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Correlates of Plasma Citrulline, a Potential Marker of Enterocyte Mass, among Children with Stunting: A Cross-Sectional Study in Uganda

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A B S T R A C T

Background: Environmental enteric dysfunction (EED) is associated with stunting. Citrulline, produced in mature enterocytes, may be a valuable biomarker of small intestinal enterocyte mass in the context of EED.
Objectives: We aimed to explore the correlates of plasma citrulline (p-cit) in children with stunting.
Methods: In a cross-sectional study using baseline data from the community-based MAGNUS (milk affecting growth, cognition and the gut in child stunting) trial (ISRCTN13093195), we explored potential correlates of p-cit in Ugandan children with stunting aged 12–59 mo. Using linear regression in univariate and multivariate models, we explored associations with socioeconomics, diet, micronutrient status, and water, sanitation, and hygiene characteristics. The influence of covariates age, fasting, and systemic inflammation were also explored.
Results: In 750 children, the mean ± standard deviation age was 32.0 ± 11.7 mo, and height-for-age *z*-score was –3.02 ± 0.74. P-cit, available for 730 children, differed according to time fasted and was 20.7 ± 8.9, 22.3 ± 10.6 and 24.2 ± 13.1 μmol/L if fasted <2, 2–5 and >5 h, respectively. Positive correlates of p-cit were age [0.07; 95% confidence interval (CI): 0.001, 0.15 μmol/L] and $\log_{10}$ serum insulin-like growth factor-1 (8.88; 95% CI: 5.09, 12.67 μmol/L). With adjustment for systemic inflammation, the association with serum insulin-like growth factor-1 reduced (4.98; 95% CI: 0.94, 9.03 μmol/L). Negative correlates of p-cit included food insecurity, wet season (–3.12; 95% CI: –4.97, –1.26 μmol/L), serum C-reactive protein (–0.15; 95% CI: –0.20, –0.10 μmol/L), serum α_1-acid glycoprotein (–5.34; 95% CI: –6.98, –3.70 μmol/L) and anemia (–1.95; 95% CI: –3.72, –0.18 μmol/L). Among the negatively correlated water, sanitation, and hygiene characteristics was lack of soap for handwashing (–2.53; 95% CI: –4.82, –0.25 μmol/L). Many associations attenuated with adjustment for inflammation.
Conclusions: Many of the correlates of p-cit are characteristic of populations with a high EED prevalence. Systemic inflammation is strongly associated with p-cit and is implicated in EED and stunting. Adjustment for systemic inflammation attenuates many associations, reflecting either confounding, mediation, or both. This study highlights the complex interplay between p-cit and systemic inflammation.

Keywords: stunting, environmental enteric dysfunction, citrulline, inflammation, water sanitation and hygiene

Introduction

Stunting is associated with poor childhood survival, developmental delays, and lower educational attainment [1]. Globally, it affects an estimated 149 million children under 5 y [2]. Multiple factors are thought to contribute to stunting, including low socioeconomic status [3], repeated infections, and inadequate diet [4]. The interplay between these factors is still poorly understood.

Environmental enteric dysfunction (EED), a subclinical inflammatory condition of the small intestine, is pervasive among children living in low-income settings and has been proposed as

Abbreviations: EED, environmental enteric dysfunction; HAZ, height or length-for-age *z*-score; p-cit, plasma citrulline; s-AGP, serum α1-acid glycoprotein; s-CRP, serum C-reactive protein; s-IGF1, serum insulin-like-growth factor-1; WASH, water; sanitation, and hygiene.

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an important contributor to stunting [5]. Biopsies obtained from adults and children living in these settings indicate morphologic changes to the small intestinal mucosa that are considered characteristic of EED: flattened villi, crypt hyperplasia, and evidence of submucosal inflammation [6]. It is widely believed that EED results from exposure to pathogens and toxins through inadequate water, sanitation, and hygiene (WASH). This perpetual state of inflammation damages tissues and leads to a loss of epithelial cells, barrier integrity, and possibly absorptive function [7], thus exacerbating or creating nutrient deficiencies [8].

To elucidate the functional implications of EED and its role in stunting, a range of biomarkers reflecting different domains or intestinal functions are currently being explored [9]. One candidate marker is citrulline, a nonprotein amino acid produced in the small intestine by enterocytes at the villus tips. In a number of intestinal disease states, plasma citrulline (p-cit) has been shown to be a marker of enterocyte mass [10]. In short bowel syndrome, p-cit has been positively correlated with intestinal length [11], and in celiac disease, it negatively correlated with the grade of disease severity [12]. Given its use as a marker in other enteropathies, p-cit may be a relevant marker of EED [8]. The objective of this study was to explore correlates of p-cit: socioeconomic factors, diet, anthropometry, inflammation, insulin-like-growth factor-1 (IGF-1), micronutrient status, season, and household WASH characteristics in children with stunting.

Methods

Study design and ethics

This cross-sectional study used baseline data from the MAGNUS (milk affecting growth, cognition and the gut in child stunting) trial. The trial aimed to assess the effects of large-quantity lipid-based nutrient supplements containing milk protein and/or whey permeate on the growth and development of children with stunting. The trial protocol [13] and main results are published elsewhere [14]. The study protocol was approved by the School of Medicine Research Ethics Committee at Makerere University and The Ugandan National Council of Science and Technology. Consultative approval was given by the Danish National Committee on Biomedical Research Ethics.

Study sites and population

Jinja, a district of the Busoga subregion, is located on the northern shores of Lake Victoria. In this region, an estimated 29% of children below 5 y are stunted [15]. Much of the agricultural land is used for commercial production of sugar cane; however, the majority of livelihoods are derived from subsistence farming, where maize is the staple crop [16].

