Tampereen yliopisto

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PLASMA CONCENTRATIONS OF INSULIN-LIKE GROWTH FACTOR 1 (IGF-1) AND IGF BINDING PROTEIN 3 (IGFBP-3) AMONG 6- TO 30-MONTH-OLD RURAL MALAWIAN CHILDREN

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Lapsuusiän kasvuun vaikuttavat sekä perintötekijät että ympäristötekijät. Ensimmäisen puolen vuoden aikana ravitsemus on voimakkaimmin lapsen kasvua säätelevä tekijä. Ravitsemustila vaikuttaa insuliinin kaltaisen kasvutekijä 1:n (IGF-1) pitoisuuksiin, joka osallistuu yhdessä kasvuhormonin kanssa kasvun säätelyyn. Matalan tulotason maissa lasten vajaaravitsemus on yleistä, minkä seurauksena lasten IGF-1-pitoisuudet ovat matalia. IGF-1-pitoisuuksiin vaikuttavat lisäksi ympäristöperäinen suoliston toimintahäiriö, hygieniataso sekä äidin biologiset ominaisuudet.

Suurin osa IGF-1-molekyyleistä kulkee verenkierrossa kiinnittyneenä sitojaproteiini 3:een (IGFBP-3), joka osallistuu biologisesti aktiivisen IGF-1:n säätelyyn. Tutkimuksessa selvitettiin plasman IGF-1- ja IGFBP-3-pitoisuuksien jakaumaa ja siihen vaikuttavia tekijöitä 6, 18 ja 30 kuukauden ikäisillä maaseudulla asuvilla malawilaislapsilla. Plasmanäytteet on kerätty vuosina 2011–2015 osana äitien ja lasten ravitsemustutkimusta.

IGF-1- ja IGFBP-3 -pitoisuudet analysoitiin fluoresoiviin väriaineisiin perustuvalla Luminex XMap –tekniikalla. IGF-1-pitoisuuksien keskiarvo 6 kuukauden iässä oli 13,1 ng/ml (n = 519); 18 kuukauden iässä 12,5 ng/ml (n = 605) ja 30 kuukauden iässä 14,5 ng/ml (n = 581). IGFBP-3-pitoisuuksien keskiarvot olivat vastaavasti 2643ng/ml (n = 474); 2676 ng/ml (n = 584) ja 2652 ng/ml (n = 580). 18 ja 30 kuukauden iässä IGF-1- ja IGFBP-3 -pitoisuuksien keskiarvot olivat tytöillä suuremmat kuin pojilla. IGF-1- ja IGFBP-3 -pitoisuudet korreloivat keskenään kaikissa ikäryhmissä. Äidin pituus korreloi lasten IGF-1- ja IGFBP-3 -pitoisuuksiin, mutta äidin painoindeksi, ikä, tulehdusparametrit ja kotitalouden varallisuus korreloivat tuloksiin vain osittain tai eivät lainkaan. Kuivakaudella plasmanäytteiden IGF-1- ja IGFBP-3 -pitoisuudet olivat useimmiten korkeammat kuin sadekaudella. Asuinpaikkakunta, juomaveden lähde ja käytettävissä olevat saniteettitilat vaikuttivat tuloksiin muutamissa vertailluissa ryhmissä. Jotta IGF-1- ja IGFBP-3 -pitoisuuksia voitaisiin hyödyntää sairauksien diagnostiikassa, tulisi käyttää vertailukelpoisesta tutkimusjoukosta saatuja viitearvoja sekä samanlaisia laboratoriomenetelmiä.

Avainsanat: kasvu, Saharan eteläpuolinen Afrikka, vajaaravitsemus, ympäristötekijät

ABBREVIATIONS

AGP	Alpha-1-acid glycoprotein
BMI	Body mass index
CRP	C-reactive protein
EBF	Exclusive breastfeeding
EED	Environmental enteric dysfunction
GH	Growth hormone
GHRH	Growth hormone releasing hormone
ICP	Infancy-childhood-puberty
IGF-1	Insulin-like growth factor 1
IGFBP-3	Insulin-like growth factor binding protein 3
IL-6	Interleukin 6
iLiNS	International Lipid-Based Nutrient Supplements
L:M ratio	Lactulose:mannitol ratio
RPM	Revolutions per minute
SD	Standard deviation
SGA	Small for gestational age
WASH	Water, sanitation and hygiene
WHO	World Health Organization

