Tampere University

Topias Torpo

Systemic inflammation and cytokine profiles in Malawian children

Lääketieteen ja terveysteknologian tiedekunta

Syventävien opintojen kirjallinen työ

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ABSTRACT

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Tausta ja ongelma: Sekä vajaaravitsemuksen että infektioiden ja inflammaation ajatellaan vaikuttavat lapsen normaaliin kasvuun ja kehitykseen. Alikehittyneillä alueilla lasten ravitsemuksessa saattaa olla puutteita ja lasten altistuminen patogeeneille ja infektiosairauksien esiintyminen on myös yleisempää. Tutkimus on kuvaileva tutkimus, jolla pyritään selvittämään, onko iLiNS-ravitsemusinterventioon osallistuvilla lapsilla poikkeavaa inflammatorista aktiivisuutta, jolla voisi edelleen olla merkitystä ravitsemukseen, kasvuun ja kehitykseen.

Metodit: Tutkimuksen tarkoituksena on määrittää vallitsevaa systeemistä inflammaatiota analysoimalla sytokiinitasoja lasten plasmanäytteistä. Näytteet on kerätty Malawissa asuvilta 781 elävänä syntyneeltä lapselta ja kymmeneltä elävänä syntyneeltä kaksoselta 6, 18 ja 30 kuukauden iässä. Näytteitä on yhteensä 1708. Tässä tutkimuksessa tarkasteltavat sytokiinit ovat IL-1b, IL-6, IL-10 ja TNF-α. Analysointi tapahtuu Luminex-tekniikalla ja Milliplex immunologisella magneettikiinnitys menetelmällä. Sytokiinitasoja verrataan aiemmissa tutkimuksissa esitettyihin tasoihin. Lisäksi analysoidaan pitoisuuksia eri iässä, sekä yhteyttä sukupuoleen ja muiden sytokiinien pitoisuuksiin.

Tulokset: Sytokiinien pitoisuudet plasmassa olivat IL-1b=1.64 pg/ml, IL-6=3.57 pg/ml, TNF-α=15.23 pg/ml, IL-10=29.18 pg/ml. Sytokiinien tasot korreloivat keskenään, voimakkaimmin IL-1β ja IL-6 (rho=0.433 p< 0.001). IL-6 ja TNF-α tasot plasmassa olivat merkittävästi korkeammat pojilla.

Keskustelu: Systeemisen inflammaation merkitys ravitsemusinterventioille on epäselvä. Aliravitsemus on yhteydessä poikkeavaan kasvuun ja kognitiiviseen kehitykseen, mikä on laaja-alainen sosiaalinen ja terveydellinen ongelma maailmanlaajuisesti. Ravitsemusinterventioilla ei ole kaikissa aineistoissa pystytty vaikuttamaan näihin. Tulosten perusteella tutkimusjoukossa havaittiin selvää inflammatorista aktiivisuutta. Aiempaan kirjallisuuteen nähden tulokset kuitenkin eroavat selvästi akuutisti sairailla lapsilla tehdyistä tutkimuksista. Tuloksia voidaan käyttää arvioitaessa laajempaa matala-asteisempien tulehduksellisten prosessien vallitsevuutta. Lisätutkimuksia tarvitaan tulosten merkityksen arvioimiseksi osana aliravitsemukseen johtavaa prosessia.

Keywords: malnutrition, nutritional intervention, child health, Malawi, global health

The originality of this thesis has been checked using the Turnitin OriginalityCheck service.

Abbreviations

AGP	α-1 acid glycoprotein
COX	Cyclooxygenase
CRP	C- reactive protein
DIC	Disseminated intravascular coagulopathy
EED	Environmental enteric dysfunction
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iLiNS	International Lipidbased nutrient supplement
JAK	Janus kinase
NF-κB	Nuclear factor-κB
SAM	Severe acute malnutrition
SOCS	Suppressors of cytokine signaling
STATs	Signal transducers and activators of transcription family proteins
TNF	Tumor Necrosis Factor
TLR	Toll-like receptors
UTI	Urinary tract infection
WHO	World health organization

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1.Introduction

Systemic inflammation is a complicated immunological phenomenon maintaining homeostasis. Inflammation is observed in clinical practice, but for more objective evaluation by measuring biochemical markers such as cytokines. As inflammation is associated with numerous health related factors, it has an important role in child health. Inadequate systemic inflammation is associated with malnutrition, infections and numerous other health related problems. In this study, we want to evaluate the systemic inflammation in children in low-income setting by analyzing the cytokine profiles in plasma.

1.1 Malnutrition

Based on World health organization (WHO) statistics in 2018 52 million children under 5 years of age are wasted, 17 million are severely wasted and 155 million are stunted. Around 45% of deaths among children under 5 years of age are linked to undernutrition. These mostly occur in low- and middle-income countries. WHO defines malnutrition as a deficiency, but also an excesses, or imbalance of intake of energy and nutrients. Malnutrition can be undernutrition, which includes wasting, stunting and underweight. Wasting is defined as having a weight-for-height more than two standard deviation below the WHO Child Growth standard median. Similarly, stunting and underweight are defined as children`s height-for-age and weight-for-age more than two SD below the WHO Child Growth Standards median. Micronutrient-related malnutrition, which includes micronutrient deficiencies, such as lack of important vitamins and minerals is a form of malnutrition. Micronutrients enable the body to produce enzymes, hormones, and other substances that are essential for proper growth and development. (WHO Multicentre Growth Reference Study Group, 2006)
To address this issue United Nations General Assembly adopted a resolution proclaiming the UN Decade of Action on Nutrition from 2016 to 2025 (www.who.int/features/qa/malnutrition/en/, 16.12.2018). The Decade aims to catalyze policy commitments that result in measurable action to address all forms of malnutrition. The aim is to ensure all people have access to healthier and more

sustainable diets to eradicate all forms of malnutrition worldwide. International Lipid-based Nutrient Supplement (iLiNS)-project is a nutritional intervention taking place in low-income setting, where prevalence of diseases and malnutrition is a large scale issue. It is suggested that such supplements could improve child growth and health in low-income settings. However, in further studies these intervention have failed to address these issues. Therefore further studies on factors, such as systemic inflammation, effecting child health are needed to clarify to problem.

