81. Sanderson IR. Growth problems in children with IBD. *Nature reviews Gastroenterology & hepatology*. 2014; 11(10): 601-10.
 82. Mbuya MN, Humphrey JH. Preventing environmental enteric dysfunction through improved water, sanitation and hygiene: an opportunity for stunting reduction in developing countries. *Maternal & child nutrition*. 2015.
 83. Arnold BF, Null C, Luby SP, Unicomb L, Stewart CP, Dewey KG, et al. Cluster-randomised controlled trials of individual and combined water, sanitation, hygiene and nutritional interventions in rural Bangladesh and Kenya: the WASH Benefits study design and rationale. *BMJ open*. 2013; 3(8): e003476.
 84. Prendergast AJ, Humphrey JH, Mutasa K, Majo FD, Rukobo S, Govha M, et al. Assessment of Environmental Enteric Dysfunction in the SHINE Trial: Methods and Challenges. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2015; 61 Suppl 7: S726-32.
 85. Fagundes Neto U, Martins MC, Lima FL, Patricio FR, Toledo MR. Asymptomatic environmental enteropathy among slum-dwelling infants. *Journal of the American College of Nutrition*. 1994; 13(1): 51-6.
 86. Gough EK, Moodie EE, Prendergast AJ, Johnson SM, Humphrey JH, Stoltzfus RJ, et al. The impact of antibiotics on growth in children in low and middle income countries: systematic review and meta-analysis of randomised controlled trials. *BMJ*. 2014; 348: g2267.
 87. Subramanian S, Huq S, Yatsunenko T, Haque R, Mahfuz M, Alam MA, et al. Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature*. 2014; 510(7505): 417-21.
 88. Goto R, Panter-Brick C, Northrop-Clewes CA, Manahdhar R, Tuladhar NR. Poor intestinal permeability in mildly stunted Nepali children: associations with weaning practices and *Giardia lamblia* infection. *The British journal of nutrition*. 2002; 88(2): 141-9.

89. Lunn PG, Erinoso HO, Northrop-Clewes CA, Boyce SA. *Giardia intestinalis* is unlikely to be a major cause of the poor growth of rural Gambian infants. *The Journal of nutrition*. 1999; 129(4): 872-7.
90. Tolboom JJ, Kabir H, Molatseli P, Anderson J, Arens T, Fernandes J. Lactose malabsorption and giardiasis in Basotho school children. *Acta paediatrica Scandinavica*. 1987; 76(1): 60-5.
91. Ignatius R, Gahutu JB, Klotz C, Steininger C, Shyirambere C, Lyng M, et al. High prevalence of *Giardia duodenalis* Assemblage B infection and association with underweight in Rwandan children. *PLoS neglected tropical diseases*. 2012; 6(6): e1677.
92. Bartelt LA, Roche J, Kolling G, Bolick D, Noronha F, Naylor C, et al. Persistent *G. lamblia* impairs growth in a murine malnutrition model. *The Journal of clinical investigation*. 2013; 123(6): 2672-84.
93. Jackson AA, Ashworth A, Khanum S. Improving child survival: Malnutrition Task Force and the paediatrician's responsibility. *Archives of disease in childhood*. 2006; 91(8): 706-10.
94. Aiken AM, Mturi N, Njuguna P, Mohammed S, Berkley JA, Mwangi I, et al. Risk and causes of paediatric hospital-acquired bacteraemia in Kilifi District Hospital, Kenya: a prospective cohort study. *Lancet*. 2011; 378(9808): 2021-7.
95. Collins S, Dent N, Binns P, Bahwere P, Sadler K, Hallam A. Management of severe acute malnutrition in children. *Lancet*. 2006; 368(9551): 1992-2000.
96. WHO, WFP, UNSSCN, UNICEF. Community-based management of severe acute malnutrition: A Joint Statement by the World Health Organization, the World Food Programme, the United Nations System Standing Committee on Nutrition and the United Nations Children's Fund. 2007. Available at: www.who.int/nutrition/topics/statement_commbased_malnutrition/en/
97. Briend A, Lacsala R, Prudhon C, Mounier B, Grellety Y, Golden MH. Ready-to-use therapeutic food for treatment of marasmus. *Lancet*. 1999; 353(9166): 1767-8.
98. UNDP-IAPSO. Emergency relief items—compendium of generic specifications, volume 1. Copenhagen: UNDP; 1995.
99. UNICEF. Supply Catalogue. 2014. <https://supply.unicef.org/>
100. Schoonees A, Lombard M, Musekiwa A, Nel E, Volmink J. Ready-to-use therapeutic food for home-based treatment of severe acute malnutrition in children from six months to five years of age. *Cochrane Database Syst Rev*. 2013; 6: CD009000.

101. UNICEF. Position paper: ready-to-use therapeutic food for children with severe acute malnutrition. 2013. Available from: http://www.unicef.org/policyanalysis/files/UNICEF-Position-Paper_Ready-To-Use-Therapeutic-Food_June2013.pdf
102. Manary MJ. Local production and provision of ready-to-use therapeutic food (RUTF) spread for the treatment of severe childhood malnutrition. Food and nutrition bulletin. 2006; 27(3 Suppl): S83-9.
103. Dibari F, Diop el HI, Collins S, Seal A. Low-cost, ready-to-use therapeutic foods can be designed using locally available commodities with the aid of linear programming. The Journal of nutrition. 2012; 142(5): 955-61.
104. Donowitz JR, Petri WA, Jr. Pediatric small intestine bacterial overgrowth in low-income countries. Trends in molecular medicine. 2015; 21(1): 6-15.
105. Isanaka S, Langendorf C, Berthe F, Gnegne S, Li N, Ousmane N, et al. Routine Amoxicillin for Uncomplicated Severe Acute Malnutrition in Children. The New England journal of medicine. 2016; 374(5): 444-53.
106. Trehan I, Goldbach HS, LaGrone LN, Meuli GJ, Wang RJ, Maleta KM, et al. Antibiotics as part of the management of severe acute malnutrition. The New England journal of medicine. 2013; 368(5): 425-35.
107. Maitland K, Berkley JA, Shebbe M, Peshu N, English M, Newton CR. Children with severe malnutrition: can those at highest risk of death be identified with the WHO protocol? PLoS medicine. 2006; 3(12): e500.
108. Irena AH, Mwambazi M, Mulenga V. Diarrhea is a major killer of children with severe acute malnutrition admitted to inpatient set-up in Lusaka, Zambia. Nutrition journal. 2011; 10: 110.
109. Nhampossa T, Sigauque B, Machevo S, Macete E, Alonso P, Bassat Q, et al. Severe malnutrition among children under the age of 5 years admitted to a rural district hospital in southern Mozambique. Public health nutrition. 2013; 16(9): 1565-74.
110. Moisi JC, Gatakaa H, Berkley JA, Maitland K, Mturi N, Newton CR, et al. Excess child mortality after discharge from hospital in Kilifi, Kenya: a retrospective cohort analysis. Bulletin of the World Health Organization. 2011; 89(10): 725-32, 32A.
111. Kerac M, Bunn J, Chagaluka G, Bahwere P, Tomkins A, Collins S, et al. Follow-up of post-discharge growth and mortality after treatment for severe acute malnutrition (FuSAM study): a prospective cohort study. PloS one. 2014; 9(6): e96030.