Mobile teams screened children from villages surrounding the study sites at Buwenge and Walukuba health centers. Those living in the catchment area, aged between 12 and 59 mo, with a length- or height-for-age *z*-score (HAZ) of <–2 according to WHO growth standards [17], were then invited to a study site for final eligibility screening and potential enrolment. Those identified with severe acute malnutrition, according to WHO criteria [18], were excluded and referred for treatment. Additional criteria for exclusion were medical complications requiring hospitalization, obvious disability, or participation in another study. Children with a history of allergy to peanuts or milk or whose family planned to move from the catchment area within 6 mo were also excluded as this would have interfered with the original MAGNUS trial. Prior to enrolment, caregivers provided written informed consent whereby understanding was assessed using a verbal questionnaire. A literate witness was present if the caregiver was illiterate.

Data collection

Baseline data were collected on the day of enrolment unless stated otherwise. Trained nutritionists used interviewer-administered questionnaires to enquire about diet, including breastfeeding status, meal variety, and frequency during the past 24 h. The diet was classified according to the minimum dietary diversity score, where ≥5 of 8 food groups should be consumed for the diet to be considered adequately diverse [19]. The same minimum dietary diversity score cutoffs were applied irrespective of age group. A medical doctor conducted a clinical assessment, which included a 14-d history of diarrhoeal symptoms and antibiotic use. Height or length and weight were measured in triplicate, and the median value was used. Height or length was measured to the nearest 0.1 cm with a wooden height board (Weigh and Measure LLC). Bodyweight was measured to the nearest 100 g using a digital double weighing scale (SECA 876).

TABLE 1
Baseline characteristics of children with stunting[1]

Parameter	value	N
Age (mo)	32 ± 11.7	750
Sex, female	45% (338)	750
Anthropometrics		
Height-for-age *z*-score	–3.02 ± 0.74	750
Weight-for-height *z*-score	–0.36 ± 0.99	749
Weight-for-age *z*-score	–1.93 ± 0.85	749
Diet		
Currently breastfeeding	13% (95)	741
Inadequate dietary diversity[2]		
12–23 mo	59% (130)	222
24–59 mo	81% (422)	524
Household		
Rural residence	45% (333)	750
Own agricultural land	41% (311)	750
Own animals	50% (379)	750
Food secure[3]	4% (33)	750
Maternal age (y)	26.4 ± 6.1	692
Maternal education, primary or less	61% (444)	726
Paraclinical		
Malaria positive (rapid test)	40% (292)	737
Anaemia[4]	65% (479)	743
Insulin-like growth factor-1 (ng/mL)	37.4 (24.2; 53.3)	740
C-reactive protein (mg/L)	1.56 (0.33; 8.25)	741
α_1-acid glycoprotein (g/L)	1.20 (0.88; 1.61)	741
Outcome		
Citrulline (μmol/L)	23.0 ± 11.7	730
Fasted <2 h	20.7 ± 8.9	30
Fasted 2–5 h	22.3 ± 10.6	404
Fasted 5+ h	24.2 ± 13.1	296

[1] Data are presented as mean ± SD, median (interquartile range), or % (*n*) and *N*.
[2] Minimum dietary diversity score: adequate is a minimum of 5 of 8 food groups (including breastmilk) eaten in the past 24 h.
[3] Household food insecurity access scale.
[4] Hemoglobin <110 g/L.

TABLE 2
Dietary and socioeconomic correlates of plasma citrulline (μmol/L) in children with stunting ($n = 730$)

	n[3]	Model 1 (Unadjusted)			Model 2 (Model 1 + age and sex)			Model 3[1] (Model 2 + fasting)			Model 4[2] (Model 3 + inflammation)		
		β	95% CI	*P* value	β	95% CI	*P* value	β	95% CI	*P* value	β	95% CI	*P* value
Residence	730	—	—	—	—	—	—	—	—	—	—	—	—
Urban	396	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Rural	334	–2.24	–3.94; –0.55	0.01	–2.56	–4.28; –0.84	0.003	–2.33	–4.49; –0.18	0.03	–0.20	–2.39; 2.00	0.86
Household food insecurity[4]	730	—	—	—	—	—	—	—	—	—	—	—	—
Food secure	33	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Mildly insecure	26	–4.35	–10.35; 1.64	0.15	–4.53	–10.51; 1.46	0.14	–4.64	–10.62; 1.33	0.13	–3.49	–9.30; 2.32	0.24
Moderately insecure	208	–5.02	–9.30; –0.73	0.02	–5.50	–9.79; –1.22	0.01	–5.42	–9.69; –1.14	0.01	–4.41	–8.57; –0.25	0.04
Severely insecure	463	–3.35	–7.46; 0.77	0.11	–3.73	–7.85; 0.39	0.08	–3.76	–7.87; 0.35	0.07	–2.68	–6.69; 1.34	0.19
Diet		—	—	—	—	—	—	—	—	—	—	—	—
Breastfeeding	726	—	—	—	—	—	—	—	—	—	—	—	—
Yes	92	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
No	634	2.46	–0.08; 5.01	0.06	1.50	–1.36; 4.35	0.30	1.37	–1.49; 4.23	0.35	2.34	–0.46; 5.15	0.10
Diet includes	730	—	—	—	—	—	—	—	—	—	—	—	—
Meat, eggs, or dairy	425	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
No meat, eggs, or dairy	305	0.31	–1.41; 2.03	0.72	0.07	–1.65; 1.80	0.93	0.42	–1.33; 2.17	0.64	1.03	–0.70; 2.75	0.24
Dietary diversity[5]	726	—	—	—	—	—	—	—	—	—	—	—	—
Adequate	185	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Inadequate	541	0.75	–1.20; 2.70	0.45	0.35	–1.63; 2.33	0.73	0.45	–1.54; 2.43	0.66	1.16	–0.78; 3.10	0.24
Meal frequency	728	—	—	—	—	—	—	—	—	—	—	—	—
3+	668	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
<3	60	0.37	–2.72; 3.46	0.82	0.35	–2.73; 3.44	0.82	0.42	–2.65; 3.50	0.79	0.78	–2.23; 3.79	0.61

Abbreviation: CI, confidence interval.
[1] Adjustment for fasting based on categorical variable <2 h, 2–5 h, and 5+ h after a meal.
[2] Inflammation: serum α_1-acid glycoprotein and serum C-reactive protein as continuous variables.
[3] May not sum ≤730 because of missing data.
[4] Based on Household Food Insecurity Access Scale.
[5] Adequate is a minimum of 5 of 8 food groups (including breastmilk) eaten in the past 24 h.