1.2 Immunology of cytokines

Cytokines are a structurally diverse group of proteins produced and released by various cells. They operate as modulators of immunological responses in autocrine, paracrine and endocrine manner. Cytokines are divided based on their essential effect on the process, pro-inflammatory and anti-inflammatory cytokines. (Janeway, 2012)

In this study we will analyze interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor α (TNF-α) and interleukin-10 (IL-10). The molecular characteristics and receptors of the cytokine analyzed in this study are shown more specifically in Table 1. These cytokine were chosen due to their essential role in inflammatory processes.

The cellular signal transduction of cytokine is initiated by binding a specific receptor (Hanada et al., 2002). The appropriate extra-cellular stimulation through receptor binding activates intra-cellular pathways. The substrate-receptor complex activates the associated Janus kinases (JAK), which phosphorylate the receptors cytoplasmic domains and the signal transducers and transcription family proteins (STAT) are activated. Pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α are modulating the progression of the inflammation through this pathway, but it is known that several growth and hormonal factors can activate JAK/STAT proteins as well. (Hanada et al., 2002)

Table 1: The molecular characteristics and receptors of the cytokine (Janeways, 2012).

Family	Cytokine	No. Of amino acids	Form	Receptors	Producer cells	Actions
IL	IL-1β	153	Monomer	CD121	Macrophages, epithelial cells	Fever, T-cell activation, macrophage activation
IL	IL-6	184	Monomer	CD126, CD130	T-cells, macrophages, endothelial cells	Fever, T-cell and B-cell growth and activation, acute phase protein production
IL	IL-10	160	Homodimer	IL-10R	Monocytes	Potent suppressant of macrophage functions
TNF	TNF-α	157	Trimer	p55, p75	Macrophages, T-cells, NK-cells	Promotes inflammation, endothelial activation

Anti-inflammatory cytokine including IL-10, suppress the activation of pro-inflammatory pathways. For example TNF-α and IL-1 with appropriate pathogen through Toll-like receptors(TLR) can activate Nuclear factor-κB (NF-κB)-protein related cascade, which regulates gene encoding of cytokine, adhesion molecules, chemokines, growth factors and inducible enzyme including cyclooxygenase-2 (COX2). (Hanada et al., 2002). IL-10 negatively regulates these pathways by activating suppressors of cytokine signaling (SOCS) proteins. Cytokine responses are regulated also through negative feedback system, which is described to effect JAK/STAT pathway by SOCS activation by JAK family. (Hanada et al., 2002)

Cytokine regulation is a dynamic process, and is effected by target cells availability and paracrine factors, eg. IL-6 can be considered both pro- and anti-inflammatory effects depending on if it is in

soluble form or if the target cells induce only receptors promoting anti-inflammatory pathways (Scheller et al., 2011). Therefore the division between cytokine is more of a guideline.

Immunological reaction are modulated by numerous biochemical pathways and these reactions and the molecules that participate are imperfectly portrayed. The guidelines of these processes however, can be described through classification of the known key particles and simplifications of the reactions.

1.3 Immunology of systemic inflammation

Effecting cellular pathways, the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α have several endocrine effects in different tissues initiating a systemic response to a pathogen. Endocrine effects include activating complement reaction and inducing acute-phase protein synthesis in liver and mobilization neutrophil in bone marrow endothelium. Pro-inflammatory cytokines are pyrogens', increasing body temperature in hypothalamus. In fat and muscle tissue all these pro-inflammatory cytokines promote katabolic reaction to provide energy for immunological reaction. (Janeway, 2012)

TNF-α initiate adaptive immune response by stimulating migration to lymph nodes and maturation of white blood cells (Janeway, 2012). TNF-α has pro-apoptotic macrophage-mediated effect (Costa et al.). IL-1β is produced by monocytes in infection and injury, and causes fever, hypotension and stimulates production of other cytokine (Costa et al., 2013). IL-6 induces several cellular pathways and targets numerous cells, most importantly it promotes acute phase response (Janeway, 2012). It has central role in severe immunological responses such as sepsis and chronic inflammation in arthritis (Costa et al., 2013). Pro-inflammatory effects of cytokine studied are presented in Table 2.

IL-10 is an anti-inflammatory cytokine released by several cell types in the body. By reducing the production of pro-inflammatory cytokine, immunological reactions are modulated to reduce the tissue damage caused by the reaction. (Janeway, 2012)

Table 2: Pro-inflammatory effects of cytokines (Janeway, 2012).

	Local effect	Systemic effect
IL-1β	Activates vascular endothelium, activates lymphocytes, local tissue destruction, increase access of effector cells	Fever, production of IL-6
TNF-α	Activates vascular endothelium and increases vascular permeability, increased entry of IgG, complement and cells to tissue and increased fluid drainage to lymph nodes	Fever, mobilization of metabolites, shock
IL-6	Lymphocyte activation, increased antibody production	Fever, induction of acute-phase protein production

Cellular processes induce systemic phenomena observed in clinical practice. Local initiation of infection or inflammation causes both biochemical and clinically observable reactions. Systemic inflammation is induced by pro-inflammatory cytokines by activating vascular endothelium and lymphocytes, allowing increased entry of immunoglobulins and complement to tissue and increased fluid drainage in lymph vessels (Janeway, 2012). Immunological responses initiated locally through single antigen-binding reaction can be detected systemically due vascular responses included. Based on the volume of the reaction it might be biochemically detected and whether the reaction causes symptoms, it can be described as clinical or sub-clinical (Rogawski et al., 2017). Systemic responses can be considered acute or chronic and be induced by inflammatory or infectious process and systemic inflammation can be portrayed by measuring levels of cytokine in plasma (Janeway, 2012).