112. Chisti MJ, Graham SM, Duke T, Ahmed T, Faruque AS, Ashraf H, et al. Post-discharge mortality in children with severe malnutrition and pneumonia in bangladesh. *PloS one*. 2014; 9(9): e107663.
113. Thurnham DI, Northrop-Clewes CA, McCullough FS, Das BS, Lunn PG. Innate immunity, gut integrity, and vitamin A in Gambian and Indian infants. *The Journal of infectious diseases*. 2000; 182 Suppl 1: S23-8.
114. Lima NL, Soares AM, Mota RM, Monteiro HS, Guerrant RL, Lima AA. Wasting and intestinal barrier function in children taking alanyl-glutamine-supplemented enteral formula. *Journal of pediatric gastroenterology and nutrition*. 2007; 44(3): 365-74.
115. Lima AA, Anstead GM, Zhang Q, Figueiredo IL, Soares AM, Mota RM, et al. Effects of glutamine alone or in combination with zinc and vitamin A on growth, intestinal barrier function, stress and satiety-related hormones in Brazilian shantytown children. *Clinics (Sao Paulo)*. 2014; 69(4): 225-33.
116. van der Merwe LF, Moore SE, Fulford AJ, Halliday KE, Drammeh S, Young S, et al. Long-chain PUFA supplementation in rural African infants: a randomized controlled trial of effects on gut integrity, growth, and cognitive development. *The American journal of clinical nutrition*. 2013; 97(1): 45-57.
117. Smith HE, Ryan KN, Stephenson KB, Westcott C, Thakwalakwa C, Maleta K, et al. Multiple micronutrient supplementation transiently ameliorates environmental enteropathy in Malawian children aged 12-35 months in a randomized controlled clinical trial. *The Journal of nutrition*. 2014; 144(12): 2059-65.
118. Ryan KN, Stephenson KB, Trehan I, Shulman RJ, Thakwalakwa C, Murray E, et al. Zinc or albendazole attenuates the progression of environmental enteropathy: a randomized controlled trial. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*. 2014; 12(9): 1507-13 e1.
119. Galpin L, Manary MJ, Fleming K, Ou CN, Ashorn P, Shulman RJ. Effect of *Lactobacillus* GG on intestinal integrity in Malawian children at risk of tropical enteropathy. *The American journal of clinical nutrition*. 2005; 82(5): 1040-5.
120. Kerac M, Bunn J, Seal A, Thindwa M, Tomkins A, Sadler K, et al. Probiotics and prebiotics for severe acute malnutrition (PRONUT study): a double-blind efficacy randomised controlled trial in Malawi. *Lancet*. 2009; 374(9684): 136-44.
121. Goto R, Mascie-Taylor CG, Lunn PG. Impact of anti-Giardia and anthelmintic treatment on infant growth and intestinal permeability in rural Bangladesh: a

- randomised double-blind controlled study. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 2009; 103(5): 520-9.
122. Bajaj JS, Heuman DM, Sanyal AJ, Hylemon PB, Sterling RK, Stravitz RT, et al. Modulation of the metabiome by rifaximin in patients with cirrhosis and minimal hepatic encephalopathy. *PloS one*. 2013; 8(4): e60042.
 123. Brewster DR, Manary MJ, Menzies IS, O'Loughlin EV, Henry RL. Intestinal permeability in kwashiorkor. *Archives of disease in childhood*. 1997; 76(3): 236-41.
 124. Schroder NW, Schumann RR. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. *The Lancet infectious diseases*. 2005; 5(3): 156-64.
 125. Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. *Nature reviews Immunology*. 2007; 7(3): 179-90.
 126. Matzinger P. The danger model: a renewed sense of self. *Science*. 2002; 296(5566): 301-5.
 127. Corridoni D, Arseneau KO, Cifone MG, Cominelli F. The dual role of nod-like receptors in mucosal innate immunity and chronic intestinal inflammation. *Frontiers in immunology*. 2014; 5: 317.
 128. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity*. 2013; 38(2): 209-23.
 129. Alsina L, Israelsson E, Altman MC, Dang KK, Ghandil P, Israel L, et al. A narrow repertoire of transcriptional modules responsive to pyogenic bacteria is impaired in patients carrying loss-of-function mutations in MYD88 or IRAK4. *Nature immunology*. 2014; 15(12): 1134-42.
 130. Roth A, Garly ML, Jensen H, Nielsen J, Aaby P. *Bacillus Calmette-Guerin* vaccination and infant mortality. *Expert review of vaccines*. 2006; 5(2): 277-93.
 131. Stensballe LG, Nante E, Jensen IP, Kofoed PE, Poulsen A, Jensen H, et al. Acute lower respiratory tract infections and respiratory syncytial virus in infants in Guinea-Bissau: a beneficial effect of BCG vaccination for girls community based case-control study. *Vaccine*. 2005; 23(10): 1251-7.
 132. Kleinnijenhuis J, van Crevel R, Netea MG. Trained immunity: consequences for the heterologous effects of BCG vaccination. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 2015; 109(1): 29-35.
 133. Freyne B, Marchant A, Curtis N. BCG-associated heterologous immunity, a historical perspective: intervention studies in animal models of infectious diseases.

- Transactions of the Royal Society of Tropical Medicine and Hygiene. 2015; 109(1): 52-61.
134. Freyne B, Marchant A, Curtis N. BCG-associated heterologous immunity, a historical perspective: experimental models and immunological mechanisms. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2015; 109(1): 46-51.
 135. Jensen KJ, Larsen N, Biering-Sorensen S, Andersen A, Eriksen HB, Monteiro I, et al. Heterologous Immunological Effects of Early BCG Vaccination in Low-Birth-Weight Infants in Guinea-Bissau: A Randomized-controlled Trial. The Journal of infectious diseases. 2015; 211(6): 956-67.
 136. Kleinnijenhuis J, Quintin J, Preijers F, Benn CS, Joosten LA, Jacobs C, et al. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. Journal of innate immunity. 2014; 6(2): 152-8.
 137. Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, et al. mTOR- and HIF-1 α -mediated aerobic glycolysis as metabolic basis for trained immunity. Science. 2014; 345(6204): 1250684.
 138. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109(43): 17537-42.
 139. Biswas SK, Lopez-Collazo E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends in immunology. 2009; 30(10): 475-87.
 140. Kox M, de Kleijn S, Pompe JC, Ramakers BP, Netea MG, van der Hoeven JG, et al. Differential ex vivo and in vivo endotoxin tolerance kinetics following human endotoxemia. Critical care medicine. 2011; 39(8): 1866-70.
 141. Pena OM, Hancock DG, Lyle NH, Linder A, Russell JA, Xia J, et al. An Endotoxin Tolerance Signature Predicts Sepsis and Organ Dysfunction at Initial Clinical Presentation. EBioMedicine. 2014; 1(1): 64-71.
 142. Lin CY, Tsai IF, Ho YP, Huang CT, Lin YC, Lin CJ, et al. Endotoxemia contributes to the immune paralysis in patients with cirrhosis. Journal of hepatology. 2007; 46(5): 816-26.
 143. Lester RT, Yao XD, Ball TB, McKinnon LR, Omange WR, Kaul R, et al. HIV-1 RNA dysregulates the natural TLR response to subclinical endotoxemia in Kenyan female sex-workers. PloS one. 2009; 4(5): e5644.

