Effects of lipid-based nutrient supplements on gut markers in stunted children: Secondary analysis of a randomised trial

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Effects of lipid-based nutrient supplements on gut markers in stunted children: Secondary analysis of a randomised trial

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Abstract

Objectives: To examine the effects of lipid-based nutrient supplements (LNS) containing milk protein (MP) and/or whey permeate (WP) on markers of intestinal inflammation and enterocyte mass among stunted children. Furthermore, to explore whether gut status modifies effects of LNS on growth and micronutrient status.

Methods: In a 2 × 2 factorial trial 12–59 months-old Ugandan children with stunting were randomized to four LNS formulations (100 g/day for 12 weeks) containing MP or soy protein and WP or maltodextrin, or to no supplementation. Linear mixed-effects models were used to explore faecal myeloperoxidase (f-MPO) and plasma citrulline (p-cit) as outcomes and modifiers of the intervention effects (ISRCTN13093195).

Results: Of 750 children, mean ± SD age was 32.0 ± 11.7 months and height-for-age *Z*-score was −3.02 ± 0.74. Neither MP nor WP had effects on p-cit or f-MPO. f-MPO decreased over time among controls (ratio of change 0.54, 95% confidence interval [CI]: 0.35, 0.84), but not among those given LNS (0.99, 95% CI: 0.79, 1.23) ($p = 0.016$). In contrast, LNS had no effect on p-cit ($p = 0.27$). The effect of LNS on cobalamin (B12) status was reduced in children with p-cit <20 μmol/L; whereby there was 20% (95% CI: 2, 35) lower increase in plasma cobalamin and 59% (95% CI: 13, 125) smaller decrease in plasma methylmalonic acid. p-cit or f-MPO did not modify the effects of LNS on growth or other micronutrient markers.

Conclusion: LNS had no effect on enterocyte mass and possibly increased intestinal inflammation. The effect of LNS on cobalamin status was reduced in those with low enterocyte mass.

KEYWORDS

citrulline, cobalamin, environmental enteric dysfunction, malabsorption, stunting

1 | INTRODUCTION

Stunting is widespread and associated with increased risk of infection, developmental delays, poor educational attainment, as well as chronic disease and reduced working capacity later in life.[1] Environmental enteric dysfunction (EED) may contribute to growth faltering.[2] EED affects the small intestine, and is characterised by villous atrophy, crypt hyperplasia and microbial translocation.[3] Data from biopsies and biomarkers suggests that EED affects a substantial proportion of children in low-income settings.[4] Poor nutrition and a high enteropathogen burden may be key drivers of EED.[5–7] In food insecure settings, the diet lacks nutrients essential for growth, development and/or intestinal repair.[8] Concurrent infections increase nutrient requirements and promote epithelial damage and inflammation,[9,10] exacerbating micronutrient deficiencies.

Recent trials that aimed to prevent stunting through improved water, sanitation and hygiene (WASH) and small-quantity lipid-based nutrient supplements (LNS), found no effect of WASH and only small effects of LNS on linear growth.[11,12] It was concluded that transformative WASH approaches would be necessary to sufficiently reduce the pathogen burden in these settings.[13] Similarly, perhaps small-quantity LNS interventions have been insufficient to meet the nutrient requirements for growth and intestinal repair in a population with EED. Consequently, there is a need to explore the interactions between diet and enteropathy.

We examined the effects of supplementation with large-quantity LNS containing milk protein (MP) and/or whey permeate (WP) on plasma citrulline (p-cit), a marker of enterocyte mass, and faecal myeloperoxidase (f-MPO), a marker of intestinal inflammation, in children with stunting. Furthermore, we explored whether the state of the gut, as measured by these markers, modified the effects of LNS on growth, haemoglobin and micronutrient status (iron, B12, folate and vitamin A).

What Is Known?

- Current nutrition interventions seem inadequate to support linear growth in children; environmental enteric dysfunction (EED) is thought to play a role.
- EED may create or exacerbate problems of malabsorption and micronutrient deficiency.

What Is New?