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1. BACKGROUND

1.1 Malnutrition among children

Definition

Terms malnutrition and undernutrition are often used to describe the same phenomenon (1) but term malnutrition refers to deficiencies, excesses, or imbalances in a person's intake of energy and/or nutrients. Forms of undernutrition are stunting, wasting, underweight and micronutrient deficiencies. (2) Undernutrition is defined as a state in which the amount of energy and nutrients from food is inadequate to meet nutritional demands of a subject (1). Stunting is defined as children's height-for-age more than two standard deviations (SD) below the WHO Child Growth Standards median. Likewise, wasting and underweight are defined as children's weight-for-height and weight-for-age more than two SD below the WHO Child Growth Standards median. (3) Micronutrient insufficiencies indicate a person has deficiencies in vitamins and minerals (2).

Prevalence

Undernutrition is a huge problem in low- and middle-income countries and especially in sub-Saharan Africa (4). About one third of children in developing countries suffer from stunting (5). In a meta-analysis which included 32 Sub-Saharan countries, the prevalence of stunting was 33.2%, the prevalence of wasting was 7.1% and the prevalence of underweight was 16.3% among children younger than five years (4). According to Unicef's statistics 42.4 % of Malawian children were stunted and 16.7% were underweight in 2013 - 14 (6). In rural Malawi incidence numbers among 0 – 36 months old children are higher: 70 % of children were stunted and 40 % were underweight (7). In the meta-analysis it was shown that prevalence of stunting is 47.1 % in Malawi. This was the second highest stunting percentage of the African countries that were included in the meta-analysis. (4)

Adverse outcomes

Adverse outcomes of malnutrition is a combination of many factors which can occur simultaneously, such as inadequate diet intake, recurrent infections, micronutrient deficiency and low income. All these factors negatively affect child growth. Take stunting as example, mortality

and morbidity due to infections, such as pneumonia and diarrhea, are higher in stunted children compared to well-nourished children. (8) Brain development is negatively affected (4) which leads to lower cognitive capacity. Development of motor skills and school attendance are poorer in stunted children (8).

Stunting in childhood has consequences for life in adulthood: economic status is lower and in some cases the risk of overweight, hypertension and cardio-vascular diseases is higher. The risk of having metabolic syndrome in adulthood has been explained by epigenetic changes in metabolism due to lack of nutrition prenatally and in early childhood. (8)

1.2 Child growth

Normal growth

The growth after birth can be divided into three phases according to the infancy-childhood-puberty (ICP) model: 1) the growth during infancy, 2) childhood and 3) puberty. During infancy the growth velocity is high: infants grow about 25 cm during the first 12 months of life. The growth of infancy continues up to first 2 – 3 years of life but the velocity is more modest. Childhood growth begins during 6 – 18 months of age which means that infancy and childhood growth phases occur simultaneously. Thus, the two phases cannot be clearly separated from each other. During puberty the growth velocity increases again: girls have the peak growth velocity at age of 12.1 years and boys at age of 14.1 years. (9)

During the first couple of months of life exclusive breastfeeding (EBF) has a positive effect on growth velocity. Growth hormone (GH) is needed already during infancy growth but the role of GH becomes more important later. After infancy the most important regulator of child growth is growth hormone – insulin-like growth factor 1 (IGF-1) system. (9) At the age of 6 – 12 months the role of GH in IGF-1 production becomes more important than nutrition. (10) Some hormones, such as thyroid hormones and sex steroids, affect growth plates in cartilage but these hormones have also important role in modulation of GH – IGF-1 –system. (9, 11) Thyroid hormone is needed for growth throughout the period of growing (9).

Growth hormone releasing hormone (GHRH) and growth hormone inhibitory hormone, called somatostatin, are produced and released by the hypothalamus. These hormones affect the

anterior pituitary gland: GHRH stimulates excretion of growth hormone and somatostatin inhibits the process. GH is released in pulsatile pattern. The regulation of GH release is based on negative feedback system: when the amount of GH is high in bloodstream the release of GHRH is diminished. This causes levels of GH diminish too. (12)

IGF-1 in normal growth and factors regulating IGF-1 concentration

Most functions of GH are mediated via IGF-1. IGF-1 production is stimulated when GH binding receptors are activated in the target organs. One of the target organs is the liver. IGF-1 produced by the liver is excreted in the circulation and this circulating IGF-1 functions as a hormone. IGF-1 stimulates cartilage of long bones to grow in length. (9, 11) The regulation of IGF-1 production is also based on negative feedback system: increased concentration of IGF-1 suppresses production of GH which in turn slows down IGF-1 production in the target organs (13). IGF-1 is also an independent growth factor affecting cell proliferation and differentiation and preventing apoptosis (14).