144. Seksik P, Sokol H, Grondin V, Adrie C, Duboc H, Pigneur B, et al. Sera from patients with Crohn's disease break bacterial lipopolysaccharide tolerance of human intestinal epithelial cells via MD-2 activity. *Innate immunity*. 2010; 16(6): 381-90.
145. Berkley JA, Lowe BS, Mwangi I, Williams T, Bauni E, Mwarumba S, et al. Bacteremia among children admitted to a rural hospital in Kenya. *The New England journal of medicine*. 2005; 352(1): 39-47.
146. Talbert A, Thuo N, Karisa J, Chesaro C, Ohuma E, Ignas J, et al. Diarrhoea complicating severe acute malnutrition in Kenyan children: a prospective descriptive study of risk factors and outcome. *PloS one*. 2012; 7(6): e38321.
147. Al-Herz W, Bousfiha A, Casanova JL, Chatila T, Conley ME, Cunningham-Rundles C, et al. Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. *Frontiers in immunology*. 2014; 5: 162.
148. von Bernuth H, Picard C, Puel A, Casanova JL. Experimental and natural infections in MyD88- and IRAK-4-deficient mice and humans. *European journal of immunology*. 2012; 42(12): 3126-35.
149. Uhlig HH. Monogenic diseases associated with intestinal inflammation: implications for the understanding of inflammatory bowel disease. *Gut*. 2013; 62(12): 1795-805.
150. Damen GM, van Krieken JH, Hoppenreijds E, van Os E, Tolboom JJ, Warris A, et al. Overlap, common features, and essential differences in pediatric granulomatous inflammatory bowel disease. *Journal of pediatric gastroenterology and nutrition*. 2010; 51(6): 690-7.
151. Hanson EP, Monaco-Shawver L, Solt LA, Madge LA, Banerjee PP, May MJ, et al. Hypomorphic nuclear factor-kappaB essential modulator mutation database and reconstitution system identifies phenotypic and immunologic diversity. *The Journal of allergy and clinical immunology*. 2008; 122(6): 1169-77 e16.
152. Smith AM, Rahman FZ, Hayee B, Graham SJ, Marks DJ, Sewell GW, et al. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *The Journal of experimental medicine*. 2009; 206(9): 1883-97.
153. Liu JZ, Anderson CA. Genetic studies of Crohn's disease: past, present and future. *Best practice & research Clinical gastroenterology*. 2014; 28(3): 373-86.
154. Kuuliala K, Lappalainen M, Turunen U, Puolakkainen P, Kemppainen E, Siitonen S, et al. Detection of muramyl dipeptide-sensing pathway defects in monocytes of

- patients with Crohn's disease using phospho-specific whole blood flow cytometry. *Scandinavian journal of clinical and laboratory investigation*. 2013; 73(6): 494-502.
155. Gordon JI, Dewey KG, Mills DA, Medzhitov RM. The human gut microbiota and undernutrition. *Science translational medicine*. 2012; 4(137): 137ps12.
 156. Kelly P, Menzies I, Crane R, Zulu I, Nickols C, Feakins R, et al. Responses of small intestinal architecture and function over time to environmental factors in a tropical population. *The American journal of tropical medicine and hygiene*. 2004; 70(4): 412-9.
 157. Korpe PS, Petri WA, Jr. Environmental enteropathy: critical implications of a poorly understood condition. *Trends in molecular medicine*. 2012; 18(6): 328-36.
 158. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature*. 2011; 474(7351): 298-306.
 159. Meresse B, Malamut G, Cerf-Bensussan N. Celiac disease: an immunological jigsaw. *Immunity*. 2012; 36(6): 907-19.
 160. Managlia E, Katzman RB, Brown JB, Barrett TA. Antioxidant properties of mesalamine in colitis inhibit phosphoinositide 3-kinase signaling in progenitor cells. *Inflammatory bowel diseases*. 2013; 19(10): 2051-60.
 161. Kennedy M, Wilson L, Szabo C, Salzman AL. 5-aminosalicylic acid inhibits iNOS transcription in human intestinal epithelial cells. *International journal of molecular medicine*. 1999; 4(4): 437-43.
 162. Bantel H, Berg C, Vieth M, Stolte M, Kruis W, Schulze-Osthoff K. Mesalazine inhibits activation of transcription factor NF-kappaB in inflamed mucosa of patients with ulcerative colitis. *The American journal of gastroenterology*. 2000; 95(12): 3452-7.
 163. Rousseaux C, Lefebvre B, Dubuquoy L, Lefebvre P, Romano O, Auwerx J, et al. Intestinal antiinflammatory effect of 5-aminosalicylic acid is dependent on peroxisome proliferator-activated receptor-gamma. *The Journal of experimental medicine*. 2005; 201(8): 1205-15.
 164. Turner D, Levine A, Escher JC, Griffiths AM, Russell RK, Dignass A, et al. Management of pediatric ulcerative colitis: joint ECCO and ESPGHAN evidence-based consensus guidelines. *Journal of pediatric gastroenterology and nutrition*. 2012; 55(3): 340-61.
 165. Gupta N, Bostrom AG, Kirschner BS, Cohen SA, Abramson O, Ferry GD, et al. Presentation and disease course in early- compared to later-onset pediatric Crohn's disease. *The American journal of gastroenterology*. 2008; 103(8): 2092-8.
 166. Wilson DC, Thomas AG, Croft NM, Newby E, Akobeng AK, Sawczenko A, et al. Systematic review of the evidence base for the medical treatment of paediatric