- There was no effect of lipid-based nutrient supplements (LNS), or ingredients milk protein and whey permeate on plasma citrulline, a marker of enterocyte mass. There was a greater increase in faecal myeloperoxidase, a marker of intestinal inflammation, among those supplemented with LNS, compared to no supplementation.
- Our data suggest that low enterocyte mass reduces the beneficial effect of LNS on B12 status.

2 | METHODS

2.1 | Study design

This intervention study examined exploratory outcomes as part of the 'Milk affecting growth, cognition and the gut in child stunting' trial (MAGNUS),[14] registered at www.isrctn.com: ISRCTN13093195. Briefly, MAGNUS was a randomised, double-blind, two-by-two factorial trial that aimed to assess the effects of large-quantity LNS containing MP and/or WP on growth and development in stunted children. The primary outcomes were height and knee-heel length, as previously published.[15]

2.2 | Ethics statement

Ethical approval was obtained from Makerere University, Ugandan National Council of Science and Technology and consultative approval was obtained from the Danish National Committee on Biomedical Research Ethics. Caregivers gave written informed consent and a literate witness was present if the caregiver was illiterate.

2.3 | Study sites and population

Between February and September 2020, mobile teams screened for childhood stunting in the villages surrounding Buwenge and Walukuba health centres, Jinja, East Central Uganda. Children living in the catchment area, aged 12–59 months with a height-for-age *Z*-score (HAZ) < –2,[16] were invited for eligibility screening. Those with severe acute malnutrition,[17] were excluded and referred for treatment. Additional exclusion criteria were complications requiring hospitalisation, history of allergy to peanuts or milk, disability impeding eating or measurement, plans to move from the area, previous enrollment of any child in the household or enrollment in another study.

2.4 | Intervention

Participants were randomly allocated to one of the four LNS interventions: +MP+WP, +MP–WP, –MP+WP,

–MP–WP, or to no supplement (1:1:1:1:1). The comparators were soy protein isolate for MP and maltodextrin for WP. The LNS was produced by Nutriset (Malaunay) as individually packaged 100 g (530–535 kcal) sachets of fortified peanut-based paste. Rations of one sachet per day were supplied to caregivers fortnightly for 12 weeks. Those not receiving LNS received laundry soap at these intervals. For further details, see Supplemental Digital Content #1 (intervention composition) and #2 (randomisation/blinding).

2.5 | Data collection

At baseline, information was collected on sociodemographics, breastfeeding, minimum dietary diversity score,[18] household food insecurity access scale,[19] and 14-day morbidity recall, using interviewer-administered questionnaires. Trained staff visited households to assess WASH characteristics.[14]

2.6 | Outcomes

The main and subgroup effects of LNS containing MP and/or WP on p-cit and f-MPO were assessed. We also assessed if (low vs. high) baseline p-cit and f-MPO modified the effects on growth and micronutrient-related outcomes. The effect estimates are differences in change between intervention groups from baseline to Week 12.

Height and weight were measured in triplicate, and the median was used. Height was measured using a wooden height board (Weigh and Measure LLC) to the nearest 0.1 cm; for those <24 months, length was measured. A knee-heel caliper (Mitutoyo), was used to collect five consecutive knee-heel length measurements, of which the median was used. Body weight was measured to the nearest 100 g using a digital double weighing scale (SECA 876, Hamburg).

2.7 | Biological sample collection and analysis

Blood and stool samples were collected at baseline and 12 weeks. The estimated time interval since the child had last eaten was recorded to indicate fasting status. A laboratory technician performed rapid diagnostic tests for malaria (SD Bioline malaria Ag Pf), HIV (Determine HIV-1/2 [Abbott], STAT-PAK [Chembio Diagnostics], and SD Bioline HIV-1/2 [Standard Diagnostics]), and haemoglobin concentration (Hb201+, HemoCue) according to the manufacturers' instructions. Haemoglobin <110 g/L defined anaemia. Stool collection kits and individual instruction on their use was given to caregivers. Samples were returned within 2 days of the scheduled visit. Approximately 1 g of stool was collected in a screw-top tube containing 4 mL of StayRNA (A&A Biotechnology) or RNAlater (Sigma-Aldrich). Stool sample consistency and history of diarrhoea were recorded using a modified four-point Bristol Stool Scale.