TABLE 3
Household water, sanitation, and hygiene correlates of plasma citrulline (μmol/L) in children with stunting ($n = 730$)

	n[3]	Model 1 (Unadjusted)			Model 2 (Model 1 + age and sex)			Model 3[1] (Model 2 + fasting)			Model 4[2] (Model 3 + inflammation)		
		β	95% CI	*P* value	β	95% CI	*P* value	β	95% CI	*P* value	β	95% CI	*P* value
Dwelling materials	—	—	—	—	—	—	—	—	—	—	—	—	—
Walls	730	—	—	—	—	—	—	—	—	—	—	—	—
Cement or brick	488	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Thatch and other[4]	242	–1.89	–3.68; –0.09	0.04	–1.78	–3.58; 0.01	0.051	–2.31	–4.15; –0.47	0.01	–2.21	–4.00; –0.42	0.02
Floor	730	—	—	—	—	—	—	—	—	—	—	—	—
Cement	280	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Dirt or mud[4]	450	–2.92	–4.65; –1.18	0.001	–3.14	–4.88; –1.40	<0.001	–2.88	–4.66; –1.09	0.002	–1.70	–3.50; 0.09	0.06
Drinking water	—	—	—	—	—	—	—	—	—	—	—	—	—
Source	730	—	—	—	—	—	—	—	—	—	—	—	—
Private tap	27	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Public tap	323	–3.02	–7.61; 1.56	0.20	–2.72	–7.29; 1.86	0.24	–2.86	–7.45; 1.72	0.22	–1.54	–6.02; 2.94	0.50
Public borehole	380	–4.22	–8.77; 0.34	0.07	–4.24	–8.79; 0.32	0.07	–3.75	–8.36; 0.85	0.11	–0.89	–5.44; 3.66	0.70
Treated	730	—	—	—	—	—	—	—	—	—	—	—	—
Yes[5]	213	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
No	517	–0.89	–2.75; 0.98	0.35	–1.11	–2.98; 0.75	0.24	–0.82	–2.72; 1.08	0.40	–0.06	–1.93; 1.81	0.95
Toilet[6]	728	—	—	—	—	—	—	—	—	—	—	—	—
Improved	409	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Not improved	319	–2.09	–3.80; –0.39	0.02	–2.27	–3.98; –0.55	0.01	–1.93	–3.69; –0.17	0.03	–0.74	–2.49; 1.02	0.41
Toilet shared	713	—	—	—	—	—	—	—	—	—	—	—	—
No	332	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Yes[7]	381	1.38	–0.35; 3.11	0.12	1.51	–0.22; 3.25	0.09	1.01	–0.89; 2.91	0.30	–0.11	–1.98; 1.76	0.91
Handwashing station	730	—	—	—	—	—	—	—	—	—	—	—	—
Soap and water	186	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Water only	222	–2.59	–4.86; –0.32	0.03	–2.67	–4.92; –0.41	0.02	–2.53	–4.82; –0.25	0.03	–1.45	–3.71; 0.81	0.21
Neither soap nor water	322	–3.08	–5.18; –0.98	0.004	–3.24	–5.34; –1.13	0.003	–2.87	–5.17; –0.57	0.01	–0.64	–2.99; 1.72	0.60
Animals[8]	727	—	—	—	—	—	—	—	—	—	—	—	—
Not kept, not observed	91	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Kept, not observed	138	1.11	–1.98; 4.20	0.48	0.95	–2.14; 4.04	0.55	1.21	–1.88; 4.31	0.44	2.59	–0.45; 5.63	0.09
Not kept, observed	241	2.02	–0.79; 4.84	0.16	1.98	–0.83; 4.79	0.17	1.48	–1.44; 4.41	0.32	0.80	–2.06; 3.66	0.58
Kept, observed	257	3.16	0.37; 5.95	0.03	2.84	0.04; 5.64	0.047	2.72	–0.08; 5.53	0.06	2.72	–0.01; 5.45	0.051

Abbreviation: CI, confidence interval.
[1] Adjustment for fasting based on categorical variable <2 h, 2–5 h, and 5+ h after a meal.
[2] Inflammation: serum α_1 -acid glycoprotein + serum C-reactive protein as continuous variables.
[3] May not sum ≤730 because of missing data.
[4] Building materials, such as sheet metal, thatch, wood planks, dirt, and mud, are classified as other materials.
[5] Yes: treatment by boiling or chemicals.
[6] Improved: flush or pour flush toilets and pit latrines with slabs; unimproved: pit latrines with no slab, hanging or bucket latrines, and open defecation.
[7] Toilet facility shared with ≥1 other household.
[8] Animals kept by household and/or observed roaming/tied up close to the living quarters.