- inflammatory bowel disease. *Journal of pediatric gastroenterology and nutrition*. 2010; 50 Suppl 1: S14-34.
167. Feagan BG, Macdonald JK. Oral 5-aminosalicylic acid for induction of remission in ulcerative colitis. *Cochrane Database Syst Rev*. 2012; 10: CD000543.
 168. Feagan BG, Macdonald JK. Oral 5-aminosalicylic acid for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev*. 2012; 10: CD000544.
 169. Ford AC, Kane SV, Khan KJ, Achkar JP, Talley NJ, Marshall JK, et al. Efficacy of 5-aminosalicylates in Crohn's disease: systematic review and meta-analysis. *The American journal of gastroenterology*. 2011; 106(4): 617-29.
 170. Gisbert JP, Gonzalez-Lama Y, Mate J. 5-Aminosalicylates and renal function in inflammatory bowel disease: a systematic review. *Inflammatory bowel diseases*. 2007; 13(5): 629-38.
 171. Sandhu BK, Fell JM, Beattie RM, Mitton SG, Wilson DC, Jenkins H. Guidelines for the management of inflammatory bowel disease in children in the United Kingdom. *Journal of pediatric gastroenterology and nutrition*. 2010; 50 Suppl 1: S1-13.
 172. Lichtenstein GR, Kamm MA. Review article: 5-aminosalicylate formulations for the treatment of ulcerative colitis--methods of comparing release rates and delivery of 5-aminosalicylate to the colonic mucosa. *Alimentary pharmacology & therapeutics*. 2008; 28(6): 663-73.
 173. Jones KD, Berkley JA. Severe acute malnutrition and infection. *Paediatrics and international child health*. 2014; 34 Suppl 1: S1-S29.
 174. Fiorino G, Peyrin-Biroulet L, Naccarato P, Szabo H, Sociale OR, Vetrano S, et al. Effects of immunosuppression on immune response to pneumococcal vaccine in inflammatory bowel disease: a prospective study. *Inflammatory bowel diseases*. 2012; 18(6): 1042-7.
 175. Calder PC. n-3 fatty acids, inflammation and immunity: new mechanisms to explain old actions. *The Proceedings of the Nutrition Society*. 2013; 72(3): 326-36.
 176. Kim W, Fan YY, Barhoumi R, Smith R, McMurray DN, Chapkin RS. n-3 polyunsaturated fatty acids suppress the localization and activation of signaling proteins at the immunological synapse in murine CD4+ T cells by affecting lipid raft formation. *J Immunol*. 2008; 181(9): 6236-43.
 177. Calder PC. Very long chain omega-3 (n-3) fatty acids and human health. *European Journal of Lipid Science and Technology*. 2014; 116(10): 1280-300.

178. Houssaini FZ, Foulon T, Iraqi MR, Payen N, Gros Lambert P. Lipids, lipoproteins, and fatty acids during infantile marasmus in the Fes area of Morocco. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*. 1999; 53(5-6): 278-83.
179. Koletzko B, Abiodun PO, Laryea MD, Bremer HJ. Fatty acid composition of plasma lipids in Nigerian children with protein-energy malnutrition. *European journal of pediatrics*. 1986; 145(1-2): 109-15.
180. Smit EN, Dijkstra JM, Schnater TA, Seerat E, Muskiet FA, Boersma ER. Effects of malnutrition on the erythrocyte fatty acid composition and plasma vitamin E levels of Pakistani children. *Acta Paediatr*. 1997; 86(7): 690-5.
181. Leichsenring M, Sutterlin N, Less S, Baumann K, Anninos A, Becker K. Polyunsaturated fatty acids in erythrocyte and plasma lipids of children with severe protein-energy malnutrition. *Acta Paediatr*. 1995; 84(5): 516-20.
182. Wolff JA, Margolis S, Bujdoso-Wolff K, Matusick E, MacLean WC, Jr. Plasma and red blood cell fatty acid composition in children with protein-calorie malnutrition. *Pediatric research*. 1984; 18(2): 162-7.
183. Holman RT, Johnson SB, Mercuri O, Itarte HJ, Rodrigo MA, De Tomas ME. Essential fatty acid deficiency in malnourished children. *The American journal of clinical nutrition*. 1981; 34(8): 1534-9.
184. Vajreswari A, Narayanareddy K, Rao PS. Fatty acid composition of erythrocyte membrane lipid obtained from children suffering from kwashiorkor and marasmus. *Metabolism: clinical and experimental*. 1990; 39(8): 779-82.
185. Franco VH, Hotta JK, Jorge SM, dos Santos JE. Plasma fatty acids in children with grade III protein-energy malnutrition in its different clinical forms: marasmus, marasmic kwashiorkor, and kwashiorkor. *Journal of tropical pediatrics*. 1999; 45(2): 71-5.
186. Lauritzen L, Carlson SE. Maternal fatty acid status during pregnancy and lactation and relation to newborn and infant status. *Maternal & child nutrition*. 2011; 7 Suppl 2: 41-58.
187. Brenna JT, Varamini B, Jensen RG, Diersen-Schade DA, Boettcher JA, Arterburn LM. Docosahexaenoic and arachidonic acid concentrations in human breast milk worldwide. *The American journal of clinical nutrition*. 2007; 85(6): 1457-64.
188. Michaelsen KF, Dewey KG, Perez-Exposito AB, Nurhasan M, Lauritzen L, Roos N. Food sources and intake of n-6 and n-3 fatty acids in low-income countries with emphasis on infants, young children (6-24 months), and pregnant and lactating women. *Maternal & child nutrition*. 2011; 7 Suppl 2: 124-40.

189. Prentice AM, Paul AA. Fat and energy needs of children in developing countries. *The American journal of clinical nutrition*. 2000; 72(5 Suppl): 1253S-65S.
190. Smit EN, Oelen EA, Seerat E, Boersma ER, Muskiet FA. Fish oil supplementation improves docosahexaenoic acid status of malnourished infants. *Archives of disease in childhood*. 2000; 82(5): 366-9.
191. Robert A, Mfilinge P, Limbu SM, Mwita CJ. Fatty Acid composition and levels of selected polyunsaturated Fatty acids in four commercial important freshwater fish species from lake victoria, Tanzania. *Journal of lipids*. 2014; 2014: 712134.
192. Ogutu-ohwayo R. Nile perch: the great lake experiment. *World conservation*. 1998; (1): 11-2.
193. Goyal A, Sharma V, Upadhyay N, Gill S, Sihag M. Flax and flaxseed oil: an ancient medicine & modern functional food. *Journal of food science and technology*. 2014; 51(9): 1633-53.
194. United Nations Human Settlements Programme. The challenge of slums: global report on human settlements. UN-HABITAT; 2003. Available at: <http://unhabitat.org/books/the-challenge-of-slums-global-report-on-human-settlements-2003/>
195. Muungano Support Trust SDI, University of Nairobi, University of California, Berkley. Mathare zonal plan, Nairobi, Kenya. Collaborative plan for informal settlement upgrading. 2012. Available at: http://www.sdinet.org/media/upload/documents/Mathare_Zonal_Plan_25_06_2012_low_res-2.pdf
196. UNHCR. Handbook for emergencies. Geneva: UNHCR; 2007.
197. German Doctors: <https://http://www.german-doctors.de/de/>
198. The Sphere Project. The Sphere Handbook. 2011. <http://www.sphereproject.org/handbook/>
199. Geri G, Passeron A, Heym B, Arlet JB, Pouchot J, Capron L, et al. Paradoxical reactions during treatment of tuberculosis with extrapulmonary manifestations in HIV-negative patients. *Infection*. 2013; 41(2): 537-43.
200. Burl S, Townend J, Njie-Jobe J, Cox M, Adetifa UJ, Touray E, et al. Age-dependent maturation of Toll-like receptor-mediated cytokine responses in Gambian infants. *PloS one*. 2011; 6(4): e18185.
201. StataCorp. PCA Postestimation. *Stata Base Reference Manual 13*. Texas: Stata Press; 2013.
202. Kaiser HF. An index of factor simplicity. *Psychometrika*. 1974; 39: 31-6.