P-cit was measured at Chalmers University of Technology, Sweden using a Siex QTRAP 6500+ system (AB Sciex) with a Nexera Ultrahigh-Performance Liquid Chromatography system (Shimadzu). Sample preparation and analysis was based on the commercial aTRAQ kit for analysis of amino acids in physiological fluids (AB Sciex),[20] with certain modifications as reported elsewhere.[21] F-MPO was analysed at the University of Copenhagen using a commercially available sandwich Enzyme Linked Immunosorbent Assay (ELISA) kit according to the manufacturer's instructions (Human Myeloperoxidase DuoSet, R&D systems, Minneapolis, MN). The following biological markers were determined by VitMin lab in Willstaedt, Germany, using sandwich-ELISA[22]: serum C-reactive protein (s-CRP) and serum α_1-acid glycoprotein (s-AGP), both markers of systemic inflammation, serum ferritin (s-ferritin) a marker of iron stores, serum soluble transferrin receptor (s-sTfR), indicating peripheral iron deficiency and serum retinol binding protein (s-RBP), an indicator of vitamin A status. Both s-ferritin and s-RBP were corrected for inflammation.[23] Plasma cobalamin (p-cobalamin), plasma methyl malonic acid (p-MMA) and plasma folate (p-folate) were determined using the Advia Centaur CP Immunoassay System (Siemens) at Aarhus University Hospital, Denmark.[24] Together, p-cobalamin and p-MMA are markers of cobalamin (vitamin B12) status. Serum insulin-like growth factor-1 (s-IGF1) was determined using the Immulite2000 (Siemens) at the University of Copenhagen, Denmark. Further description of sample collection and processing are described in Supplemental Digital Content #3.

2.8 | Statistical methods

Data were double entered using EpiData Software (Epidata Association). *Z*-scores were calculated using the STATA module zscore06.[25] Statistical analyses were made using R version 4.1.2[26] based on intention-to-treat (ITT). Analyses were adjusted for baseline level of age, sex, season and the following random effects: site, id (only in case of repeated measurements over time), and batch number (only for models with citrulline as outcome). The inclusion of id random effects in the analyses provided results that were comparable to change score analyses. Missing values were not imputed. Right-skewed outcomes were logarithm transformed, implying that results should be understood in terms of fold changes or ratios of the fold changes for stratum-specific values over time. P-cit

was corrected for fasting status and inflammation[21] (Supplemental Digital Content #4).

Linear mixed models were used to assess the effects of LNS containing MP or WP on the change in p-cit and f-MPO from baseline to 12 weeks. First, we tested for interactions between MP versus WP. If no interaction, we tested for effects of +MP versus −MP and +WP versus −WP on p-cit and f-MPO. Additionally, we compared LNS irrespective of ingredient against the unsupplemented group. Sensitivity analysis was conducted to evaluate whether exclusion of those with a history of diarrhoea impacted the estimated effects of LNS.

We assessed for effect modification using linear mixed models with interaction between intervention and (i) sex, (ii) breastfeeding status, (iii) severity of stunting being moderate (≥−3 HAZ) or severe (<−3 HAZ), grade of systemic inflammation being high for (iv) s-CRP (>10 mg/L) or (v) s-AGP (≥1.2 g/L), (vi) recent diarrhoea and (vii) stool sample classified as diarrhoea. Additionally, we explored whether baseline p-cit <20 μmol/L and f-MPO > 2000 ng/L[21] modified the effects of LNS on growth and micronutrient status. Due to the exploratory nature of this study, we did not adjust for multiplicity. p-Values < 0.05 were considered significant.

3 | RESULTS

Between February and September 2020, 7611 children were screened for stunting; 1112 were referred for eligibility screening and 750 were included in the trial (Supplemental Digital Content #5). Of those, 55% ($n = 412$) were male, the mean ± SD age was 32.0 ± 11.7 months and HAZ was −3.02 ± 0.74. Half of children ($n = 368$) had s-AGP > 1.2 g/L, 22% ($n = 163$) had s-CRP > 10 mg/L and 64% ($n = 479$) had anaemia. Twenty-six percent ($n = 194$) met dietary diversity requirements. The majority had access to an improved water source ($n = 748$), but only 4% ($n = 29$) to a private tap. The randomization resulted in baseline equivalence (Table 1).