IGF-1 production is regulated by several factors, for example growth hormone concentrations, age, pubertal status, liver function, and nutritional status (15, 16). At birth the IGF-1 concentration is relatively low but it increases during childhood. By the beginning of puberty and at the end of puberty the IGF-1 concentrations are on the same levels as during adulthood. During a few years of puberty the concentrations are 2 – 3 times higher than in adults. (11) IGF-1 is produced by the liver so liver function affects IGF-1 concentration (16).

The role of nutrition in IGF-1 concentration

IGF-1 concentrations are lower in malnourished subjects compared to well nourished subjects: IGF-1 concentrations are lower in people with significant energy or protein deficiency. It has been studied that amount of nutrition affects IGF-1 concentrations also within normal IGF-1 range. (13) The quality of nutrition may also affect IGF-1 concentrations: formula fed infants had higher IGF-1 concentrations at ages of 3, 6 and 12 months compared to breastfed infants. The reasons for this difference are not clear: faster weight gain or larger amount of adipose tissue in formula fed infants are some possible explanations. (17) Milk feeding practices may also affect the concentration of biologically available IGF-1. This has been suggested since there have been no differences in IGF binding protein 3 concentrations in formula fed and breastfed infants. (10) It is

also possible that IGF-1 production is increased by higher protein intake (18). It is not known if the total protein intake or the amount of essential amino acids are more significant for IGF-1 production (13). The role of protein may be important for growth among malnourished children: it has been suggested that GH stimulation in malnourished subjects is more dependent on amount of dietary protein than total amount of energy (13).

It has been studied that low IGF-1 concentrations in stunted 1 year old children might be caused by suboptimal nutrition. Besides protein and amino acids, also the amount of carbohydrates in diet affect IGF-1 concentrations. If carbohydrate-rich foods were the main source of energy, the amount of vegetables and protein, such as dairy products, were low. Thus, the quality of diet was suboptimal. The role of fat in diet should be also taken into account: It has been suggested that low amount of fat in diet leads to deficiency of essential fatty acids. Also in this case, the quality of nutrition is poor which might lead to stunting and low IGF-1 concentrations. In addition, some minerals such as zinc, potassium, calcium, and magnesium concentrations correlate positively with IGF-1 concentrations. (13)

Other factors affecting child growth

Environmental factors have a huge impact on a child's growth during the first two years of life. These factors include frequent infections, maternal characteristics such as nutritional status, availability of health care services, feeding practices, hygiene and sanitation. (8)

Infections

Infections affect the regulation of IGF-1 –axis. IGF-1 production by the liver is suppressed during intercurrent infections (e.g. pneumonia, diarrhea) and during chronic inflammatory diseases (e.g. Crohn's disease, juvenile idiopathic arthritis). Suppressed IGF-1 production caused by frequent infections can deteriorate child's growth. There are also other mechanisms that affect growth during infections, for example anorexia, increased nutrition demand, malabsorption, and catabolism. Acute-phase reactions also suppress nutrients transport from the bowel to other tissues. Pro-inflammatory cytokine concentrations and other markers of immune activation increase during infections while IGF-1 concentrations decrease, (19) though the correlation is not always consistent (20).

Recurrent infections can be one cause for growth problems. On the other hand, it has been studied that malnourished children have more infections compared to well-nourished children. Infections among malnourished children are more frequent, more severe and longer-lasting. However, causes and consequences of infections and impaired growth have not been confirmed. (8)

Diarrhea is a common infection in childhood. About 25 % of stunted children in developing countries had had at least five episodes of diarrhea by age of 24 months. It is not clear whether diarrhea causes stunting or not. (8) It is common that after diarrhea and other infections children have a time period of faster growing, so called catch-up growth (15). Thus, evaluating consequences of diarrhea is difficult. On the other hand, it has been noticed that children with typical diarrheal burden were 0.38 cm shorter at age of 2 years compared to children without diarrhea. (8)