203. Azevedo JP. Factortest: Stata module to perform tests for appropriateness of factor analysis. 2003. Available at: <https://ideas.repec.org/c/boc/bocode/s436001.html>
204. World Bank. World Development Indicators 2015. Available from: http://data.worldbank.org/topic/poverty - tp_wdi
205. McCall MB, Netea MG, Hermesen CC, Jansen T, Jacobs L, Golenbock D, et al. Plasmodium falciparum infection causes proinflammatory priming of human TLR responses. J Immunol. 2007; 179(1): 162-71.
206. Boeuf PS, Loizon S, Awandare GA, Tetteh JK, Addae MM, Adjei GO, et al. Insights into deregulated TNF and IL-10 production in malaria: implications for understanding severe malarial anaemia. Malaria journal. 2012; 11: 253.
207. Noor AM, Kinyoki DK, Mundia CW, Kabaria CW, Mutua JW, Alegana VA, et al. The changing risk of Plasmodium falciparum malaria infection in Africa: 2000-10: a spatial and temporal analysis of transmission intensity. Lancet. 2014; 383(9930): 1739-47.
208. Heikens GT, Manary M. 75 years of Kwashiorkor in Africa. Malawi medical journal : the journal of Medical Association of Malawi. 2009; 21(3): 96-8.
209. Golden MH. Issues in kwashiorkor. Lancet. 1994; 343(8892): 292.
210. Briend A. Kwashiorkor: still an enigma - the search must go on. CMAM Forum Technical Brief; 2014. Available at: www.cmamforum.org/Pool/Resources/Kwashiorkor%2c-still-an-enigma-CMAM-Forum-Dec-2014.pdf
211. Amadi B, Kelly P, Mwiya M, Mulwazi E, Sianongo S, Changwe F, et al. Intestinal and systemic infection, HIV, and mortality in Zambian children with persistent diarrhea and malnutrition. Journal of pediatric gastroenterology and nutrition. 2001; 32(5): 550-4.
212. Bachou H, Tylleskar T, Downing R, Tumwine JK. Severe malnutrition with and without HIV-1 infection in hospitalised children in Kampala, Uganda: differences in clinical features, haematological findings and CD4+ cell counts. Nutrition journal. 2006; 5: 27.
213. Prendergast A, Bwakura-Dangarembizi MF, Cook AD, Bakeera-Kitaka S, Natukunda E, Nahirya Ntege P, et al. Hospitalization for severe malnutrition among HIV-infected children starting antiretroviral therapy. AIDS. 2011; 25(7): 951-6.
214. Kopylov U, Rosenfeld G, Bressler B, Seidman E. Clinical utility of fecal biomarkers for the diagnosis and management of inflammatory bowel disease. Inflammatory bowel diseases. 2014; 20(4): 742-56.

215. De Jong HK, Achouiti A, Koh GC, Parry CM, Baker S, Faiz MA, et al. Expression and function of S100A8/A9 (calprotectin) in human typhoid fever and the murine *Salmonella* model. *PLoS neglected tropical diseases*. 2015; 9(4): e0003663.
216. Lorenz F, Marklund S, Werner M, Palmqvist R, Wahlin BE, Wahlin A. Fecal calprotectin as a biomarker of intestinal graft versus host disease after allogeneic hematopoietic stem cell transplantation. *Scientific reports*. 2015; 5: 7920.
217. Marks MA, Rabkin CS, Engels EA, Busch E, Kopp W, Rager H, et al. Markers of microbial translocation and risk of AIDS-related lymphoma. *AIDS*. 2013; 27(3): 469-74.
218. Powrie F, Uhlir H. Animal models of intestinal inflammation: clues to the pathogenesis of inflammatory bowel disease. *Novartis Foundation symposium*. 2004; 263: 164-74; discussion 74-8, 211-8.
219. MohanKumar K, Kaza N, Jagadeeswaran R, Garzon SA, Bansal A, Kurundkar AR, et al. Gut mucosal injury in neonates is marked by macrophage infiltration in contrast to pleomorphic infiltrates in adult: evidence from an animal model. *American journal of physiology Gastrointestinal and liver physiology*. 2012; 303(1): G93-102.
220. Cotton JA, Motta JP, Schenck LP, Hirota SA, Beck PL, Buret AG. *Giardia duodenalis* infection reduces granulocyte infiltration in an in vivo model of bacterial toxin-induced colitis and attenuates inflammation in human intestinal tissue. *PloS one*. 2014; 9(10): e109087.
221. Strutz F, Heller G, Krasemann K, Krone B, Muller GA. Relationship of antibodies to endotoxin core to mortality in medical patients with sepsis syndrome. *Intensive care medicine*. 1999; 25(5): 435-44.
222. Barclay GR. Endogenous endotoxin-core antibody (EndoCAb) as a marker of endotoxin exposure and a prognostic indicator: a review. *Progress in clinical and biological research*. 1995; 392: 263-72.
223. Doherty JF, Golden MH, Remick DG, Griffin GE. Production of interleukin-6 and tumour necrosis factor- α in vitro is reduced in whole blood of severely malnourished children. *Clin Sci (Lond)*. 1994; 86(3): 347-51.
224. Munoz C, Arevalo M, Lopez M, Schlesinger L. Impaired Interleukin-1 and Tumor-Necrosis-Factor Production in Protein-Calorie Malnutrition. *Nutrition Research*. 1994; 14(3): 347-52.
225. Palacio A, Lopez M, Perez-Bravo F, Monkeberg F, Schlesinger L. Leptin levels are associated with immune response in malnourished infants. *The Journal of clinical endocrinology and metabolism*. 2002; 87(7): 3040-6.