TABLE 4
Anthropometric and clinical correlates of plasma citrulline (μmol/L) in children with stunting ($n = 730$)

	n[3]	Model 1 (Unadjusted)			Model 2 (Model 1 + age and sex)			Model 3[1] (Model 2 + fasting)			Model 4[2] (Model 3 + inflammation)		
		β	95% CI	*P* value	β	95% CI	*P* value	β	95% CI	*P* value	β	95% CI	*P* value
Anthropometrics	—	—	—	—	—	—	—	—	—	—	—	—	—
Height-for-age, *z*-score[4]	730	0.03	–1.12; 1.17	0.96	–0.20	–1.36; 0.95	0.73	–0.33	–1.49; 0.83	0.57	–1.23	–2.39; –0.07	0.04
<–2 to –3	424	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
<–3	306	0.73	–0.99; 2.45	0.41	1.01	–0.72; 2.73	0.25	1.17	–0.55; 2.90	0.18	2.15	0.44; 3.86	0.01
Weight-for-height, *z*-score[4]	729	0.37	–0.48; 1.23	0.39	0.21	–0.65; 1.08	0.63	0.32	–0.54; 1.19	0.46	0.26	–0.58; 1.11	0.54
≥–2	692	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
<–2	37	–1.42	–5.29; 2.45	0.47	–0.95	–4.83; 2.93	0.63	–1.15	–5.02; 2.73	0.56	–1.23	–4.98; 2.53	0.52
Weight-for-age, *z*-score[4]	729	0.22	–0.78; 1.22	0.66	0.13	–0.86; 1.13	0.79	0.18	–0.82; 1.17	0.73	–0.26	–1.25; 0.72	0.60
≥–2	408	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
<–2	621	–0.93	–2.64; 0.78	0.29	–0.79	–2.50; 0.92	0.36	–0.86	–2.56; 0.85	0.32	–0.15	–1.83; 1.52	0.86
Clinical and paraclinical	—	—	—	—	—	—	—	—	—	—	—	—	—
Diarrhea5	730	—	—	—	—	—	—	—	—	—	—	—	—
No	531	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Yes	199	–1.90	–3.80; –0.004	0.049	–1.67	–3.58; 0.25	0.09	–1.91	–3.82; 0.01	0.051	–1.00	–2.89; 0.89	0.30
Antibiotics[5]	468	—	—	—	—	—	—	—	—	—	—	—	—
No	256	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Yes	212	–0.38	–2.48; 1.73	0.73	–0.31	–2.42; 1.80	0.77	–0.53	–2.72; 1.67	0.64	–1.46	–3.62; 0.70	0.18
Malaria rapid test	720	—	—	—	—	—	—	—	—	—	—	—	—
Negative	434	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
Positive	286	–2.95	–4.67; –1.23	<0.001	–3.22	–4.95; –1.49	<0.001	–2.95	–4.73; –1.18	0.001	–0.59	–2.47; 1.30	0.54
Serum CRP, mg/L	724		—	—	—	—	—	—	—	—	—	—	—
<5	479	Reference	—	—	Reference	—	—	Reference	—	—	—	—	—
5–10	86	–2.21	–4.83; 0.41	0.10	–2.16	–4.77; 0.46	0.11	–1.91	–4.54; 0.71	0.15	—	—	—
10–20	60	–5.66	–8.73; –2.59	<0.001	–5.76	–8.82; –2.70	<0.001	–5.64	–8.70; –2.58	<0.001	—	—	—
20 +	99	–6.93	–9.40; –4.46	<0.001	–7.07	–9.54; –4.60	<0.001	–6.93	–9.41; –4.45	<0.001	—	—	—
Serum AGP, g/L	724	—	—	—	—	—	—	—	—	—	—	—	—
<0.8	135	Reference	—	—	Reference	—	—	Reference	—	—	—	—	—
0.8–1.2	232	–3.26	–5.69; –0.83	0.01	–3.37	–5.80; –0.95	0.01	–3.46	–5.89; –1.02	0.01	—	—	—
1.2 +	357	–6.63	–8.90; –4.37	<0.001	–6.56	–8.83; –4.30	<0.001	–6.47	–8.77; –4.18	<0.001	—	—	—
Log_{10} (serum IGF-1), ng/mL	730	9.08	5.63; 12.53	<0.001	8.66	4.86; 12.47	<0.001	8.88	5.09; 12.67	<0.001	4.98	0.94; 9.03	0.02

Abbreviations: AGP, α_1-acid glycoprotein; CI, confidence interval; CRP, C-reactive protein; IGF-1, insulin-like-growth factor-1.
[1] Adjustment for fasting based on categorical variable <2 h, 2–5 h, and 5+ h after a meal.
[2] Inflammation: serum AGP and serum CRP as continuous variables.
[3] May not sum ≤730 because of missing data.
[4] Continuous variable.
[5] History in the 14 d prior.

TABLE 5
Micronutrient correlates of plasma citrulline (μmol/L) in children with stunting ($n = 730$)

		Model 1 (Unadjusted)			Model 2 (Model 1 + age and sex)			Model 3[1] (Model 2 + fasting)			Model 4[2] (Model 3 + inflammation)		
	n[3]	β	95% CI	*P* value	β	95% CI	*P* value	β	95% CI	*P* value	β	95% CI	*P* value
Hemoglobin, g/L	727	—	—	—	—	—	—	—	—	—	—	—	—
110 +	256	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
<110	471	–2.40	–4.15; –0.65	0.01	–2.16	–3.92; –0.39	0.02	–1.95	–3.72; –0.18	0.03	–0.33	–2.12; 1.45	0.71
Serum ferritin, μg/L[4]	724	—	—	—	—	—	—	—	—	—	—	—	—
25+	113	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
12–24	230	–0.10	–2.74; 2.54	0.94	0.10	–2.54; 2.74	0.94	0.24	–2.40; 2.87	0.86	1.08	–1.47; 3.64	0.41
<12	381	–0.71	–3.17; 1.75	0.57	0.07	–2.50; 2.64	0.96	–0.21	–2.79; 2.36	0.87	0.72	–1.79; 3.22	0.57
Serum sTfR, mg/L	724	—	—	—	—	—	—	—	—	—	—	—	—
≤8.3	272	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
>8.3	452	–2.00	–3.76; –0.25	0.03	–1.48	–3.33; 0.36	0.11	–1.49	–3.33; 0.35	0.11	–0.59	–2.40; 1.21	0.52
Plasma cobalamin, pmol/L	706	—	—	—	—	—	—	—	—	—	—	—	—
222+	539	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
148–221	142	0.33	–1.84; 2.50	0.76	0.43	–1.73; 2.59	0.70	0.84	–1.34; 3.02	0.45	1.29	–0.86; 3.43	0.24
<148	25	1.92	–2.78; 6.63	0.42	2.11	–2.57; 6.80	0.38	2.69	–2.01; 7.38	0.26	3.13	–1.46; 7.72	0.18
Serum RBP, μmol/L[4]	724	—	—	—	—	—	—	—	—	—	—	—	—
0.7+	597	Reference	—	—	Reference	—	—	Reference	—	—	Reference	—	—
<0.7	127	–0.99	–3.23; 1.25	0.39	–0.96	–3.20; 1.27	0.40	–0.89	–3.13; 1.34	0.43	1.21	–3.37; 0.96	0.27

