Inflammatory biomarkers in HIV-infected children hospitalized for severe malnutrition in Uganda and Zimbabwe

Andrew J PRENDERGAST[1,2], Chipo BEREJENA[3], Godfrey PIMUNDU[4], Annie SHONHAI[3], Mutsa BWAKURA-DANGAREMBIZI[3], Victor MUSIIME[4,5], Alexander J SZUBERT[1], Adrian D COOK[1], Moira J SPYER[1], Patricia NAHIRYA-NTEGE[6], Adeodata KEKITIINWA[7], Diana M GIBB[1], Nigel KLEIN[8], A Sarah WALKER[1]

[1]MRC Clinical Trials Unit at UCL, London, UK

[2]Blizard Institute, Queen Mary University of London, UK

[3]University of Zimbabwe, Harare, Zimbabwe

[4]Joint Clinical Research Centre, Kampala, Uganda

[5] College of Health Sciences, Makerere University, Kampala, Uganda

[6]MRC/UVRI Uganda Research Unit on AIDS, Entebbe, Uganda

[7]Baylor-Uganda, Mulago Hospital, Kampala, Uganda

[8]UCL Great Ormond Street Institute of Child Health, London, UK

Corresponding author and requests for reprints to: Professor Andrew Prendergast, Blizard Institute, 4 Newark Street, London E1 2AT. Tel +44 207 882 2269. Fax +44 207 882 2195. Email a.prendergast@qmul.ac.uk

Short title: Inflammatory biomarkers and malnutrition

Word count: 1791 words

Abstract: 250 words

Funding: This work was supported by the Medical Research Council, the Department for International Development (UK Aid), the Wellcome Trust (grant 108065/Z/15/Z to AJP), and ViiV Healthcare/GlaxoSmithKline (donation of drugs and funding of viral load assays). The MRC Clinical Trials Unit at UCL receives core support from the MRC [MC_UU_12023/26].

INTRODUCTION

Malnutrition and immunosuppression frequently co-exist among children with advanced HIV in sub-Saharan Africa, and both are risk factors for mortality after antiretroviral therapy (ART) initiation [1]. We previously reported that 11% of children starting ART with severe immunosuppression and low weight-for-age in the ARROW trial in Uganda and Zimbabwe were hospitalized with severe malnutrition (marasmus, kwashiorkor or marasmic kwashiorkor) in the first few weeks after ART initiation [2]. Children frequently had co-infections, and high mortality: 20% and 32% of those who developed oedematous and non-oedematous malnutrition, respectively, died in the first six months of ART.

The pathogenesis of malnutrition in children with HIV remains poorly understood, and the underlying mechanistic pathways may differ from HIV-uninfected children [3]. Both HIV infection and malnutrition are characterized by immune dysfunction, inflammation, co-infections, enteropathy and metabolic disturbance, and the complex interplay between these factors needs to be better elucidated, particularly when children initiate ART. The cause of oedema in children with kwashiorkor remains enigmatic; in HIV-infected children, oedema tends to occur in those with higher CD4 counts, suggesting a degree of immune competence is required [4]. We previously hypothesized that early onset of severe malnutrition (and, in particular, oedema in half the cases) after ART initiation was due to immune restoration in children with very low baseline CD4 counts [2]. We further speculated that this phenomenon was a manifestation of the immune reconstitution inflammatory syndrome (IRIS), which has manifold presentations [5]. In particular, changes in the inflammatory milieu among children starting ART in the context of baseline malnutrition and advanced HIV may underlie the onset of severe malnutrition.

Here, we set out to address this hypothesis using stored biospecimens from children in the ARROW trial in Uganda and Zimbabwe [6].

METHODS

The design and outcomes of the ARROW trial have previously been reported [6]. Briefly, ARROW was a randomized trial of monitoring and first-line ART strategies among 1207 HIV-infected children in Uganda and Zimbabwe enrolled between 2007-2008 (ISCRTN24791884). Eligible children were aged 3 months to 17 years and met World Health Organization (WHO) criteria for ART initiation at the time.