226. Azevedo ZM, Luz RA, Victal SH, Kurdian B, Fonseca VM, Fitting C, et al. Increased production of tumor necrosis factor-alpha in whole blood cultures from children with primary malnutrition. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica* [et al]. 2005; 38(2): 171-83.
227. Malave I, Vethencourt MA, Chacon R, Quinones D, Rebrij C, Bolivar G. Production of interleukin-6 in cultures of peripheral blood mononuclear cells from children with primary protein-calorie malnutrition and from eutrophic controls. *Annals of Nutrition and Metabolism*. 1998; 42(5): 266-73.
228. Monk JM, Steevels TA, Hillyer LM, Woodward B. Constitutive, but not challenge-induced, interleukin-10 production is robust in acute pre-pubescent protein and energy deficits: new support for the tolerance hypothesis of malnutrition-associated immune depression based on cytokine production in vivo. *International journal of environmental research and public health*. 2011; 8(1): 117-35.
229. Fock RA, Vinolo MA, Blatt SL, Borelli P. Impairment of the hematological response and interleukin-1beta production in protein-energy malnourished mice after endotoxemia with lipopolysaccharide. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica* [et al]. 2012; 45(12): 1163-71.
230. de Oliveira DC, Hastreiter AA, Mello AS, de Oliveira Beltran JS, Oliveira Santos EW, Borelli P, et al. The effects of protein malnutrition on the TNF-RI and NF-kappaB expression via the TNF-alpha signaling pathway. *Cytokine*. 2014; 69(2): 218-25.
231. Fock RA, Vinolo MA, Crisma AR, Nakajima K, Rogero MM, Borelli P. Protein-energy malnutrition modifies the production of interleukin-10 in response to lipopolysaccharide (LPS) in a murine model. *Journal of nutritional science and vitaminology*. 2008; 54(5): 371-7.
232. Fock RA, Vinolo MA, de Moura Sa Rocha V, de Sa Rocha LC, Borelli P. Protein-energy malnutrition decreases the expression of TLR-4/MD-2 and CD14 receptors in peritoneal macrophages and reduces the synthesis of TNF-alpha in response to lipopolysaccharide (LPS) in mice. *Cytokine*. 2007; 40(2): 105-14.
233. Anstead GM, Chandrasekar B, Zhang Q, Melby PC. Multinutrient undernutrition dysregulates the resident macrophage proinflammatory cytokine network, nuclear factor-kappaB activation, and nitric oxide production. *Journal of leukocyte biology*. 2003; 74(6): 982-91.

234. Reynolds JV, Redmond HP, Ueno N, Steigman C, Ziegler MM, Daly JM, et al. Impairment of macrophage activation and granuloma formation by protein deprivation in mice. *Cellular immunology*. 1992; 139(2): 493-504.
235. Diamanti A, Panetta F, Basso MS, Forgione A, Colistro F, Bracci F, et al. Diagnostic work-up of inflammatory bowel disease in children: the role of calprotectin assay. *Inflammatory bowel diseases*. 2010; 16(11): 1926-30.
236. Nordlander S, Pott J, Maloy KJ. NLR4 expression in intestinal epithelial cells mediates protection against an enteric pathogen. *Mucosal immunology*. 2014; 7(4): 775-85.
237. Song-Zhao GX, Srinivasan N, Pott J, Baban D, Frankel G, Maloy KJ. Nlrp3 activation in the intestinal epithelium protects against a mucosal pathogen. *Mucosal immunology*. 2014; 7(4): 763-74.
238. Pearson C, Uhlig HH, Powrie F. Lymphoid microenvironments and innate lymphoid cells in the gut. *Trends in immunology*. 2012; 33(6): 289-96.
239. Mack DR, Langton C, Markowitz J, LeLeiko N, Griffiths A, Bousvaros A, et al. Laboratory values for children with newly diagnosed inflammatory bowel disease. *Pediatrics*. 2007; 119(6): 1113-9.
240. Republic of Kenya Ministry of Health. National Guideline for Integrated Management of Acute Malnutrition. 2008.
241. Savory DJ. Reference ranges for serum creatinine in infants, children and adolescents. *Annals of clinical biochemistry*. 1990; 27 (Pt 2): 99-101.
242. Emergency Medicine Compendium. Available from:
<http://www.medicines.org.uk/emc/medicine/20977/SPC/Pentasa+Sachet+2g/>
243. Ryan, P. RALLOC: Stata module to design randomized controlled trials. EconPapers. 2011. Available at: <http://EconPapers.repec.org/RePEc:boc:bocode:s319901>
244. NIH Division of Microbiology and Infectious Diseases Pediatric Toxicity Tables. 2007. Available at:
<http://www.niaid.nih.gov/LabsAndResources/resources/DMIDClinRsrch/Documents/dmidpedtox.pdf>
245. Howie SR. Blood sample volumes in child health research: review of safe limits. *Bulletin of the World Health Organization*. 2011; 89(1): 46-53.
246. Russo M, Martinelli M, Sciorio E, Botta C, Miele E, Vallone G, et al. Stool consistency, but not frequency, correlates with total gastrointestinal transit time in children. *The Journal of pediatrics*. 2013; 162(6): 1188-92.

247. World Medical Association. Declaration of Helsinki. 2000. Available from: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2566407/pdf/11357217.pdf>
248. Henderson P, Casey A, Lawrence SJ, Kennedy NA, Kingstone K, Rogers P, et al. The diagnostic accuracy of fecal calprotectin during the investigation of suspected pediatric inflammatory bowel disease. *The American journal of gastroenterology*. 2012; 107(6): 941-9.
249. Walters TD, Griffiths AM. Mechanisms of growth impairment in pediatric Crohn's disease. *Nature reviews Gastroenterology & hepatology*. 2009; 6(9): 513-23.
250. Bannerjee K, Camacho-Hubner C, Babinska K, Dryhurst KM, Edwards R, Savage MO, et al. Anti-inflammatory and growth-stimulating effects precede nutritional restitution during enteral feeding in Crohn disease. *Journal of pediatric gastroenterology and nutrition*. 2004; 38(3): 270-5.
251. Kirschner BS, Sutton MM. Somatomedin-C levels in growth-impaired children and adolescents with chronic inflammatory bowel disease. *Gastroenterology*. 1986; 91(4): 830-6.
252. Ryan KN, Stephenson KB, Trehan I, Shulman RJ, Thakwalakwa C, Murray E, et al. Zinc or Albendazole Attenuates the Progression of Environmental Enteropathy: A Randomized Controlled Trial. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*. 2014.
253. Kapel N, Roman C, Caldari D, Sieprath F, Canioni D, Khalfoun Y, et al. Fecal tumor necrosis factor-alpha and calprotectin as differential diagnostic markers for severe diarrhea of small infants. *Journal of pediatric gastroenterology and nutrition*. 2005; 41(4): 396-400.
254. Murch SH, Lamkin VA, Savage MO, Walker-Smith JA, MacDonald TT. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. *Gut*. 1991; 32(8): 913-7.
255. Street ME, Volta C, Ziveri MA, Zanacca C, Banchini G, Viani I, et al. Changes and relationships of IGFS and IGFBPS and cytokines in coeliac disease at diagnosis and on gluten-free diet. *Clinical endocrinology*. 2008; 68(1): 22-8.
256. van der Meer RW, Hammer S, Lamb HJ, Frolich M, Diamant M, Rijzewijk LJ, et al. Effects of short-term high-fat, high-energy diet on hepatic and myocardial triglyceride content in healthy men. *The Journal of clinical endocrinology and metabolism*. 2008; 93(7): 2702-8.
257. Kenya Open Data. Poverty rate by district. 2014. Available from: <http://www.opendata.go.ke/Poverty/Poverty-Rate-by-District/i5bp-z9aq>

258. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*. 1959; 37(8): 911-7.
259. Browning LM, Walker CG, Mander AP, West AL, Madden J, Gambell JM, et al. Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish. *The American journal of clinical nutrition*. 2012; 96(4): 748-58.
260. Naismith DJ. Kwashiorkor in western Nigeria: a study of traditional weaning foods, with particular reference to energy and linoleic acid. *The British journal of nutrition*. 1973; 30(3): 567-76.
261. Chen SC, Dickerman, S. Iron, thyroid hormone and essential fatty acid status of Honduran preschoolers. *Nutrition Research*. 1985; 5: 21-30.
262. Taylor GO. Serum triglycerides and fatty acids in kwashiorkor. *The American journal of clinical nutrition*. 1971; 24(10): 1212-5.
263. Decsi T, Zaknun D, Zaknun J, Sperl W, Koletzko B. Long-chain polyunsaturated fatty acids in children with severe protein-energy malnutrition with and without human immunodeficiency virus-1 infection. *The American journal of clinical nutrition*. 1995; 62(6): 1283-8.
264. Hodson L, Skeaff CM, Fielding BA. Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Progress in lipid research*. 2008; 47(5): 348-80.
265. Brossard N, Croset M, Normand S, Pousin J, Lecerf J, Laville M, et al. Human plasma albumin transports [13C]docosahexaenoic acid in two lipid forms to blood cells. *Journal of lipid research*. 1997; 38(8): 1571-82.
266. Gordon RS, Jr., Cherkes A. Unesterified fatty acid in human blood plasma. *The Journal of clinical investigation*. 1956; 35(2): 206-12.
267. Owen OE, Schramm VL. Lipid metabolism during starvation: hepatic energy balance and ketogenesis. *Biochemical Society transactions*. 1981; 9(4): 342-4.
268. Raclot T. Selective mobilization of fatty acids from adipose tissue triacylglycerols. *Progress in lipid research*. 2003; 42(4): 257-88.
269. Raclot T, Groscolas R. Selective mobilization of adipose tissue fatty acids during energy depletion in the rat. *Journal of lipid research*. 1995; 36(10): 2164-73.
270. Caspar-Bauguil S, Montastier E, Galinon F, Frisch-Benarous D, Salvayre R, Ritz P. Anorexia nervosa patients display a deficit in membrane long chain poly-unsaturated fatty acids. *Clin Nutr*. 2012; 31(3): 386-90.

271. Zak A, Vecka M, Tvrzicka E, Hruby M, Novak F, Papezova H, et al. Composition of plasma fatty acids and non-cholesterol sterols in anorexia nervosa. *Physiological research / Academia Scientiarum Bohemoslovaca*. 2005; 54(4): 443-51.
272. Joglekar CV, Fall CH, Deshpande VU, Joshi N, Bhalerao A, Solat V, et al. Newborn size, infant and childhood growth, and body composition and cardiovascular disease risk factors at the age of 6 years: the Pune Maternal Nutrition Study. *Int J Obes (Lond)*. 2007; 31(10): 1534-44.
273. Prendergast AJ, Humphrey JH. The stunting syndrome in developing countries. *Paediatrics and international child health*. 2014; 34(4): 250-65.
274. Calder PC, Dangour AD, Diekman C, Eilander A, Koletzko B, Meijer GW, et al. Essential fats for future health. *Proceedings of the 9th Unilever Nutrition Symposium, 26-27 May 2010. European journal of clinical nutrition*. 2010; 64 Suppl 4: S1-13.
275. Kenya National Bureau of Statistics. Kenya Demographic and Health Survey 2014: Key Indicators. 2015. Available from: <https://dhsprogram.com/pubs/pdf/PR55/PR55.pdf>
276. Nackers F, Broillet F, Oumarou D, Djibo A, Gaboulaud V, Guerin PJ, et al. Effectiveness of ready-to-use therapeutic food compared to a corn/soy-blend-based pre-mix for the treatment of childhood moderate acute malnutrition in Niger. *Journal of tropical pediatrics*. 2010; 56(6): 407-13.
277. Food and Agriculture Organization of the United States. Fats and fatty acids in human nutrition: Report of an expert consultation. Rome: FAO; 2010. Available at: http://www.who.int/nutrition/publications/nutrientrequirements/fatsandfattyacids_humannutrition/en/
278. Sewell GW, Hannun YA, Han X, Koster G, Bielawski J, Goss V, et al. Lipidomic profiling in Crohn's disease: abnormalities in phosphatidylinositols, with preservation of ceramide, phosphatidylcholine and phosphatidylserine composition. *The international journal of biochemistry & cell biology*. 2012; 44(11): 1839-46.
279. Postle AD, Henderson NG, Koster G, Clark HW, Hunt AN. Analysis of lung surfactant phosphatidylcholine metabolism in transgenic mice using stable isotopes. *Chemistry and physics of lipids*. 2011; 164(6): 549-55.
280. Dondorp AM, Angus BJ, Chotivanich K, Silamut K, Ruangveerayuth R, Hardeman MR, et al. Red blood cell deformability as a predictor of anemia in severe falciparum malaria. *The American journal of tropical medicine and hygiene*. 1999; 60(5): 733-7.

281. Thienprasert A, Samuhaseneetoo S, Popplestone K, West AL, Miles EA, Calder PC. Fish oil n-3 polyunsaturated fatty acids selectively affect plasma cytokines and decrease illness in Thai schoolchildren: a randomized, double-blind, placebo-controlled intervention trial. *The Journal of pediatrics*. 2009; 154(3): 391-5.
282. Koletzko B, Lien E, Agostoni C, Bohles H, Campoy C, Cetin I, et al. The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations. *Journal of perinatal medicine*. 2008; 36(1): 5-14.
283. Carlson SE. Early determinants of development: a lipid perspective. *The American journal of clinical nutrition*. 2009; 89(5): 1523S-9S.
284. Grantham-McGregor S. A review of studies of the effect of severe malnutrition on mental development. *The Journal of nutrition*. 1995; 125(8 Suppl): 2233S-8S.
285. Brenna JT, Salem N, Jr., Sinclair AJ, Cunnane SC. alpha-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. *Prostaglandins, leukotrienes, and essential fatty acids*. 2009; 80(2-3): 85-91.
286. Brenna JT, Carlson SE. Docosahexaenoic acid and human brain development: Evidence that a dietary supply is needed for optimal development. *Journal of human evolution*. 2014.
287. Grindel A, Staps F, Kuhnt K. Cheek cell fatty acids reflect n-3 PUFA in blood fractions during linseed oil supplementation: a controlled human intervention study. *Lipids in health and disease*. 2013; 12: 173.
288. Gibson RA, Neumann MA, Lien EL, Boyd KA, Tu WC. Docosahexaenoic acid synthesis from alpha-linolenic acid is inhibited by diets high in polyunsaturated fatty acids. *Prostaglandins, leukotrienes, and essential fatty acids*. 2013; 88(1): 139-46.
289. Gregory MK, Gibson RA, Cook-Johnson RJ, Cleland LG, James MJ. Elongase reactions as control points in long-chain polyunsaturated fatty acid synthesis. *PloS one*. 2011; 6(12): e29662.
290. Carlson SE, Werkman SH, Peeples JM, Cooke RJ, Tolley EA. Arachidonic acid status correlates with first year growth in preterm infants. *Proceedings of the National Academy of Sciences of the United States of America*. 1993; 90(3): 1073-7.
291. Gibson RA, Muhlhausler B, Makrides M. Conversion of linoleic acid and alpha-linolenic acid to long-chain polyunsaturated fatty acids (LCPUFAs), with a focus on pregnancy, lactation and the first 2 years of life. *Maternal & child nutrition*. 2011; 7 Suppl 2: 17-26.