In addition to the adequate response to a pathogen, systemic inflammation may lead to severe manifestations of which disseminated intravascular coagulopathy (DIC) is considered one of the most

severe (Costa et al.). Other manifestation vary to sub-clinical to mild in symptom but have wide-spread effects on vaccine responses, child growth, cognitive development, obesity, diabetes and metabolic syndrome (Rogawski et al., 2017).

1.4 Measuring immunological reaction

Immunological pathways are known through numerous cellular and biochemical models. Detecting these processes in clinical and research practices in individuals is possible by using specific indicators. In clinical practice the C- reactive protein (CRP) is most commonly used to model inflammation. Various other processes have an effect on these markers. Acute infection and several other factors must be considered for adequate assessment such as age, sex and chronic illness. For more profound examination we model more independent variables, such as cytokines due to their modulatory qualities. (Janeway, 2012)

There are many commercial technologies to measure immunological markers. Technics are usually based on the absorption or reflection of light, different techniques are not automatically comparable (DuPont, 2005). There is no golden standard for such technic.

1.5 Systemic inflammation with children in low-income setting

Numerous factors effecting systemic inflammation (Hanada et. al., 2002). Initiation of such process can be endogenous or more commonly exogenous, in which case pathogens are central. Endogenous processes are considered to be less significant in low-income setting, as the burden of pathogens is greater. This aspect requires further studies. Considering exposure to pathogens, one of the most important is nutrition and food source. Not only is the state of nutrition is considered to effect both infection and inflammation but in low-income setting the sources of both food and water can be contaminated and housing and sanitation can effect morbidity (Attia et al., 2016).

The most important food and water source for infants is breast milk. WHO universally recommend that infants receive breast milk exclusively until 6 months and along with other safe and nutritious

complementary foods at least until child is 2 years old. Contamination is problematic with complementary food. Infections of mothers and other community are considered to have an effect in child health.

Although inflammatory processes are physiological response to pathogens, inadequate or excessive immunological response and systemic inflammation is considered a possible cause of disease (Costa et al., 2013). Costa et al. studied exaggerated innate immune response and systemic inflammation mediated by cytokines in severe leishmaniasis or Kala-azar. Study showed that infants under 2 years old had higher levels of pro-inflammatory cytokine and therefore higher risk of death due response that may lead to DIC and other severe manifestation. (Costa et al., 2013) Such manifestations can be considered most excessive, but it is suggested that lower level of inflammation can have adverse effects as well. Campbell et al. studied systemic inflammation in 18-month old infants, in low-income setting in rural Bangladesh using several markers, but focusing on CRP or α-1 acid glycoprotein (AGP). Study suggests that elevated level of systemic inflammation is associated with recent morbidities and sociodemographic characteristics such as education and income. Systemic inflammation was associated stronger to acute respiratory infection markers than gastro-enteric markers. (Campbell et. al., 2017) Previous studies focusing on portraying inflammatory processes by measuring levels of cytokines in children under 2 years old are listed in Table 3.

Table 3: Previous studies focusing on measuring cytokine levels of children under 2 years old

Author	Year	Subject	Markers	Age-group	country
Kassir et. al.	2001	UTI	IL-1β, IL-6	under 29 months	USA
Costa et. al.	2013	Kala-azar	IL-1β, IL-6, TNF-α, IL-10	under 24 months	Brazil
Attia et. al.	2016	SAM	IL-6, TNF	6-59 months	Malawi
Campbell et. al.	2017	EED	CRP, AGP	18 months	Bangladesh

Rogawski et al. suggest that systemic inflammation caused by continuous exposure to pathogens is related to enteropathy which may lead to several adverse effect (Roqawski et al., 2017). In low-income setting systemic inflammation is associated with malnutrition and gastro-enteric damage and mortality (Attia et al, 2016). Association to cytokine levels is stronger however to acute infection than chronic infection and studies show stronger association to respiratory infections than gastro-enteric ones (Campbell et al., 2013). In urinary tract infections (UTI) cytokine levels adequately portray infection in infants and levels are normalize shortly after due anti-inflammatory intervention in high-income setting (Kassir et al., 2001). Infants with no acute clinical infection, cytokine levels that seem elevated might suggest an inadequate immunological response.

1.6 Aims of the study

The aims of this study was to evaluate the systemic inflammation in infants in low-income setting by analyzing the cytokine profiles IL-1β, IL-6, IL-10 and TNF-α. Results were compared with previous studies focusing on children of early age.

2. Methods

2.1 Study population

Our study is part of The International Lipid-Based Nutrient Supplements Project-DYAD-Malawi (iLiNS-DYAD-M), a nutritional intervention taking place in low-income settings. ILiNS-project is a randomized controlled trial of nutrient supplementation, to test the effect of lipid based nutrient supplement (LNS) on pregnancy and child outcomes (LNS Study plan, 2010). This study is based on data from iLiNS-DYAD, conducted in rural Malawi, Mangochi district. Pregnant women were identified in the antenatal clinics of health centers. 1391 pregnant women were enrolled and 781 live-born Malawian children in follow-up from birth to 2.5 years of age. We used peripheral venous blood samples collected during study follow-up visits at age of 6, 18 and 30 months (26, 78 and 130 weeks). Sample of the visit was not collected in case of severe acute illness.