3.1 | The effect of milk in LNS

There was no interaction between MP and WP for p-cit or f-MPO ($p > 0.2$), hence the main effect of each intervention was assessed. Neither MP nor WP had an effect on p-cit (MP: −0.97 μmol/L, 95% confidence interval [CI]: −2.87, 0.93; WP: 1.07 μmol/L, 95% CI: −0.83, 2.97) or f-MPO (MP: 1.03, 95% CI: 0.67, 1.60; WP: 1.03 μmol/L, 95% CI: 0.66, 1.59). Over the 12 weeks, p-cit decreased in both the supplemented and unsupplemented groups, and there was no effect of the LNS per se on p-cit (1.30 μmol/L, 95% CI: −1.03; 3.64) (Table 2). For f-MPO there was no change among supplemented (ratio of change 0.99, 95% CI: 0.79, 1.23), but a decrease over time in the control group (ratio of change 0.54, 95% CI: 0.35, 0.84) ($p = 0.016$). Adjustment for diarrhoea did not change the effect estimates, and there were no differences between the unadjusted and adjusted models for both outcomes. In sensitivity analysis where children with a history of diarrhoea in the past 2 weeks were excluded (removed baseline $n = 199$, endpoint $n = 36$), the effect estimate remained unchanged for p-cit. However, the ratio between the relative change in f-MPO between LNS and no supplementation changed considerably from 1.82 (95% CI: 1.12, 2.96) (Table 2) to 1.20 (95% CI: 0.71, 2.12) after removing those with a history of diarrhoea (baseline $n = 207$, endpoint $n = 38$).

3.2 | Subgroup effects

Among those receiving LNS, MP was associated with a 4.69 (95% CI: 0.88, 8.50) μmol/L smaller decline in p-cit for girls (Supplementary Digital Content #6). There was no difference in the effect of LNS according to systemic inflammation indicated by s-CRP and s-AGP (Table 3). However, LNS increased f-MPO in those with recent or current diarrhoea, but not in those without diarrhoea (interaction, $p = 0.02$ and 0.04). There were no other subgroup effects of LNS or LNS containing MP or WP on p-cit and f-MPO (Supplementary Digital Content #6, #7 and #8).

3.3 | Gut as a modifier of the effect of LNS

At baseline, 26% ($n = 194$) had f-MPO > 2000 ng/mL and 15% ($n = 109$) had p-cit <20 μmol/L. Neither p-cit nor f-MPO modified the effects of the LNS intervention on growth outcomes (Supplemental Digital Content #9). In those receiving LNS compared to the unsupplemented controls, p-cobalamin increased by 138 (95% CI: 111, 164) pmol/L and p-MMA decreased by 33 (95% CI, 27–39) μmol/L over the 12 weeks, as reported elsewhere.[27] However, the effect of LNS on both markers of cobalamin status was modified by baseline p-cit (interaction $p = 0.008$ and 0.03) (Table 4). The interaction reflects a 20% (95% CI: 2, 35) lower effect of LNS on p-cobalamin and a 59% (95% CI: 13, 125) greater effect on p-MMA in those with low baseline p-cit. Likewise, baseline f-MPO > 2000 ng/mL tended to reduce the effects of LNS on markers of cobalamin status, but was not significant (interaction $p = 0.07$ and 0.10). p-cit or f-MPO did not modify the effect of LNS on other micronutrients (iron, folate or vitamin A).