292. Clark KJ, Makrides M, Neumann MA, Gibson RA. Determination of the optimal ratio of linoleic acid to alpha-linolenic acid in infant formulas. *The Journal of pediatrics*. 1992; 120(4 Pt 2): S151-8.
293. Taha AY, Cheon Y, Faurot KF, Macintosh B, Majchrzak-Hong SF, Mann JD, et al. Dietary omega-6 fatty acid lowering increases bioavailability of omega-3 polyunsaturated fatty acids in human plasma lipid pools. *Prostaglandins, leukotrienes, and essential fatty acids*. 2014; 90(5): 151-7.
294. Liou YA, King DJ, Zibrik D, Innis SM. Decreasing linoleic acid with constant alpha-linolenic acid in dietary fats increases (n-3) eicosapentaenoic acid in plasma phospholipids in healthy men. *The Journal of nutrition*. 2007; 137(4): 945-52.
295. Hsieh JC, Liu L, Zeilani M, Ickes S, Trehan I, Maleta K, et al. High-Oleic Ready-to-Use Therapeutic Food Maintains Docosahexaenoic Acid Status in Severe Malnutrition. *Journal of pediatric gastroenterology and nutrition*. 2015; 61(1): 138-43.
296. von Schacky C. Omega-3 fatty Acids in cardiovascular disease - An uphill battle. *Prostaglandins, leukotrienes, and essential fatty acids*. 2014.
297. McNamara RK. Evaluation of docosahexaenoic acid deficiency as a preventable risk factor for recurrent affective disorders: current status, future directions, and dietary recommendations. *Prostaglandins, leukotrienes, and essential fatty acids*. 2009; 81(2-3): 223-31.
298. Luxwolda MF, Kuipers RS, Sango WS, Kwesigabo G, Dijck-Brouwer DA, Muskiet FA. A maternal erythrocyte DHA content of approximately 6 g% is the DHA status at which intrauterine DHA biomagnifications turns into bioattenuation and postnatal infant DHA equilibrium is reached. *European journal of nutrition*. 2012; 51(6): 665-75.
299. Makhoul Z, Kristal AR, Gulati R, Luick B, Bersamin A, Boyer B, et al. Associations of very high intakes of eicosapentaenoic and docosahexaenoic acids with biomarkers of chronic disease risk among Yup'ik Eskimos. *The American journal of clinical nutrition*. 2010; 91(3): 777-85.
300. Glaser C, Lattka E, Rzehak P, Steer C, Koletzko B. Genetic variation in polyunsaturated fatty acid metabolism and its potential relevance for human development and health. *Maternal & child nutrition*. 2011; 7 Suppl 2: 27-40.
301. Jones K, Hunten-Kirsch B, Laving AM, Munyi CW, Ngari M, Mikusa J, et al. Mesalazine in the initial management of severely acutely malnourished children with environmental enteric dysfunction: a pilot randomized controlled trial. *BMC medicine*. 2014; 12(1): 133.

302. Colombel JF, Sands BE, Rutgeerts P, Sandborn W, Danese S, D'Haens G, et al. The safety of vedolizumab for ulcerative colitis and Crohn's disease. *Gut*. 2016.
303. Monteleone G, Neurath MF, Ardizzone S, Di Sabatino A, Fantini MC, Castiglione F, et al. Mongersen, an oral SMAD7 antisense oligonucleotide, and Crohn's disease. *The New England journal of medicine*. 2015; 372(12): 1104-13.
304. Coutinho BP, Oria RB, Vieira CM, Sevilleja JE, Warren CA, Maciel JG, et al. *Cryptosporidium* infection causes undernutrition and, conversely, weanling undernutrition intensifies infection. *The Journal of parasitology*. 2008; 94(6): 1225-32.
305. Brown EM, Wlodarska M, Willing BP, Vonaesch P, Han J, Reynolds LA, et al. Diet and specific microbial exposure trigger features of environmental enteropathy in a novel murine model. *Nature communications*. 2015; 6: 7806.
306. Monteleone G, MacDonald TT. Manipulation of cytokines in the management of patients with inflammatory bowel disease. *Annals of medicine*. 2000; 32(8): 552-60.
307. Rezaie A, Kuenzig ME, Benchimol EI, Griffiths AM, Otley AR, Steinhart AH, et al. Budesonide for induction of remission in Crohn's disease. *Cochrane Database Syst Rev*. 2015; 6: CD000296.
308. Ciacci C, Maiuri L, Russo I, Tortora R, Bucci C, Cappello C, et al. Efficacy of budesonide therapy in the early phase of treatment of adult coeliac disease patients with malabsorption: an in vivo/in vitro pilot study. *Clinical and experimental pharmacology & physiology*. 2009; 36(12): 1170-6.
309. Soendergaard C, Riis LB, Nielsen OH. Collagenous sprue: a coeliac disease look-alike with different treatment strategy. *BMJ case reports*. 2014; 2014.
310. Pasic S, Ristic G, Djuricic S, Prokic D, Zdravkovic S. Celiac-like sprue in Nijmegen breakage syndrome: successful treatment with budesonide. *Journal of investigational allergology & clinical immunology*. 2014; 24(2): 128-9.
311. Leffler DA, Kelly CP, Green PH, Fedorak RN, DiMarino A, Perrow W, et al. Larazotide acetate for persistent symptoms of celiac disease despite a gluten-free diet: a randomized controlled trial. *Gastroenterology*. 2015; 148(7): 1311-9 e6.
312. Fernandes M, Stein A, Newton CR, Cheikh-Ismail L, Kihara M, Wulff K, et al. The INTERGROWTH-21st Project Neurodevelopment Package: a novel method for the multi-dimensional assessment of neurodevelopment in pre-school age children. *PloS one*. 2014; 9(11): e113360.

313. Abubakar A, Holding P, van Baar A, Newton CR, van de Vijver FJ. Monitoring psychomotor development in a resource-limited setting: an evaluation of the Kilifi Developmental Inventory. *Annals of tropical paediatrics*. 2008; 28(3): 217-26.