2.2 Laboratory analysis

Trained laboratory staff centrifuged the whole blood at 3,000 RPM for 15 minutes and separated plasma into storage cryovials. The storage vial were placed upright in freezer boxes and put in a -20°C freezer for temporary storage at satellite clinics. Within 48 hours, plasma samples were transported to the main laboratory in Mangochi for long term storage at -80°C before they were shipped in dry ice to Tampere where the laboratory analysis were done. 1708 plasma samples from 6-, 18- and 30-month were analyzed soon after de-frosting. Samples were processed one batch of 78 samples at the time and divided into 9 different samples for further analyze. Each sample was measured to specific volume to use in a single marker analyze.

We used Milliplex immune-assay kit based on Luminex xMap technology to measure levels of IL-1β, IL-6, IL-10 and TNF-α in plasma. Technic is based on antibody-immobilized magnetic beads. (Elshal et. al.)

2.3 Statistical analysis

The data was analysis using R version 3.5.1. (2017/07/02). Cytokine levels are not normally distributed. We analyzed quantify association between cytokine levels using Spearman correlation, and we considered the levels un-dependent because of the long 6 to 12 month time period between visits. We also tested each correlation between different cytokines at each visit separately.

We analyzed the association between gender and cytokine level using Mann-Whitney test. We tested the association on all visits separately and together because of the missing values. For statistical significance we used P-value <0.05.

2.4 Ethical consideration

For this study we requested access to use iLiNS-DYAD data. The project is conducted according Good Clinical Practice guidelines and the ethical standards of the Helsinki Declaration. Project was approved by the College of Medicine Research and Ethics Committee, University of Malawi, and the Ethics

Committee of Pirkanmaa Hospital District, Finland. Informed consent was collected from each participant. An independent data safety and monitoring board monitored the incidence of suspected serious adverse events (SAEs) during the trial.

3. Results

We analyzed 1708 samples of all participants from 6-, 18- and 30-month clinical visits. Not all participants had plasma samples on all three visits. The analysis was therefore done both individually on each cytokine on each visit and all visits included. Table 4 shows descriptive mean, SD, median, maximum and minimum IL-1β (A), IL-6 (B), IL-10 (C) and TNF-α (D) concentrations at 6-, 18- and 30 month Malawian children separately and all combined. The number of samples on each visit and all combined also were shown as count in the Table 4.

Table 4: IL-1β (A), IL-6 (B), IL-10 (C) and TNF-α (D) concentrations at 6-, 18- and 30 month Malawian children separately and all combined.

A. IL-1β (pg/ml)

Visit	**26**	**78**	**130**	**All**
Mean	2.24	1.41	1.33	1.64
SD	5.29	0.96	0.81	3.03
Median	1.46	1.27	1.19	1.31
Max	79.91	12.63	9.34	79.91
Min	0.06	0.24	0.15	0.06
Count	**516**	**606**	**581**	**1703**

B. IL-6 (pg/ml)

Visit	**26**	**78**	**130**	**All**
Mean	6.27	2.63	2.15	3.57
SD	30.83	14.77	3.86	19.28
Median	2.45	1.32	1.22	1.55
Max	609.31	358.98	42.28	609.31
Min	0.11	0.09	0.02	0.02
Count	**510**	**606**	**580**	**1696**

C. IL-10 (pg/ml)

Visit	**26**	**78**	**130**	**All**
Mean	23.46	27.31	36.15	29.17
SD	52.49	67.99	103.91	78.69
Median	15.15	13.67	12.39	13.62
Max	1062.55	924.82	1931.14	1931.14
Min	0.62	1.11	1.56	0.62
Count	**511**	**606**	**580**	**1697**

D. TNF-α (pg/ml)

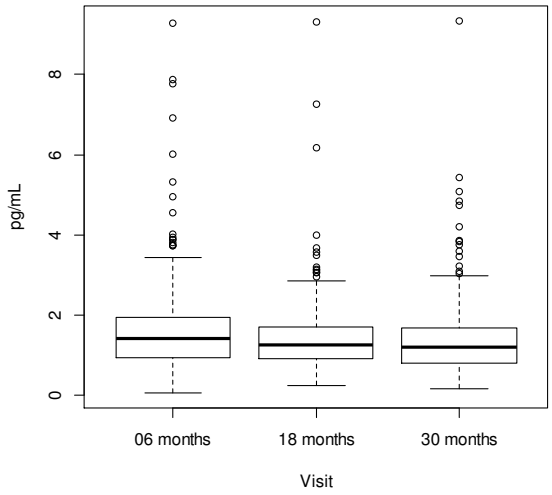
Visit	**26**	**78**	**130**	**All**
Mean	20.6	13.47	12.29	15.23
SD	21.32	9.58	7.49	14.22
Median	16.52	11.75	10.65	12.68
Max	266.03	154.31	61.18	266.03
Min	0.19	1.89	2.12	0.19
Count	**516**	**606**	**580**	**1702**

Figure 1 shows median values with standard deviation of ±1 SD and ±2 SD. An individual box plot was formed for each visit. Values were plotted without outlier values. There were 6, 60, 77 and 5 outlier values for TNF-α, IL-6, IL-10 and IL-1β respectively.