Abbreviations: CI, confidence interval; RBP, retinol-binding protein; sTfR, soluble transferrin receptor.
[1] Adjustment for fasting based on categorical variable <2 h, 2–5 h, and 5+ h after a meal.
[2] Inflammation: serum α_1-acid glycoprotein + serum C-reactive protein as continuous variables.
[3] May not sum ≤730 because of missing data.
[4] Corrected for inflammation according to Cichon et al. [26].

Home visit and WASH assessment

With permission, study staff accompanied the caregiver and participating child home to conduct a WASH assessment. Dwellings were classified as urban if the township exceeded 2000 persons. Staff used interviewer-administered questionnaires to enquire about family characteristics, education, income, and household food insecurity. Food insecurity during the previous 30 d was assessed using the Household Food Insecurity Access Scale, and responses were later classified according to severity [20]. Characteristics evaluated as part of the WASH assessment were building materials used for the dwelling walls and floor, the availability of clean and accessible water, basic sanitation services, and hygiene practices. The assessment was supported by an interviewer-administered questionnaire about toilet use, animal ownership (chicken, cow, goat, pig, sheep, dog, cat), and water access and treatment. Where possible, questionnaire responses were confirmed through observation. The Joint Monitoring Program for Water Supply, Sanitation, and Hygiene Household Wash Strategy informed the WASH assessment and its classifications for drinking water source and toilet type [21]. Building materials used for the walls were classified as either brick or cement or other: sheet metal, thatch, and mud. For the floors, building materials were classified as cement or other, wooden planks, mud, or mud mixed with dung or dirt. Brick and cement were considered improved materials. Water treatment was adequate when reported as boiling or using appropriate chemical treatment. Treatment was inadequate if not reported or if reported as filtering through a cloth or leaving in the sun. Sharing was indicated when >1 household shared the same toilet facility. Handwashing was assessed by the presence of a handwashing station with water and/or soap.

Biologic sample collection

Using a butterfly kit, 6 mL of venous blood was collected into 4 mL serum, lithium-heparin, and EDTA blood collection tubes. Caregivers were encouraged (not required) to bring the child in a fasted state, attending the visit in the early morning before feeding. At the time of the blood draw, phlebotomists recorded the estimated time interval since the child had last been fed. All children received porridge after blood sampling. Samples were transported for processing at room temperature (20°C–25°C). Approximately 200 μL of whole blood was used for rapid diagnostic tests (RDT) for malaria (SD Bioline Malaria Ag Pf; Abbott), HIV using serial RDT (Determine HIV-1/2; Abbott, STAT-PAK; Chembio Diagnostics, Inc, and SD Bioline HIV-1/2; Standard Diagnostics, Inc), and hemoglobin (Hb) concentration (Hb201+; HemoCue) according to the manufacturer's instructions. Hb < 110 g/L was used as a definition of anemia [22], and results were used to direct treatment where appropriate. The remaining blood was centrifuged (EBA200; Hettich) at 2300 x *g* for 10 min before the serum and plasma aliquots were transferred to cryotubes for storage at –20°C. Serum and plasma samples were transferred within 7 d to the Integrated Biorepository of H3Africa Uganda (IBRH3AU) in Kampala for storage at –80°C. Samples were shipped on dry ice to the University of Copenhagen and subsequently to laboratories in Sweden, Germany, and Denmark for analysis.

Analysis of p-cit

The p-cit was assayed by ultrahigh-performance liquid chromatography (UHPLC)-tandem mass spectrometry by using a commercially available amine-reactive isotope-coded tags (aTRAQ reagent) kit for Amino Acid Analysis of Physiological Fluids (AB Sciex) at the Chalmers University of Technology, Gothenburg, Sweden. Analysis was conducted with some modifications to the kit protocol. In brief, to prepare the samples for analysis, 20 μL of 10% sulfosalicylic acid containing 400 μmol/L norleucine was added to 80 μL of each plasma sample. Samples were vortex mixed and centrifuged before 10 μL of the supernatant was mixed with 40 μL of labeling buffer containing 0.45 M borate buffer, pH 8.4, and 20 μL of norvaline. From this, 10 μL of supernatant was recovered, and 5 μL of diluted aTRAQ Δ8-reagent was added and subsequently mixed before incubation at room temperature for 30 min. Thereafter, 5 μL of hydroxylamine was added, and the solution was incubated for a further 15 min at room temperature. A volume of 32 μL of reconstituted aTRAQ Internal Standard solution and 400 μL of water was added to the reaction mixture. This solution was mixed and transferred into vials for analysis by UHPLC-tandem mass spectrometry. A quality control plasma sample was used to assess within and between batch precision. This control sample was injected at the beginning, at every tenth sample, and at the end of each batch.