TABLE 1 Baseline characteristics of 750 children with stunting randomised to LNS with or without milk protein or whey permeate or to no supplement.[a]

	LNS											
	Milk protein[b]				Whey permeate[b]				LNS		No supplement	
	n	+	*n*	–	*n*	+	*n*	–	*n*	+	*n*	–
Age, months	299	32.4 ± 11.5	301	31.5 ± 12.0	301	32.6 ± 12.1	299	31.2 ± 11.4	600	31.9 ± 11.8	150	32.3 ± 11.7
Sex, male	299	52% (156)	301	57% (172)	301	53% (159)	299	56% (169)	600	55% (328)	150	56% (84)
Anthropometrics												
Height-for-age	299	−3.02 ± 0.74	301	−3.04 ± 0.73	301	−3.06 ± 0.75	299	−2.99 ± 0.72	600	−3.03 ± 0.73	150	−2.99 ± 0.75
Weight-for-height	299	−0.27 ± 1.03	300	−0.42 ± 0.93	301	−0.34 ± 0.95	298	−0.35 ± 1.01	599	−0.35 ± 0.98	150	−0.43 ± 1.03
Weight-for-age	299	−1.87 ± 0.91	300	−1.97 ± 0.79	301	−1.94 ± 0.84	298	−1.90 ± 0.87	599	−1.92 ± 0.86	150	−1.97 ± 0.83
Clinical												
Diarrhoea in 2 weeks prior	299	24% (73)	301	29% (88)	301	24% (71)	299	30% (90)	600	27% (161)	150	32% (48)
Serum C-reactive protein, mg/L	294	1.45 [7.54]	297	1.74 [9.12]	296	1.51 [7.65]	295	1.51 [8.93]	591	1.51 [8.24]	150	1.64 [5.55]
Serum α1-acid glycoprotein, g/L	294	1.24 [0.77]	297	1.18 [0.67]	296	1.20 [0.67]	295	1.22 [0.76]	591	1.20 [0.72]	150	1.16 [0.80]
Haemoglobin, g/L	296	103.7 ± 14.7	297	103.4 ± 14.3	297	103.4 ± 15.3	296	103.6 ± 13.7	593	103.5 ± 14.5	150	104.6 ± 15.4
Diet												
Currently breastfed	298	14% (42)	300	13% (38)	299	16% (48)	299	11% (32)	598	13% (80)	148	10% (15)
Diet includes milk, meat or eggs	299	57% (172)	301	59% (177)	301	52% (157)	299	64% (192)	600	58% (349)	150	61% (91)
Minimum dietary diversity[c]	298	27% (80)	300	25% (76)	299	26% (77)	299	26% (79)	598	26% (156)	148	26% (38)
Household												
Urban residence	299	55% (166)	301	56% (170)	301	57% (172)	299	55% (164)	600	56% (336)	150	53% (79)
Maternal age, years	273	26.8 ± 6.3	282	25.9 ± 5.7	278	26.4 ± 5.8	277	26.2 ± 6.3	555	26.3 ± 6.0	137	26.6 ± 6.3
Mother education, primary or less	289	62% (178)	292	62% (182)	296	67% (197)	285	57% (163)	581	62% (360)	145	58% (84)
Food secure[d]	299	5% (16)	301	5% (14)	301	4% (11)	299	6% (19)	600	5% (30)	150	2% (3)
WASH characteristics												
Drinking water not treated	299	71% (213)	301	68% (204)	301	70% (212)	299	69% (205)	600	69% (417)	150	65% (98)
Soap available for hand washing	299	29% (86)	301	24% (72)	301	27% (82)	299	25% (76)	600	26% (158)	150	25% (38)

Abbreviation: LNS, lipid-based nutrient supplements.

[a]Data are mean ± standard deviation, median [interquartile range] or % (*n*).

[b]Milk protein and comparator soy protein isolate, whey permeate and comparator maltodextrin.

[c]Positive score is minimum five of eight food groups eaten in past 24 h (Coates et al.[19]).

[d]Household food insecurity access scale (USAID, FANTA v.3).

TABLE 2 The effect of 12 weeks of supplementation with versus without MP and with versus without WP and the effect of LNS compared with no supplementation on markers p-cit and f-MPO in children with stunting.