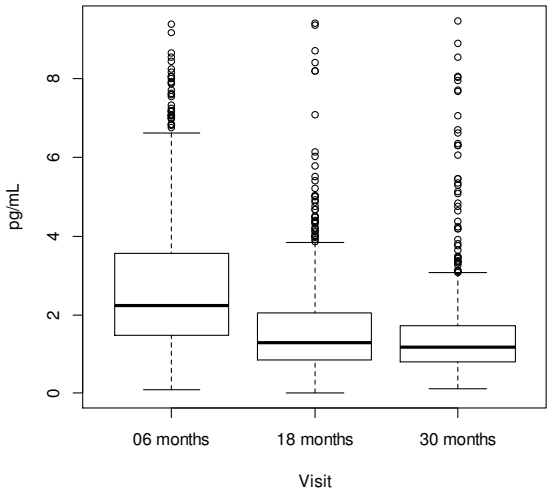
We analyzed the correlation of the levels of these cytokine using Spearmans test. The correlation between IL-1β and IL-6 was the strongest (rho=0.43 $p< 0.001$). There was no significant correlation between IL-1β and TNF-α (rho=0.04 p-value = 0.14). Due to missing values we also tested each cytokine correlation in all visits and each visit individually and results were similar (Figure 2). Results are portrayed in scatterplot (Figure 2).

Figure 1. IL-1β (A), IL-6 (B), IL-10 (C), TNF-α (D) concentrations at 6-, 18- and 30-month Malawian children.

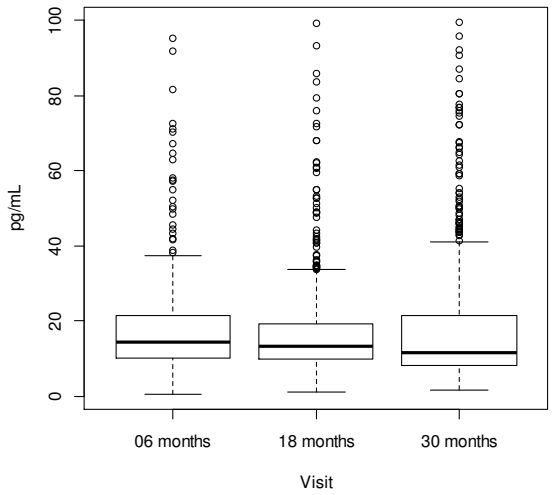
A IL-1β



B IL-6



C IL-10



D TNF-α

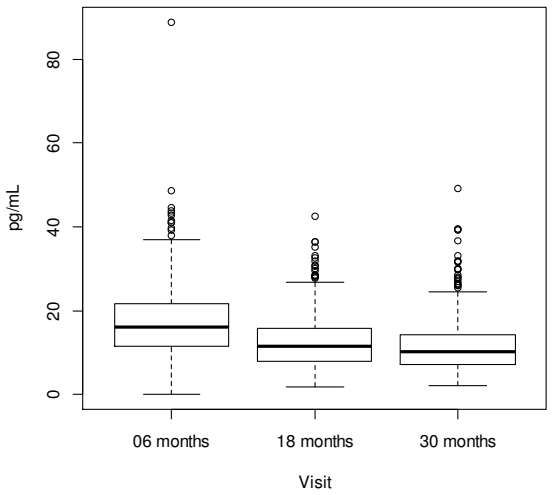
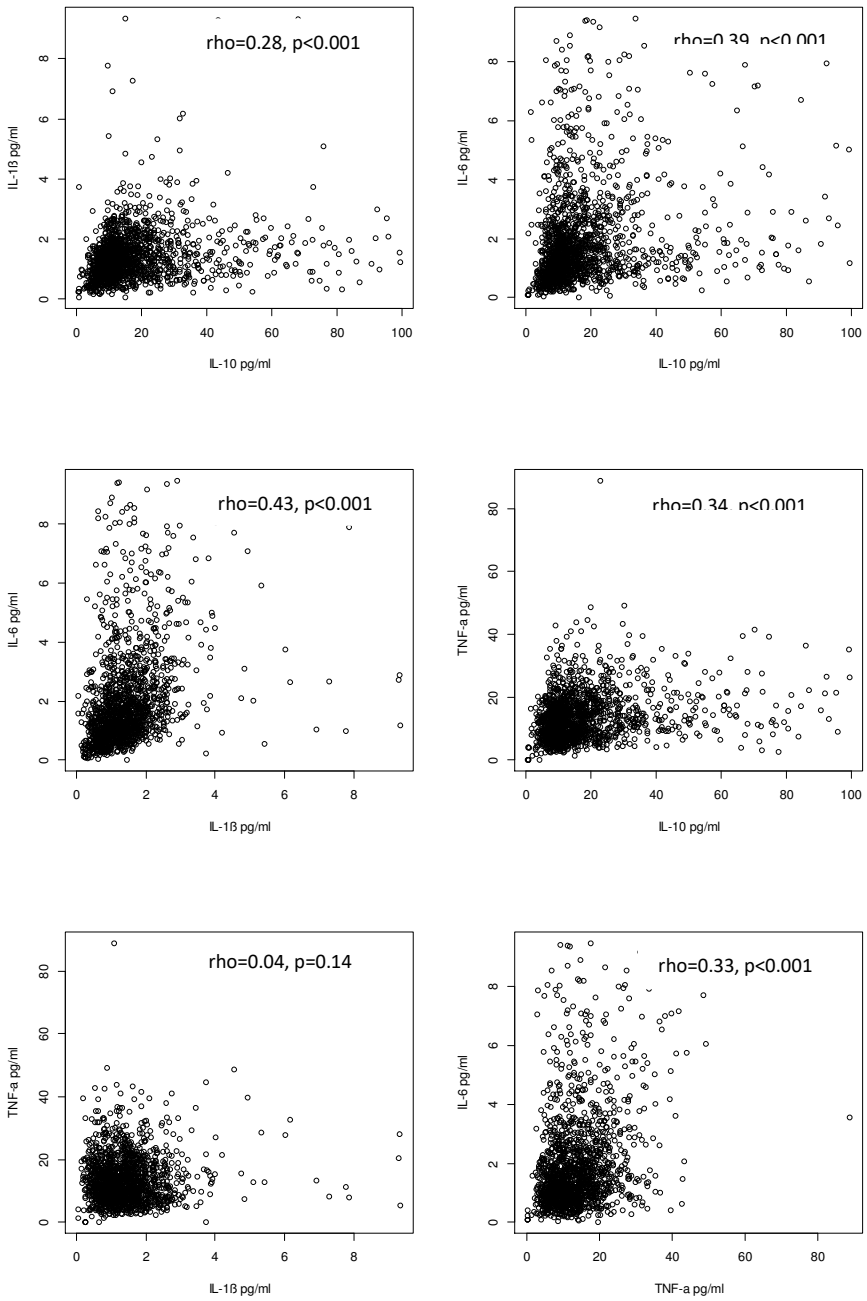