Samples were analyzed using a Siex QTRAP 6500+ system (AB Sciex) with a Nexera UHPLC system (Shimadzu). A volume of 2 μL of the sample was separated on a Waters BEH C18 column (150 × 2.1 mm, 1.7 μm) held at 50°C using the following gradient: 0 min 2% B, 0–2.5 min 2–40% B, 2.5–3.9 min 40% B, 3.9–4.2 min 40–90% B, and 4.2–6.0 min 90% B. Mobile phases were water (A) and methanol (B), both containing 0.1% formic and 0.01% heptafluorobutyric acids (total flow 0.4 mL/min). The mass spectrometer was set to monitor the transitions 324.2–121.1 m/z for citrulline and 316.2–113.1 m/z for the citrulline internal standard. The following ion source parameters were used: CUR 30, CAD MED, IS 5500, TEM 500, GS1 60, and GS2 50, and compound parameters DP 30, EP 10, CE 30, and CXP 5.

Other biomarkers

We used serum C-reactive protein (s-CRP) as a fast- and serum α_1-acid glycoprotein (s-AGP) as a slow-reacting marker of the acute-phase response. To explore the relationship between inflammation and p-cit, we used dummy variables for s-CRP based on the cutoffs 5, 10, and 20 mg/L, and for s-AGP based on the cutoffs 0.8 and 1.2 g/L, as previously suggested [23].

Serum ferritin (s-ferritin) <12 μg/L and serum soluble transferrin receptor >8.3 mg/L were indicators of depleted iron stores and peripheral iron deficiency, respectively. Serum retinol-binding protein (s-RBP) <0.7 μmol/L was a proxy of low vitamin A status. The aforementioned were analyzed by VitMin laboratories in Willstaedt, Germany, using sandwich-ELISA [24]. Cobalamin was analyzed from plasma samples using the Advia Centaur CP Immunoassay System (Siemens) at the Department of Clinical Medicine, Aarhus University Hospital, Denmark. Plasma cobalamin of <148 pmol/L and 148–221 pmol/L indicated low and marginal concentrations, respectively [25]. Serum IGF1(s-IGF1) was determined with the Immulite2000 (Siemens) at the

Department of Nutrition, Exercise and Sports, University of Copenhagen, Denmark, and was log10 transformed for statistical analysis.

Data handling and statistical methods

Data were double entered using EpiData software version 3.1 (Epidata, Odense, Denmark). The *z*-scores were calculated using STATA software (STATA, College Station, Texas, US), Igrowup package (UNICEF, Data and Analytics, 2019). Statistical analyses were made using R version 4.1.2 (R-Core team, 2021, Vienna, Austria). We assessed associations between potential correlates and p-cit using multiple linear regression. The selection of potential correlates was based on a conceptual framework (Supplemental Figure 1) and included both proximal (e.g., inflammation) and distal (e.g., residence, WASH) variables. The correlates explored were as follows: age, sex, rural residence, diet (breastfeeding status, animal-source foods, diet diversity, and meal frequency), food insecurity (Household Food Insecurity Access Scale classification), anthropometrics (HAZ, Weight-for-height *z*-score, and weight-for-age *z*-score), clinical and paraclinical factors (s-IGF1, history of diarrhea and antibiotics, positive malaria RDT, s-CRP, s-AGP), household WASH characteristics (materials used for dwelling roof and floor, drinking water source, water treated, toilet improved, toilet shared, handwashing practices, and animals owned and/or observed close to living quarters) and micronutrient status (plasma cobalamin, s-RBP, s-ferritin, Hb, serum soluble transferrin receptor and iron deficiency anemia). The s-ferritin and s-RBP were corrected for inflammation, as described by Cichon et al. [26].

We fit 4 models: a univariate model (model 1) and multivariate models adjusted for age and sex (model 2), age, sex and fasting (model 3, considered the main analysis), and finally, age, sex, fasting and inflammation (model 4, an extra model to evaluate if and to which extent inflammation confounded or mediated the association with p-cit). To adjust for fasting, the time since the child's last feed, according to caregiver reports, was classified into 3 groups: <2, 2–5, and >5 h. We adjusted for inflammation using continuous data from inflammatory markers s-AGP and s-CRP.

Finally, in a post hoc subanalysis, we used Pearson's χ^2 test to determine whether there were changes in household handwashing practices before and after community-level COVID-19 restrictions were enforced in Uganda. Because of the explorative nature of this study, we did not adjust for multiplicity, and no imputations were made. Data were presented as β coefficients with 95% confidence intervals (CIs) and *P* values. A *P* value of <0.05 was considered significant.

Results

Between February and September 2020, 7611 children were screened for stunting; 1112 were referred for eligibility screening, and of these, 750 were enrolled (Supplemental Figure 2). The mean ± SD age was 32.0 ± 11.7 mo with 30% (222) below 24 mo. The mean ± SD HAZ was –3.02 ± 0.74 (Table 1). Eight children (1%) had positive serology for HIV. Thirteen percent ($n = 95$) were breastfed, and for those no longer breastfeeding, the mean ± SD age at cessation was 15.8 ± 6.2 mo. Dietary diversity was inadequate among 74% ($n = 552$), and inadequacy was more prevalent among those above than those below 24 mo (81% vs. 56%, $P < 0.001$). Forty-five percent ($n = 333$) lived in rural areas; the mean ± SD household size was 5.4 ± 2.1, and 41% (311) owned agricultural land.

The p-cit was determined from 730 (97%) of the children enrolled (Supplementary Figure 2). The 20 children without p-cit data had similar baseline characteristics, except that the majority were from rural areas (95%). Mean ± SD p-cit was 23.0 ± 11.7 μmol/L with 39% (288) and 5% (39) of p-cit values between 20 μmol/L and 10 μmol/L and below 10 μmol/L, respectively. Compared with children who had fasted for >5 h, those who fasted <2 and 2–5 h had 3.24 (95% CI: –1.14, 7.62) and 2.04 (95% CI: 0.29, 3.79) μmol/L lower p-cit, respectively.