Anthropometry and clinical evaluation was undertaken at trial screening, at enrolment and at follow-up visits. Weight was converted into weight-for-age Z-scores using UK reference values [8], because WHO reference data did not cover the full age range of enrolled children. Before enrolment, children were categorized as having kwashiorkor (60-80% weight-for-age with oedema), marasmus (<60% weight-for-age without oedema), or marasmic kwashiorkor (<60% weight-for-age with oedema), according to the Wellcome classification in use at the time [7]. Children with severe malnutrition who were clinically stable, had no concurrent infections and good appetite, or whose caregivers refused admission, received supplementary feeding at home with high-energy milk (or ready-to-use therapeutic food, from 2008). Children who were clinically unstable, had evidence of infections or poor appetite were admitted to hospital for management as previously described [2].

Following written informed consent, children started abacavir, lamivudine and either efavirenz or nevirapine, together with cotrimoxazole prophylaxis; children randomized to an induction-maintenance regimen also received zidovudine for the first 36 weeks. No child had oedema at the time of ART initiation. After starting ART, children were hospitalized for

severe malnutrition if they developed oedema, loss of appetite and/or infections requiring treatment. Hospitalized children received standard-of-care inpatient nutritional rehabilitation and continued ART, as previously described [2].

Cryopreserved plasma samples from enrolment (pre-ART) or screening (maximum 30 days before enrolment) were retrieved for all children who were subsequently hospitalized with severe malnutrition, and a subgroup of participants who did not develop severe malnutrition enrolled in an immunology sub-study [9]. Among children hospitalized for severe malnutrition, plasma samples from 4 weeks post-ART were also retrieved, where available. We measured plasma inflammatory markers (C-reactive protein (CRP), tumour necrosis factor-α (TNFα), interleukin-6 (IL-6) and soluble CD14 (sCD14)) by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Oxford, UK), according to the manufacturer's instructions. Viral load was measured using the Abbott m2000sp/rt (Uganda) or Roche COBAS Amplicor Monitor v1.5 (Zimbabwe). CD4 counts were measured in real-time and expressed as CD4-for-age, calculated as the ratio of the child's CD4 count to the count expected in a healthy child of the same age.

Baseline factors were compared between groups with and without subsequent hospitalization for severe malnutrition using Mann-Whitney tests and fractional polynomial logistic regression. Independent associations were identified using multivariable fractional polynomial logistic regression. Within-child differences in cytokines between weeks 0 and 4 were compared using paired Wilcoxon sign-rank tests. All analyses were undertaken using Stata 15.1 (StataCorp).

The trial was approved by ethics committees in Uganda, Zimbabwe and the United Kingdom.

RESULTS

Of 1207 children enrolled in ARROW, 39 (3%) were hospitalized for severe malnutrition, a median of 28 days (IQR 14,36) post-ART initiation for marasmus (n=19) and 26 days (IQR 14,56) post-ART for kwashiorkor or marasmic kwashiorkor (n=20). Pre-ART inflammatory biomarkers were available for 613 (51%) children, including all 39 who were hospitalized with severe malnutrition (6% of those with biomarkers). As previously reported [2], children who developed severe malnutrition were younger than those who did not (median 2.3 (IQR 1.6,8.0) versus 6.3 (2.5,9.7) years; p=0.003) and had lower baseline weight-for-age Z-scores (median -4.8 (-5.7,-3.9) versus -2.2 (-3.2,-1.3); p<0.0001) and CD4-for-age (median -2.5 (-5.9,-1.6) versus -1.9 (-2.9,-1.3); p=0.02).