Figure 2. Cytokine correlation scatterplots with rho and P-value.



We tested the association between gender and cytokine level using Willcoxon test. Statistically significant association between gender and cytokine were found at 6-, 18- and 30-month and all combined Malawian children (Table 5). The cytokine levels by gender were shown in Figure 3 with all visits combined. Level of cytokine is statistically different at some visits between the genders. There is no continuous trend however. IL-1β is higher with girls at 18-, 30-month and all together. Levels of IL-10 and TNF-α are higher in all visits on boys than girls. IL-6 is higher with girls at 18- and 30-moths, but lower than boys at 6-moths and all combined.

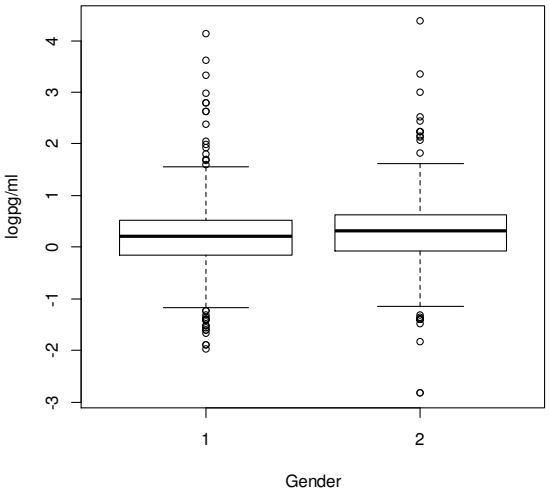
Table 5. IL-1β, IL-6, IL-10 and TNF-α concentration according to gender at 6-, 18- and 30-month Malawian children and all combined.

Cytokine	Visit	Gender	Mean	SD	P-value
IL-1β (pg/ml)	**6-mon**	Boy	**2.41**	5.32	0.174
		Girl	2.11	5.29	
	18-mon	Boy	1.37	0.87	<0.001
		Girl	**1.46**	1.03	
	30-mon	Boy	1.18	0.72	<0.001
		Girl	**1.49**	0.85	
	All	Boy	1.61	3.01	<0.001
		Girl	**1.67**	3.06	
IL-6 (pg/ml)	**6-mon**	Boy	**8.09**	43.91	<0.001
		Girl	4.65	8.92	
	18-mon	Boy	1.99	2.51	0.0295
		Girl	**3.23**	20.43	
	30-mon	Boy	1.99	3.52	<0.001
		Girl	**2.31**	4.16	
	All	Boy	**3.79**	24.06	<0.001
		Girl	3.36	13.38	
IL-10 (pg/ml)	**6-mon**	Boy	**26.33**	73.13	<0.001
		Girl	20.87	20.54	
	18-mon	Boy	**29.57**	75.92	<0.001
		Girl	25.19	59.61	
	30-mon	Boy	**36.37**	127.95	<0.001
		Girl	35.94	74.31	
	All	Boy	**30.97**	96.44	<0.001
		Girl	27.51	57.38	
TNF-α (pg/ml)	**6-mon**	Boy	**20.81**	21.89	<0.001
		Girl	20.41	20.84	
	18-mon	Boy	**13.93**	8.68	<0.001
		Girl	13.03	10.35	
	30-mon	Boy	**12.81**	7.08	<0.001
		Girl	11.81	7.83	
	All	Boy	**15.57**	14.04	<0.001
		Girl	14.91	14.37	

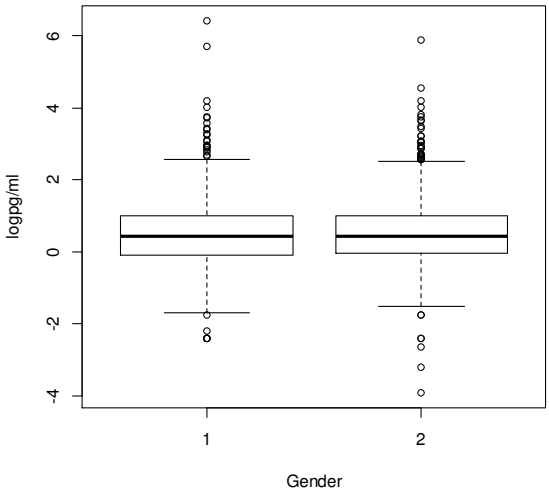
Figure 3. Cytokine levels and gender.

Boxplots showing level of cytokine in plasma. All visits are combined. Gender 1 equals male, gender 2 female. Level of cytokine portrayed in log. Plot A IL-10, B IL-1 β, C IL-6, D TNF-α.