Increasing age in months was associated with a 0.08 (95% CI: 0.004, 0.15) μmol/L increase in p-cit, whereas there were no differences in p-cit by sex (1.46 μmol/L; 95% CI: –0.25, 3.16). After adjustment for age, sex, and fasting, the rural residence was associated with a 2.33 (95% CI: 0.18, 4.49) μmol/L lower p-cit (Table 2, model 3), but the association disappeared with adjustment for inflammation (model 4). Food insecurity was a correlate of p-cit but only became statistically significant in those who were moderately food insecure (–5.42 μmol/L, 95% CI: –9.69, –1.14). Neither breastfeeding nor any other dietary factors, including dietary diversity and consumption of animal-source foods, were associated with p-cit.

Of the WASH characteristics, the use of building materials other than brick and cement was associated with lower p-cit (Table 3, model 3), and these associations were only slightly attenuated with further adjustment for inflammation (model 4). The use of a nonimproved toilet system was associated with 1.93 (95% CI: 0.17; 3.69) μmol/L lower p-cit, but the association disappeared with adjustment for inflammation. There was no difference according to toilet sharing, the source of drinking water, or water treatment methods. Compared with households with both soap and water available for handwashing, lacking 1 or both was associated with lower p-cit (model 3), but this disappeared with adjustment for inflammation. There was a pause in study recruitment during April and May 2020 because of the implementation of national COVID-19 restrictions across Uganda. Our subanalysis of the handwashing practices of enrolled households showed that a greater proportion were using soap for handwashing after COVID-19 restrictions were enforced (9% compared with 30%, $P < 0.001$) (Supplemental Table 1). Compared with the months after these restrictions were implemented, those included before had a 3.81 (95% CI: 1.53, 6.09) μmol/L lower p-cit (Supplemental Table 2, model 3), which was reduced to 2.72 (95% CI: 0.45, 5.00) μmol/L after accounting for inflammation (model 4). Likewise, recruitment during the wet season was associated with 3.12 (95% CI: 1.26, 4.97) μmol/L lower p-cit, which reduced to 2.13 (95% CI: 0.29, 3.98) μmol/L with adjustment for inflammation. Keeping animals was not associated (1.25 μmol/L, 95% CI: –0.52, 3.02), but after adjustment for inflammation, the estimate increased to 2.15 (95% CI: 0.42, 3.89) μmol/L higher p-cit. Observation of animals close to the residence was not associated.

A history of diarrhea and positive malaria RDT were associated with lower p-cit (model 3), but both associations were attenuated with additional adjustment for inflammation (Table 4, model 4). With adjustment for age, sex, and fasting, HAZ was not associated with p-cit. However, an inverse association appeared with adjustment for inflammation: a 1.23 (95% CI: 0.07, 2.39) μmol/L lower p-cit per unit increase in HAZ or a 2.15 (95% CI: 0.44, 3.86) μmol/L higher p-cit in children with severe compared with moderate stunting (model 4). Neither wasting nor underweight were associated with p-cit. There was a positive association with s-IGF1. For each log10 increase in s-IGF1, there was an 8.88 (95% CI: 5.09, 12.67) μmol/L higher p-cit (Table 4). This association remained after adjustment for inflammation, albeit somewhat reduced (4.98 μmol/L, 95% CI: 0.94, 9.03).

Anemia was associated with 1.95 (95% CI: 0.18, 3.72) μmol/L lower p-cit (Table 5, model 3), but this disappeared after adjustment for inflammation (model 4). None of the other markers used to define micronutrient deficiencies were associated with p-cit. Further adjustment for season did not change any of the associations reported in Tables 2, 3, and 4 (model 4).

Discussion

We found that food insecurity, malaria, diarrhea, systemic inflammation, low s-IGF1, anemia, the wet seasons, and selected WASH characteristics, including the use of a nonimproved toilet system and the lack of soap for handwashing, were associated with lower p-cit. Most associations attenuated, however, with additional adjustment for systemic inflammation.

We found that fewer hours fasted was associated with lower p-cit. Those who had fasted for <2 and 2–5 h had lower concentrations than those who had fasted for >5 h. In line with this, others have observed a transient postprandial decline in p-cit that returned to basal levels after 3–4 h [27,28]. Ideally, p-cit should be measured in the fasted state [27]. However, few studies among children in low-income settings require fasting, as it may not be feasible. To account for fasting in this study, we adjusted for the approximate number of hours since the last meal.

An increase in p-cit with age has been reported previously [29,30], by some accounts reaching a plateau at around 5 y [29]. The age dependence is thought to reflect the maturation of the intestinal-renal axis and the timed expression of the enzymes involved [31]. However, no true reference intervals for citrulline exist and no cutoffs have been established to indicate reduced enterocyte mass in children under 5 y. The most frequently used cutoff is 20.0 μmol/L [10]. This cutoff has been shown to correlate with lower grades of histopathologic damage in pediatric patients with celiac disease [12] but also to predict the severity of short bowel syndrome in adults by distinguishing between permanent and transient intestinal failure [27]. On the contrary, some report that a threshold of 15.0 μmol/L in children [32] and adults [33] indicates a loss of function, whereas others use thresholds below 10.0 μmol/L [34,35]. In this study, 44% of the children had p-cit below 20.0 μmol/L and just over 5% below 10 μmol/L, indicating that this population is experiencing some form of reduced enterocyte mass. A recent study among children aged 2–5 y in Madagascar and the Central African Republic reported that children with stunting, 2.5% and 8.8%, had p-cit below 7 μmol/L, respectively [34]. Another study in Lao among younger children aged 6–24 mo, 37% of whom were stunted, reported that 5.3% had p-cit below 17 μmol/L [36]. In Burkina Faso, 70% of children aged 6–24 mo had p-cit below 14 μmol/L [37]. Because of the variation in cutoffs and analytic methods used, it is difficult to compare the results of these studies directly. However, there seems to be some reduction in p-cit in children with stunting. Validation against other EED markers or biopsies would improve understanding of relevant cutoffs in stunted populations. In the research context, p-cit may be used together with functional tests to better understand malabsorption and mucosal repair in the context of EED.