Children who developed severe malnutrition, compared to those who did not, had significantly higher baseline CRP (median 13.5 (IQR 5.5,41.1) versus 4.1 (1.4,14.4) mg/L, respectively; p=0.003) and IL-6 (median 9.2 (6.7,15.6) versus 5.9 (4.6,9.3) pg/mL; p<0.0001), but similar overall TNFα (median 22.2 (16.1,26.6) versus 22.6 (18.9,28.0) pg/mL; p=0.39), sCD14 (median 2.2 (1.7,2.7) versus 2.1 (1.7,2.6) mg/L; p=0.79) and HIV viral load (median 5.6 (5.2,5.9) versus (5.3 (4.8,5.8) $\log_{10}$ copies/mL, p=0.07). However, there was evidence that univariable associations between TNFα and subsequent hospitalization for severe malnutrition were non-linear and, allowing for this, the risk of hospitalization increased markedly at lower TNFα (p=0.01). In a multivariable model, higher pre-ART IL-6, lower pre-ART TNFα and lower pre-ART weight-for-age were independently associated with subsequent hospitalization for severe malnutrition (Table 1). Relationships between baseline IL-6 or TNFα and odds of severe malnutrition were non-linear (Figure 1A and 1B). There was no independent effect of pre-ART CD4 (p=0.38), age (p=0.22), CRP (p=0.66), sCD14 (p=0.29) or viral load (p=0.69).

Among the 39 children who were hospitalized for severe malnutrition, 17 had bio-specimens available for analysis at week 4. There was a significant rise between weeks 0 and 4 in IL-6 (median difference +6.6 (IQR 3.0,24.2) pg/mL, p=0.01; Figure 1C), CRP (median difference +31.7 (IQR 23.2,65.0) mg/L, p=0.0008; Figure 1D) and sCD14 (median difference +0.9 (IQR 0.4,1.4) mg/L, p=0.003), and a significant fall in TNFα (median difference -3.7 (IQR -10.9,-0.2) pg/mL, p=0.003; Figure 1E) and HIV viral load (median difference -2.6 (IQR -2.9,-2.0) log RNA copies/mL, p<0.0001; Figure 1F). Changes in biomarkers between weeks 0-4 were not significantly different between children who developed marasmus versus kwashiorkor or marasmic kwashiorkor (Fig 1C-E; p>0.14 for all).

DISCUSSION

The combination of low weight and baseline inflammatory status at the time of ART initiation was associated with subsequent hospitalization for severe malnutrition in this cohort of Zimbabwean and Ugandan children with HIV. Children who were hospitalized for severe malnutrition, approximately one month after ART initiation, had higher baseline IL-6 and lower baseline TNFα than children who did not develop severe malnutrition. Baseline IL-6 and TNFα were more strongly associated with severe malnutrition than baseline CD4 count or viral load, providing further evidence for the importance of the inflammatory milieu at the time of ART initiation [9]. These findings highlight the complex interplay between nutritional status, inflammation and immune restoration in children with HIV, and suggest that interventions to modulate inflammation might have a role in improving outcomes on ART.

The patterns of inflammatory biomarkers in this study were complex and associations with severe malnutrition were non-linear. Baseline CRP and IL-6 were both higher in children who were hospitalized for severe malnutrition compared to those who were not, and rose

further on ART. We have previously shown in this same cohort that high IL-6 was associated with on-ART mortality independently of CD4-for-age [9]. The current study provides further evidence that high IL-6 at ART initiation is deleterious in HIV-infected children. However, in contrast to IL-6, baseline TNFα was similar overall in children who did and did not develop severe malnutrition, but lower (rather than higher) levels at baseline were associated with subsequent malnutrition. In those developing severe malnutrition, TNFα concentrations dropped further during the first weeks of ART. These findings highlight the network of cytokines that are differentially dysregulated in advanced HIV. TNFα and IL-6 are both pleiotropic, but have distinct properties. TNFα is a stereotypical pro-inflammatory cytokine produced by monocytes and macrophages, which promotes Th1-polarized immune responses [10]. By contrast, IL-6 is a multifunctional cytokine produced by multiple cell types, which promotes acute phase responses (such as hepatic CRP production), but is Th2-polarizing [11] and can have anti-inflammatory effects [12]. Unlike IL-6, circulating levels of TNFα might not reflect biological activity in tissues [13].

We lack understanding of the causes of inflammation in HIV-infected children. Acute infections detected at the screening visit were treated prior to ART initiation in ARROW, although unidentified or unresolved infections may have contributed to heightened baseline inflammation. There are also subclinical drivers of inflammation in children with HIV, which may be distinct for different cytokines. For example, TNFα seems to be driven by viral load more than does IL-6 [9]. Furthermore, children who are already undernourished at ART initiation may be less able to produce TNFα, as shown in severe malnutrition previously [14]. The relative concentrations of circulating IL-6 and TNFα impact the functionality of the adaptive immune response [15] and may therefore influence the pattern of immune reconstitution and clinical outcomes on ART.