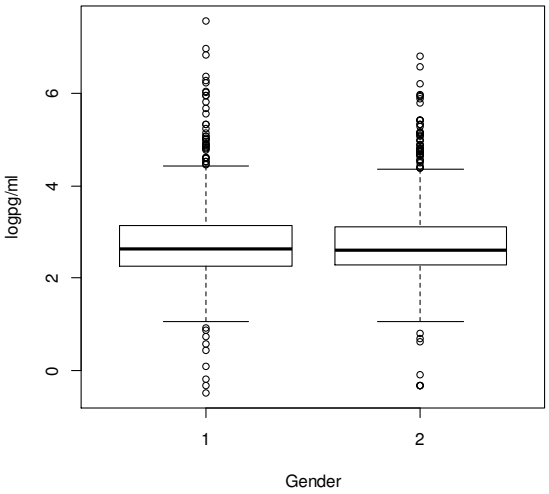
A IL-1β



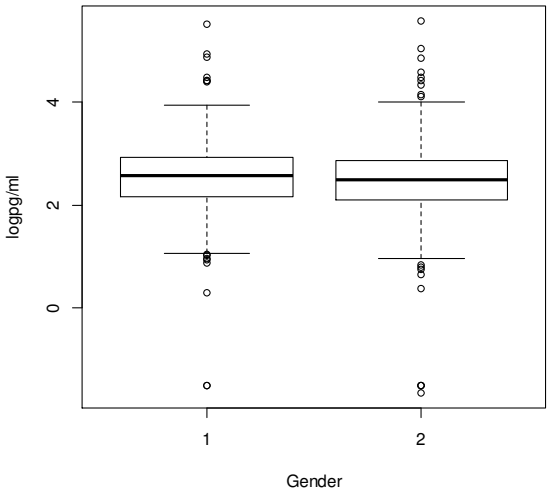
B IL-6



C IL-10



D TNF-α



Discussion

Our study investigated the distribution of IL-1β, IL-6, IL-10 and TNF-α concentrations among 6-, 18- and 30-month-old Malawian children. We also assessed if these cytokine levels were different in boys and girls. Most significant finding of our study was to show that results are in line with previous studies as the levels of cytokine correlate with each other. The overall levels can be considered accurate and believable for our study population.

The levels of cytokine are statistically different at several visits between the genders in our study. Previous studies show alteration in cytokine levels and inflammatory responses between the genders which might explain the results. These differences have been observed clearly before sexual maturation in both viral, bacterial and parasitic infections. Differences vary with age, which suggest that sex hormones may affect the immunological responses. Infants have been suggested to have more variance, as the prevalence of disease is higher. As infants have immature immunity, the balance between immunological reactivity and tolerance might be more easily compromised. In general, immunological reactions are considered biochemically stronger in males, which can be partially explained by the observation of stronger anti-inflammatory responses in females. These are simplifications however, and require further research. (Muenchhoff et. al., 2014)

In our results overall levels of pro-inflammatory cytokines IL-6 (3.79 pg/ml vs. 3.36 pg/ml, $p<0.001$) and TNF-α (15.57 pg/ml vs. 11.81 pg/ml, $p<0.001$) were higher with boys, which is in line with previous studies (Muenchhoff et. al., 2014). With IL-1β levels were higher with females, however results at all visits were not statistically significant. This might be explained with immunological variance in infants.

With adults, there are significant differences between immunological responses and clinical outcomes, but results in different studies might vary. Eg. Nasir et. al. and Pietropaoli et. al. studied mortality in sepsis and its relationship to gender. Nasir et. al. suggest that males have significantly stronger IL-6 response and higher mortality in sepsis, however Pietropaoli et. al. showed different results, showing higher mortality in sepsis with women in intensive care unit. Both studies suggested that the difference between immunological responses is caused by difference in hormonal responses between genders in adult patients, however such mechanism is inadequately portrayed in literature.

These results are not fully comparable to our results, as hormonal regulation differs with children, however Nasir et. al. report similar IL-6 response to our results. (Nasir et. al., 2015, Pietropaoli et. al., 2010)

Other factors explaining the results could be different food or water source or different exposure to pathogens, however there´s no data at this point showing this with children under 3 years old in our study population. In low-income setting Mustafa et. al. have shown gender related differences between immunological responses in male and female. The pro-inflammatory response was higher with males than females and adults than children, but no significant difference between genders in children (Mustafa et. al., 2015). These results differ from ours as the study was conducted with patients with tuberculosis, which might explain the difference. The statistical significance we found in cytokine levels between the genders in generally healthy children might not be clinically significant, as the levels of cytokine are low overall compared to previous studies (Costa et. al., 2013).

The correlation between the levels of cytokines in our study is consistent with previous study. In Costa et al. the results are similar to ours, but their results show stronger correlation between cytokine. F. ex. Pro-inflammatory cytokines had strong positive correlation IL-1β to IL-6 rho=0,66; p<0,001 and IL-1β to TNF-α rho=0,75; p<0,001. Because of the acute infection and higher concentration of cytokine, this difference might suggest that in severe infection the pro-inflammatory processes are effecting each other causing stronger correlation. (Costa et. al., 2013)

In our study population prevalence of infections is considered more common than in high-income setting, which can elevate overall levels of cytokines. Subsequently removing acute infection should decrease cytokine levels, as Kassir et al. measured systemic inflammation on acute pyelonephritis in high-income setting. Study focused on UTIs in children at high-income setting, infections considered to be caused by fecal E. coli. The levels of cytokine showed significant elevation during acute infection, but were normalized within days after anti-inflammatory intervention. The levels of cytokine in physiological state should show significant decrease as infection is passing. In our study population in low-income setting this might explain elevated cytokine levels. Kassir et al. used Immunotech enzyme-linked immunosorbent assay kit, in which the lower limits of detection is