Outcome	Supplement		Baseline N	Baseline Mean ± SD	12 weeks N	12 weeks Mean ± SD	Change	95% CI	Difference in change[a] Δ	95% CI	p
p-cit, μmol/L[b]	LNS	–MP	291	28.0 ± 9.4	287	25.8 ± 7.8	–1.38	–1.38; –3.29	–	–	–
		+MP	287	28.9 ± 10.6	289	25.9 ± 8.2	–2.34	–4.28; –0.41	–0.97	–2.87; 0.93	0.32
	LNS	–WP	288	28.2 ± 10.2	284	25.1 ± 7.4	–2.77	–4.68; –0.86	–	–	–
		+WP	290	28.6 ± 9.8	292	26.6 ± 8.5	–1.70	–3.61; 0.22	1.07	–0.83; 2.97	0.27
	None	–	146	30.0 ± 15.7	145	26.0 ± 8.0	–3.92	–6.19; –1.65	–	–	–
	LNS	LNS	578	28.4 ± 10.0	576	25.7 ± 8.0	–2.61	–3.98; –1.25	1.30	–1.03; 3.64	0.27
				Median (IQR)		Median (IQR)					
Faecal myeloperoxidase, ng/mL[c]	LNS	–MP	298	436 (135; 1663)	292	394 (121; 1407)	1.10	0.75; 1.61	–	–	–
		+MP	297	535 (200; 2171)	292	494 (200; 1813)	1.13	0.77; 1.66	1.03	0.67; 1.60	0.89
	LNS	–WP	297	457 (140; 2029)	292	451 (155; 1488)	1.25	0.85; 1.82	–	–	–
		+WP	298	530 (179; 1687)	292	453 (159; 1541)	1.28	0.87; 1.87	1.03	0.66; 1.59	0.91
	None	–	149	536 (170; 2674)	148	305 (116; 1167)	0.54	0.35; 0.84	–	–	–
	LNS	LNS	595	497 (159; 1951)	584	452 (157; 1498)	0.99	0.79; 1.23	1.82	1.12; 2.96	0.016

Abbreviations: CI, confidence interval; f-MPO, faecal myeloperoxidase; LNS, lipid-based nutrient supplements; MP, milk protein; p-cit, plasma citrulline; WP, whey permeate.

[a]Data are presented as mean difference in change (p-cit) or the ratio of change (f-MPO) between the intervention and comparison group, with 95% CIs and *p*-values based on linear mixed effects models adjusted for baseline values, age, sex, season and random effects; site, ID and batch number (p-cit only). The estimation of change determined by the model is not always equal to the difference in means calculated on raw data. All *p*-values for interaction between p-cit or f-MPO and MP and WP were >0.2.

[b]Corrected for fasted state and systemic inflammation.

[c]Log-transformed.

TABLE 3 Interaction between 12 weeks of supplementation with lipid-based nutrient supplements and systemic inflammation or recent diarrhoea on markers p-cit and f-MPO in children with stunting.[a]

	Inflammation s-CRP ≤10 mg/L >10 mg/L			Inflammation s-AGP ≤1.2 g/L >1.2 g/L			Recent history of diarrhoea[b] No diarrhoea Diarrhoea			Diarrhoeal stool sample No diarrhoea Diarrhoea		
Outcome	**p-int**		**95% CI**	**p-int**		**95% CI**	**p-int**		**95% CI**	**p-int**		**95% CI**
p-cit,	0.56	1.77	−0.81; 4.36	0.92	1.69	−1.46; 4.83	0.88	0.27	−2.35; 2.89	0.82	1.46	−1.20; 4.12
µmol/L[c]		−0.21	−6.34; 5.92		1.44	−2.23; 5.11		1.01	−7.88; 9.90		0.76	−4.50; 6.01
f-MPO,	0.20	2.06	1.20; 3.53	0.83	1.91	0.99; 3.68	0.04	1.22	0.71; 2.11	0.02	1.34	0.78; 2.33
ng/mL[d]		0.86	0.26; 2.91		1.71	0.80; 3.62		7.53	1.40; 40.47		5.65	1.97; 16.20

Abbreviations: CI, confidence interval; f-MPO, faecal myeloperoxidase; p-cit, plasma citrulline; P-int, *p*-value for interaction; s-AGP, serum α1-acid glycoprotein; s-CRP, serum C-reactive protein.

[a]Analysed using a linear mixed effects models adjusted for the baseline value, age, sex, season, and random effects; site, ID and batch (p-cit only). Data are *p*-value for interaction for the mean difference in change (p-cit) or the ratio of the fold change (f-MPO) over time between stratum specific values and 95% CIs.