We were unable to formally diagnose IRIS with the data available in this study [16], although children hospitalized for severe malnutrition had a characteristic pattern of whole blood changes, with a sharp fall in viral load and striking rise in CRP and IL-6 during the first few weeks of ART [5]. In adult cohorts, IRIS is typically associated with a rise in pro-inflammatory cytokines such as IL-6 and TNFα [5], although studies variously report pre-ART cytokine concentrations that are lower [17, 18] or higher [19, 20] than those not developing IRIS. We only measured a limited range of inflammatory biomarkers; the interacting network of pro- and anti-inflammatory mediators is likely to be critical in determining the clinical course after ART initiation.

Multiple processes drive wasting in HIV [21]. Viral replication increases resting energy expenditure, which is elevated even further with secondary infections [22]. In 142 HIV-infected Zambian adults with low body mass index (<16 kg/m^2), CD4 count (<50 cells/mm^3) or both, elevated pre-ART CRP was associated with mortality over the first 12 weeks of ART [23]. Failure to reduce inflammation after ART initiation in malnourished adults in Tanzania and Zambia led to impaired recovery of lean mass, suggesting that inflammation is a key determinant of both nutritional depletion and recovery [24]. The combination of poor nutrition and inflammation is similarly deleterious in other chronic diseases such as cirrhosis [25], cancer [26], heart failure [27] and chronic kidney disease [28]. A recent study of HIV-uninfected children in Malawi [29] showed that systemic and intestinal inflammation were independent predictors of mortality in severe acute malnutrition, confirming the importance of the inflammatory milieu in malnutrition, even outside the setting of HIV.

Collectively, our findings confirm that pre-ART inflammatory biomarkers are important independent predictors of clinical outcomes after ART initiation, and suggest that inflammation contributes to the pathogenesis of severe malnutrition in HIV-infected

children. There is a critical need to better understand the interplay between nutritional, immune and inflammatory pathways to inform future interventions. Our findings suggest that modulating the inflammatory milieu at ART initiation could improve the outcome of HIV-infected children in sub-Saharan Africa, particularly among those with the most advanced disease.

AUTHOR CONTRIBUTIONS

Study design: AJP, DMG, NK, ASW

Recruitment of participants: MB-D, VM, PN-N, AK

Trial oversight: DMG, ASW

Laboratory work: CB, GP, AS, MJS

Analysis: AJS, ADC, ASW

Interpretation: AJP, MB-D, VM, PH-N, DMG, NK, ASW

Drafted manuscript: AJP, ASW

Revised manuscript: AJP, CB, GP, AS, MB-D, VM, AJS, ADC, MJS, PN-N, AK, DMG, NK

ACKNOWLEDGEMENTS

We thank the children, caregivers, and staff from all of the centres participating in the ARROW trial; and the ARROW Trial Steering Committee for access to data.

CONFLICTS OF INTEREST

None declared.

FUNDING

This work was supported by the Medical Research Council, the Department for International Development (UK Aid), the Wellcome Trust (grant 108065/Z/15/Z to AJP), and ViiV

Healthcare/GlaxoSmithKline (donation of drugs and funding of viral load assays).

REFERENCES

1. Sutcliffe CG, van Dijk JH, Bolton C, Persaud D, Moss WJ. Effectiveness of antiretroviral therapy among HIV-infected children in sub-Saharan Africa. *Lancet Infect Dis* 2008,**8**:477-489.
2. 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**:951-956.
3. Heikens GT, Bunn J, Amadi B, Manary M, Chhagan M, Berkley JA*, et al.* Case management of HIV-infected severely malnourished children: challenges in the area of highest prevalence. *Lancet* 2008,**371**:1305-1307.
4. 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. *Nutr J* 2006,**5**:27.
5. Mori S, Levin P. A brief review of potential mechanisms of immune reconstitution inflammatory syndrome in HIV following antiretroviral therapy. *Int J STD AIDS* 2009,**20**:447-452.
6. ARROW Trial Team, Kekitiinwa A, Cook A, Nathoo K, Mugyenyi P, Nahirya-Ntege P*, et al.* Routine versus clinically driven laboratory monitoring and first-line antiretroviral therapy strategies in African children with HIV (ARROW): a 5-year open-label randomised factorial trial. *Lancet* 2013,**381**:1391-1403.
7. Wellcome Working Party. Classification of infantile malnutrition. Lancet 1970; 296:302 – 303.