15pg/ml for IL-1β and 3pg/ml for IL-6, respectively. Study shows the importance of specific and accurate method of analysis, which has been subjected in our study. (Kassir et. al., 2001)

Children with no acute clinical infection, cytokine levels that seem elevated might suggest an inadequate or excessive immunological response. As there are no cut-off values universally or in our population, the absolute values do not state whether the level of inflammation is elevated. In our study the samples were collected from children with no acute symptoms or mild symptoms. As the lower limit of cytokine concentration in Luminex technic is low, the results show values close to zero. This technic gives us a realistic values of prevalence of systemic inflammation. (Eshal et. al., 2016)

In the high-end levels of cytokines, the values suggest early infection. Pathological dysfunction of the process is extensive elevation of cytokine levels as stated in Costa et. al 2013. They studied acute severe Kala-azar in low-income setting. IL-6 concentration was much higher with children under 2 year old (62.6pg/ml) than our results (3.57 pg/ml). Costa et al. also showed IL-10 levels been significantly lower with HIV-infected patients (15.0pg/ml vs. 38.1pg/ml; p=0,01). An estimated 170 000 children are living with HIV in Malawi (Bendabenda et. al., 2017) Our results do not suggest lower IL-10 values (overall mean IL-10=29.2 pg/ml). The analysis technic used in Costa et al. was ELISA, which is considered to give lower scores on cytokine levels than Luminex (DuPont et al., 2005) The results are therefore not directly comparable, but it suggest that levels of cytokine in our study population are not generally elevated prominently.

In addition, previous study has shown that elevated level of systemic inflammation (CRP and AGP) is associated with recent morbidities and sociodemographic characteristics such as education and income. Systemic inflammation was associated stronger to acute respiratory infection markers than gastro-enteric markers. (Campbell et. al., 2017)

Stronger association to systemic inflammation is with acute infection, but more chronical conditions has been suggested to relate to gastro-intestinal infections. On the contrary to Kassir et al., the levels of cytokine might not show as clear decrease in low-income setting. This can be caused by subsequent infections, but it has been suggested that the physiological anti-inflammatory reaction can be altered due to continuous exposure to pathogens (Rogawski et al., 2017). This could be related to our study, as a factor elevating cytokine levels. The systemic inflammation is related to enteropathy

which may lead to several adverse effects. They also suggest that conditions and pathogenesis behind these conditions is difficult to recognize due to its sub-clinical features. Analyzing momentary factors such as plasma levels of cytokine, we might fail to recognize long-term conditions. (Roqawski et al., 2017)

The systemic inflammation in severe acute malnutrition related to diarrheal diseases in low-income setting has been studied by Attia et. al. In their study, cytokine levels were determined by Luminex human cytokine magnetic bead assay, but cytokines were not the main focus of their study. They found that levels of IL-6 and TNF-α were associated with diarrhea and higher mortality. They also showed high levels of fecal calprotectin suggesting intestinal damage, correlating with cytokine levels. The study suggest that the cytokine response in such condition is strong. It seems that the values are much more elevated than in our study (IL-6=18 pg/ml vs. 3.57 pg/ml and TNF-α=40 pg/ml vs. 15.23 pg/ml). In infection the physiological energy consumption is greater and pro-inflammatory cytokine have several catabolic effects in muscle and fat tissue. Also intestinal damage and inflammation can have adverse effect on absorption of nutrients in intestinal tract which affects nutrition. (Attia et. al., 2016)

A proper food source is the most important factor in malnutrition. WHO universally recommend that infants receive breast milk exclusively until 6 months and along with other safe and nutritious complementary foods at least until child is 2 years old. In rural Malawi breast feeding initiation is nearly universal, but complementary foods are low in nutrients, eg. maize porridge. This might make them susceptible to exposure to pathogens and possibly elevate cytokine levels. (Kumwenda et al., 2014)

In low-income setting, children are exposed to both unhygienic food and water sources and lack of sanitation. Preterm care and health facilities in rural Malawi has been suggested to lack knowledge on infant healthcare, which also contributes child health (Gondwe et al., 2016). Maternal infections, which can be more common in low-income setting, have adverse effects on child heath. Eg. in rural Malawi maternal parasite infections such as P falciparum may have a prevalence of 32%, other common infections effecting an infant can be tricomoniasis, vaginal candidiasis or UTI (Nkhoma et al., 2017). All these factors can be considered to effect cytokine levels in our study.

Potential effects of environment and illness on child development are modelled by Prado et al. 2017. Environmental factors such as poverty, education, poor sanitation and lack of clean food and water source effect not only infants, but burden mother with illness and undernutrition. Stress, depression and cognition are associated with each other, effecting mothers ability to give care to child. This effects child growth, cognitive development, prevalence of infections and other illness. (Prado et al., 2017)

Malnutrition is a large scale global issue and it has been addressed in numerous interventions by many organizations and nations. It is important factor considering future global health challenges because of the major role it has in child health. Due to its widespread adverse effects, it is important we understand it thoroughly. Malnutrition is associated with systemic inflammation in numerous ways, and our results suggest portraying inflammatory phenomena through biochemical markers can further extend understanding on malnutrition. Previous studies focus widely on the most adverse outcomes associated with systemic inflammation. Though our results are in line with previous studies, they differ much from results focusing on acute clinically addressed morbidity and malnutrition. Our results do not exclude the elevated prevalence of systemic inflammation as suggested by Rogawski et al. Further studies are required to portray the role of sub-clinical processes in nutritional interventions and malnutrition.

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