[b]Diarrhoea in last 14 days.

[c]Corrected for fasted state and systemic inflammation, p-cit at baseline ($n = 724$) and endpoint ($n = 721$).

[d]Log transformed, f-MPO at baseline ($n = 744$) and endpoint ($n = 732$).

TABLE 4 Baseline p-cit < 20 µmol/L and f-MPO > 2000 ng/mL as modifiers of the effects of 12 weeks of supplementation with lipid-based nutrient supplements on micronutrient status.[a]

		p-cit low[b,c] ≥20 µmol/L <20 µmol/L				f-MPO high ≤2000 ng/mL >2000 ng/mL		
Outcome	**Baseline *n*[d]**	**p-int**		**95% CI**	**Baseline *n*[e]**	**p-int**		**95% CI**
Plasma cobalamin,[f] pmol/L	700	0.03	1.51	1.41; 1.61	713	0.07	1.51	1.40; 1.63
			1.20	0.99; 1.46			1.33	1.18; 1.49
Plasma methylmalonic acid,[f] nmol/L	714	0.008	0.62	0.55; 0.70	727	0.10	0.63	0.55; 0.71
			0.99	0.71; 1.37			0.77	0.63; 0.94
Serum ferritin,[c,f] µg/L	724	0.41	1.40	1.20; 1.63	735	0.57	1.47	1.23; 1.74
			1.70	1.10; 2.61			1.34	1.03; 1.74
Haemoglobin,[g] g/L	721	0.56	4.07	1.25; 6.89	737	0.18	3.33	0.17; 6.48
			6.52	−1.32; 14.36			7.25	2.43; 12.06
Plasma folate,[g] nmol/L	675	0.86	9.51	6.93; 12.1	686	0.42	9.17	6.30; 12.03
			10.23	2.82; 17.65			11.33	6.96; 15.69
Serum soluble transferrin receptor[f] mg/L	724	0.21	0.87	0.79; 0.97	735	0.10	0.81	0.72; 0.90
			0.72	0.55; 0.95			0.96	0.81; 1.14
Serum retinol binding protein,[c,g] µmol/L	724	0.32	−0.07	−0.13; −0.01	735	0.56	−0.07	−0.13; −0.005
			0.02	−0.14; 0.18			−0.03	−0.13; 0.06

Abbreviations: CI, confidence interval; f-MPO, faecal myeloperoxidase; p-cit, plasma citrulline; P-int, *p* value for interaction.

[a]Produced from linear mixed effects models adjusted for the baseline value, age, sex, season, and random effects; site and ID.

[b]Corrected for fasted state.

[c]Corrected for systemic inflammation.

[d]p-cit < 20 µmol/L ~15% of this value.

[e]f-MPO > 2000 ng/mL ~26%.

[f]Outcome log transformed and data presented as *p*-value for interaction for the ratio of the fold change between stratum specific values and 95% CIs.

[g]Data are *p*-value for interaction for the mean difference in change between stratum specific values and 95% CIs.

4 | DISCUSSION

Although we provided a large-quantity LNS, the lack of effect on EED markers is not dissimilar to previous studies providing small-quantity LNS. Two large birth cohort trials in Zimbabwe and Bangladesh in which children were randomised to small-quantity LNS from 6 to 18 months, concluded that LNS did not impact any EED biomarkers, including p-cit.[28] and f-MPO.[28,29] Likewise, a trial in Bangladesh providing daily small- to

medium-quantity LNS for children from 6 months, reported that EED markers including f-MPO were not improved at 18 months.[30] In this study, f-MPO decreased over time to a greater extent in the unsupplemented group. Adjustment for diarrhoea did not change the estimate, whereas removal of diarrhoeal samples in sensitivity analysis reduced the effect estimate and a difference could no longer be seen between groups. Interestingly, we found a greater increase in f-MPO among those receiving LNS who had current or recent diarrhoea and this may be an indicator that LNS has a local inflammatory effect, as reported by others.[31] This may be due to emulsifiers.[32,33] or the high fat content of LNS.[34]

This large-quantity LNS was in itself inadequate to improve markers of enterocyte mass and intestinal inflammation. In vivo models have demonstrated that neither an insufficient diet nor exposure to bacteria in themselves are a cause of enteropathy, but it is the interaction between these two factors that seems to drive EED.[5] For populations living in conditions of frequent pathogen exposure, chronic inflammation and perhaps malabsorption, it is expected that recommended nutrient intakes are insufficient. It is likely that a transformative WASH approach combined with quality nutrition is required to prevent or improve EED.