8. Freeman JV, Cole TJ, Chinn S, Jones PR, White EM, Preece MA. Cross sectional stature and weight reference curves for the UK, 1990. *Arch Dis Child* 1995,**73**:17-24.

9. Prendergast AJ, Szubert AJ, Berejena C, Pimundu G, Pala P, Shonhai A*, et al.* Baseline Inflammatory Biomarkers Identify Subgroups of HIV-Infected African Children With Differing Responses to Antiretroviral Therapy. *J Infect Dis* 2016,**214**:226-236.

10. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989,**7**:145-173.

11. Diehl S, Rincon M. The two faces of IL-6 on Th1/Th2 differentiation. *Mol Immunol* 2002,**39**:531-536.

12. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF*, et al.* IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* 1998,**101**:311-320.

13. Stenvinkel P, Ketteler M, Johnson RJ, Lindholm B, Pecoits-Filho R, Riella M*, et al.* IL-10, IL-6, and TNF-alpha: central factors in the altered cytokine network of uremia--the good, the bad, and the ugly. *Kidney Int* 2005,**67**:1216-1233.

14. Carlos Munoz MS, Marianela Avevalo MT, Marcelo Lopez MT, L. S. Impaired interleukin-1 and tumor necrosis factor production in protein-calorie malnutrition. *Nutrition Research* 1994,**14**:347-352.

15. Angelone DF, Wessels MR, Coughlin M, Suter EE, Valentini P, Kalish LA*, et al.* Innate immunity of the human newborn is polarized toward a high ratio of IL-6/TNF-alpha production in vitro and in vivo. *Pediatr Res* 2006,**60**:205-209.

16. Muller M, Wandel S, Colebunders R, Attia S, Furrer H, Egger M*, et al.* Immune reconstitution inflammatory syndrome in patients starting antiretroviral therapy for HIV infection: a systematic review and meta-analysis. *Lancet Infect Dis* 2010,**10**:251-261.

17. Goovaerts O, Jennes W, Massinga-Loembe M, Ceulemans A, Worodria W, Mayanja-Kizza H*, et al.* LPS-binding protein and IL-6 mark paradoxical tuberculosis immune reconstitution inflammatory syndrome in HIV patients. *PLoS One* 2013,**8**:e81856.

18. Ravimohan S, Tamuhla N, Steenhoff AP, Letlhogile R, Nfanyana K, Bellamy SL*, et al.* Immunological profiling of tuberculosis-associated immune reconstitution inflammatory syndrome and non-immune reconstitution inflammatory syndrome death in HIV-infected adults with pulmonary tuberculosis starting antiretroviral therapy: a prospective observational cohort study. *Lancet Infect Dis* 2015,**15**:429-438.

19. Boulware DR, Hullsiek KH, Puronen CE, Rupert A, Baker JV, French MA*, et al.* Higher levels of CRP, D-dimer, IL-6, and hyaluronic acid before initiation of antiretroviral therapy (ART) are associated with increased risk of AIDS or death. *J Infect Dis* 2011,**203**:1637-1646.

20. Grant PM, Komarow L, Lederman MM, Pahwa S, Zolopa AR, Andersen J*, et al.* Elevated interleukin 8 and T-helper 1 and T-helper 17 cytokine levels prior to antiretroviral therapy in participants who developed immune reconstitution inflammatory syndrome during ACTG A5164. *J Infect Dis* 2012,**206**:1715-1723.

21. Koethe JR, Heimburger DC, PrayGod G, Filteau S. From Wasting to Obesity: The Contribution of Nutritional Status to Immune Activation in HIV Infection. *J Infect Dis* 2016,**214 Suppl 2**:S75-82.