There was a strong negative association between markers of systemic inflammation (s-CRP and s-AGP) and p-cit that warrants further consideration. Similar negative associations have been reported in studies among children who are both stunted or at risk of stunting [34,36]. In addition, studies of other inflammatory conditions widely report the same inverse association between markers of systemic inflammation and p-cit [38–40], with few not finding an association [33,41]. Citrulline and arginine are closely related, and this may partly explain the association with systemic inflammation. Citrulline is the main precursor for endogenous arginine synthesis. Arginine, a conditionally essential amino acid, has a complex metabolism but plays an important immune-modulating role in many tissues as the main precursor in nitric oxide synthesis. It has been suggested that for critically ill patients, low p-cit may result from increased requirements for arginine to produce nitric oxide during the inflammatory process [42] and, in such cases, may not be a clear indication of low enterocyte mass. On the contrary, requirements for nutrients such as arginine increase during a state of chronic intestinal inflammation and/or growth, and if they remain unmet, this can damage the intestinal mucosa [43]. Given the complex relationship that exists between citrulline and systemic inflammation, we chose to include an additional adjustment to account for the differences in p-cit that may be confounded by or mediated through systemic inflammation. When analyzing p-cit, Wessells et al. [36] chose to exclude children with s-CRP >10 mg/L. However, this risks excluding a proportion of children with intestinal damage and concurrent systemic inflammation. Low p-cit could result from increased use of arginine in the nitric oxide cycle or loss of enterocyte mass in a state of nutrient deficiency, inflammation, or a combination of these.

After adjustment for inflammation, associations with malaria and diarrhea disappeared. This was expected as these conditions are known to lead to systemic inflammation. Nevertheless, it is also possible that both malaria and diarrhea have a direct effect on the gut via inflammation and exposure to intestinal pathogens, as reported by others [43]. We found that associations related to minimizing infections, such as handwashing with soap and improved toilet facilities, were attenuated after accounting for the effects of inflammation. In the context of EED, the most widely accepted pathway of damage to the enterocytes is via exposure to pathogens that cause local [44] and subsequent systemic inflammation [5]. These WASH associations could be a reflection of this pathway. Interestingly, a greater proportion began handwashing with soap after COVID-19 lockdowns, and this was associated with increased p-cit. The effect was not

completely explained by inflammation, however, and seasonality may have played a role, where lower p-cit was associated with the wetter seasons. This is in line with what others have reported on seasonality for different EED markers [45].

We found a positive association between the growth factor s-IGF1 and p-cit. Linear growth and IGF-1 are both known to be downregulated in the presence of inflammation [46]. Thus, we expected attenuation of the association in the inflammation-adjusted model. Although partially reduced, the association with s-IGF1 remained significant. Although no causal inferences can be made, it is at least biologically plausible that increases in s-IGF1 may encourage enterocyte proliferation and rejuvenation of intestinal crypts [47] and thus increase p-cit. In contrast, we did not find an association between HAZ and p-cit, which was contrary to some studies [34,48] but not all [49,50]. Surprisingly, after adjusting for inflammation, a negative association appeared between HAZ and p-cit. This may be a chance finding. Moreover, in the current study, we compared children with moderate stunting to children with severe stunting. If we had a nonstunted comparison group, the associations in the different models may have been clearer.

Dietary diversity was inadequate among a large proportion of children, and whereas poor dietary intake is thought to play a role in EED, dietary factors were not associated with p-cit. Of the micronutrient deficiencies explored, it was surprising that low serum cobalamin was not associated with p-cit, given its role in DNA synthesis and cell division [51]. However, low serum cobalamin was not highly prevalent in this cohort. Still, we found a positive association between Hb and p-cit. A similar finding had been reported in a recent study including both children with and without stunting [34]; we added to this by showing that the relationship was driven by systemic inflammation. There was a tendency for socioeconomic factors such as food insecurity and poorer living standards to be associated with lower p-cit, after adjustment for inflammation, supporting that poverty is associated with EED [8].

The strengths of this study were its large sample size, well-characterized participants, the nearly complete dataset, and the novel use of adjustments for fasting and inflammation to improve the use of p-cit as a potential marker of enterocyte mass. On the contrary, the cross-sectional design limits any causal inference. Furthermore, we made many comparisons, which could have resulted in chance findings. The lack of nonstunted children was a limitation, as well as the lack of other functional EED markers. Finally, incompletely filled anticoagulant blood collection tubes may have affected the p-cit results.

In conclusion, we found that a number of characteristics expected in populations with EED were correlated with lower p-cit. These correlates included food insecurity, systemic inflammation, low s-IGF1, low Hb, and some WASH characteristics, including a lack of soap for handwashing. Both younger age and fewer hours fasted were associated with lower p-cit, and so should be accounted for in the analysis. Finally, adjustment for systemic inflammation attenuated many associations, reflecting confounding, mediation, or both. We propose that p-cit may be a useful marker of EED in low-income settings. However, this study highlights the complex interplay between p-cit and systemic inflammation that warrants further investigation so as to improve the use and validity of p-cit for future research.

Author contributions

The authors' responsibilities were as follows– EM, HP, HF, and BG: designed the research; RM, JM, EM, HP, BG, and OS: conducted the research; HP and CR: performed statistical analysis; HP wrote the paper; BG had primary responsibility for final content, and all authors: read and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

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Data availability

The Ugandan Act on Data Protection and Privacy and the European Act on General Data Protection Regulation does not allow for personal data to be made available to other researchers without prior written approval from relevant institutions and authorities. For further information, please contact the corresponding author.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2023.12.027.

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