Interestingly, the results of two markers, p-cobalamin and p-MMA indicate that the beneficial effect of LNS on cobalamin status is reduced in those with low baseline p-cit. Cobalamin is absorbed as a complex with intrinsic factor (IF) in the terminal ileum, part of the intestine reportedly unaffected by EED.[8] Therefore, rather than low enterocyte mass per se being responsible for the reduced effect on cobalamin status, it is more likely driven by other factors associated with low enterocyte mass. These factors could be for example, the excessive presence of bacteria or parasites in the small intestine.[35] Children living in low-income settings are predisposed to small intestinal bacterial overgrowth[5] and intestinal parasitic infections. Bacterial overgrowth has consistently been associated with stunting[9,37,38] and has been implicated in EED.[36,39] Although the role of bacterial overgrowth in EED remains unclear,[38,40] it is hypothesised to drive local inflammation, epithelial damage and impaired absorptive function.[41–43] Indeed, bacterial overgrowth is a well-established cause of cobalamin deficiency. Bacteria in the upper small intestine outcompete host IF and sequester cobalamin, thus reducing the amount of IF-bound cobalamin that reaches the terminal ilium for absorption.[44] Likewise, small intestinal helminths (e.g., *Ascaris lumbricoides* and hookworm) are associated with EED,[45] and likely compete for host cobalamin. Our current findings may be reflecting an association between reduced enterocyte mass and cobalamin-sequestering pathogens in the proximal small intestine.

Still, some malabsorption of cobalamin may be directly related to loss of enterocyte mass. Patients with untreated coeliac disease commonly experience villous atrophy as a result of chronic intestinal inflammation and a greater proportion are reported to have insufficient cobalamin status compared to the normal population.[46,47] Since cobalamin is essential for DNA synthesis and cell division, malabsorption has implications for rapidly dividing cells, including the small intestinal enterocytes. As such, malabsorption of cobalamin may be contributing to the pathogenesis of EED, perhaps also worsening malabsorption.

Interestingly, in both this study[48] and a study among young children in Burkina Faso, we found an association between a positive malaria test and low serum cobalamin status.[49] We speculate that the relationship is due to an uptake of cobalamin from the blood by the plasmodium parasite, known to express cobalamin-dependent methionine synthase.[50]

The strengths of this study were the factorial randomised design, the large and near complete data set, the nonsupplemented controls, and the use of both p-cobalamin and p-MMA as markers of cobalamin status. We find it unlikely that laundry soap given to families of unsupplemented children conferred an effect on the gut, given the lack or modest effects of intensive WASH interventions in large trials in Kenya, Zimbabwe and Bangladesh.[11] Another limitation was the use of only two EED markers, and that stool samples were collected in fixative (RNAlater and StayRNA), which may have increased the variation in f-MPO results. In addition, differences between groups may be underestimated since we did not measure f-MPO according to the dry weight of the sample or exclude diarrhoeal samples from stool analysis. Diarrhoea can dilute the sample and so reduce the amount of stool markers measured in the sample. It is also a limitation that we may have had limited power to detect an interaction, as the sample size of the trial was based on power to detect differences between any two of the five groups.

In conclusion, there was no effect of MP or WP contained in large-quantity LNS, on markers of intestinal inflammation or enterocyte mass. There was no effect of the LNS itself on enterocyte mass but a possible negative effect of LNS on intestinal inflammation. Interestingly, the beneficial effect of LNS on cobalamin status was reduced in those with low enterocyte mass, and this may be related to cobalamin-sequestering by pathogens. Cobalamin malabsorption may be contributing to the pathogenesis of EED. There is a need to understand the interplay between EED and malabsorption.

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CONFLICT OF INTEREST STATEMENT

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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