22. Melchior JC, Raguin G, Boulier A, Bouvet E, Rigaud D, Matheron S*, et al.* Resting energy expenditure in human immunodeficiency virus-infected patients: comparison between patients with and without secondary infections. *Am J Clin Nutr* 1993,**57**:614-619.

23. Koethe JR, Blevins M, Nyirenda C, Kabagambe EK, Shepherd BE, Wester CW*, et al.* Nutrition and inflammation serum biomarkers are associated with 12-week

mortality among malnourished adults initiating antiretroviral therapy in Zambia. *J Int AIDS Soc* 2011,**14**:19.

24. PrayGod G, Blevins M, Woodd S, Rehman AM, Jeremiah K, Friis H*, et al.* A longitudinal study of systemic inflammation and recovery of lean body mass among malnourished HIV-infected adults starting antiretroviral therapy in Tanzania and Zambia. *Eur J Clin Nutr* 2016,**70**:536.

25. Tandon P, Raman M, Mourtzakis M, Merli M. A practical approach to nutritional screening and assessment in cirrhosis. *Hepatology* 2017,**65**:1044-1057.

26. Kumar A, Torres ML, Cliby WA, Kalli KR, Bogani G, Aletti G*, et al.* Inflammatory and Nutritional Serum Markers as Predictors of Peri-operative Morbidity and Survival in Ovarian Cancer. *Anticancer Res* 2017,**37**:3673-3677.

27. Nakagomi A, Kohashi K, Morisawa T, Kosugi M, Endoh I, Kusama Y*, et al.* Nutritional Status is Associated with Inflammation and Predicts a Poor Outcome in Patients with Chronic Heart Failure. *J Atheroscler Thromb* 2016,**23**:713-727.

28. Kalantar-Zadeh K, Kopple JD, Block G, Humphreys MH. A malnutrition-inflammation score is correlated with morbidity and mortality in maintenance hemodialysis patients. *Am J Kidney Dis* 2001,**38**:1251-1263.

29. Attia S, Versloot CJ, Voskuijl W, van Vliet SJ, Di Giovanni V, Zhang L*, et al.* Mortality in children with complicated severe acute malnutrition is related to intestinal and systemic inflammation: an observational cohort study. *Am J Clin Nutr* 2016,**104**:1441-1449.

Table 1: Independent predictors of hospitalisation for severe malnutrition after ART initiation

Pre-ART	**Odds ratio (OR)**	**(95% CI)**	**p**
Weight-for-age (per unit higher)	0.39	(0.29-0.53)	<0.0001
IL-6, pg/mL*			0.04
3	0.10	(0.01-1.08)	
4	0.40	(0.27-0.59)	
5	0.72	(0.69-0.76)	
6	1.00	-	
8	1.39	(1.32-1.46)	
10	1.62	(1.45-1.80)	
15	1.88	(1.57-2.26)	
20	1.98	(1.60-2.45)	
30	2.06	(1.62-2.61)	
TNFα, pg/mL*			0.0004
12.5	6.82	(3.81-12.24)	
17.5	1.79	(1.69-1.88)	
22.5	1.00	-	
27.5	0.74	(0.73-0.75)	
32.5	0.63	(0.61-0.65)	
37.5	0.57	(0.54-0.60)	

*Best fitting transform inverse square root (Figure 1). OR estimated at values shown.

Note: including 599 children (12 missing IL-6, 10 missing TNFα).

FIGURE LEGENDS

Figure 1: Inflammatory biomarkers and risk of severe malnutrition

Adjusted odds ratio (with 95% confidence interval) of hospitalization for severe malnutrition by baseline (pre-ART) (A) IL-6 concentration (pg/mL) and (B) TNFα concentration (pg/mL). Among 17 children hospitalized for severe malnutrition with available plasma from both baseline (pre-ART) and 4 weeks post-ART, changes in (C) IL-6 (pg/mL), (D) CRP (mg/L), (E) TNFα (pg/mL) and (F) log HIV RNA viral load (copies/mL) are shown, split by non-oedematous malnutrition (marasmus), shown in triangles, and oedematous malnutrition (kwashiorkor or marasmic kwashiorkor), shown in squares. Hollow symbols indicate children without week 4 samples.