Maternal characteristics

Characteristics of a mother affect intrauterine growth of a child: placental development, maternal nutritional status and endocrine and metabolic signals have been proven to affect a child's growth. Thus, stunting can begin already before birth. It has been estimated that stunting has begun during antenatal period in about 20 % of stunted children. If a mother is stunted herself or is affected by malnutrition there is a higher risk that a child will be stunted as well. The risk of mortality and problems with pregnancy are more common in children of stunted mothers. Children of stunted mothers are more often small for gestational age (SGA). Those born preterm (before 37^{th} week of pregnancy) and SGA are higher risk for poor growth and mortality. Mortality, underweight and stunting during infancy and childhood are negatively associated with maternal height. (8)

There might be common factors that affect growth pre- and antenatally but these factors are not clearly known yet. One proposed mechanism is inflammation: mothers with high inflammatory markers, such as C-reactive protein (CRP), had more often stunted infants compared to mothers who had normal levels of CRP. Thus, inflammation might be a link between prenatal and antenatal stunting. (8) Alpha-1 acid glycoprotein (AGP) is another inflammatory marker which rises in response to inflammatory process in the body (21). AGP levels rise slower and stay elevated longer compared to CRP levels (5).

Feeding practices

Exclusive breastfeeding (EBF) has been proven to decrease mortality and morbidity. In addition, EBF improves cognitive capacity but the effect for growth is not clear. It has been considered that the role of EBF is not significant if growth failure has begun prenatally and continues during the first six months of life. (8)

Socioeconomic status

Stunting is also associated with low socioeconomic status: inadequate food intake is the main reason for poor growth in communities where people have low socioeconomic status. Changes is socioeconomic factors have an impact on child's growth. It has been noticed improvement in linear growth during economic development in China and growth is improved among those who move from a country with low socio-economic status to a country with better socio-economic conditions. (8)

Water, sanitation and hygiene

Water, sanitation and hygiene (WASH) have a huge impact on child's health. It has been suggested that poor WASH conditions might be reason for childhood underweight due to diarrhea and undernutrition. It has been proven in several studies that improvement in water supply and sanitation leads to better growth in childhood. (22) However, there is also a study showing that improved WASH conditions alone did not improve child growth. But when improvement in WASH conditions was combined with nutrition counselling there was a positive effect on child linear growth in rural Bangladesh. (23)

1.3 Environmental enteric dysfunction

Introduction

Many factors can cause undernutrition, e.g. inadequate food intake, frequent infections and inflammation in gastrointestinal tract (24). Recently, environmental enteric dysfunction (EED) has been shown to be associated with growth failure in low-income settings. EED is an inflammatory

condition in which the permeability of the small intestine increases and the absorption of nutrients is deteriorated. EED and stunting among infants and children are strongly connected but the causality of EED and stunting is not clear. It has been suggested that infections and malnutrition might be reasons for EED but probably the condition is multifactorial. Some other proposed reasons for EED are nutritional deficiency (vitamin A, zinc) and micro-organisms. EED is more common in low- and middle-income countries than in high-income countries. (25)

The small intestine physiology

The small intestine is the major site of absorption of nutrients. The area of absorption in the small intestine is increased because of three anatomical features: 1) the small intestine wall is folded, 2) there are villi (length of 1 mm) on the surfaces of the folds and 3) there are microvilli on the surfaces of villi. Thus, the total area of absorption in the small intestine is large. The small intestine also secretes enzymes that are needed for complete digestion. When digestion is completed nutrients are transported from the lumen of the small intestine. Finally nutrients end up into bloodstream and are delivered to various body parts where nutrients can be utilized. Besides nutrients, also water and ions are absorbed in the small intestine. (26)

Moreover, the small intestine has an important role in the local immune response. The small intestine protects body from harmful antigens that are in the contents of the small intestine. The normal bacterial flora of the small intestine and the mucosal lining protect the body from harmful agents in the gut lumen. There are lymphatic cells that are activated by pathogens in the small intestine. There are also antibacterial agents secreted by the crypts around the villi. The epithelial cells in the small intestine lumen are tightly connected to each other which prevents crossing of harmful agents from the lumen to the other parts of the body. (26)

Pathophysiology of EED

There are six aspects in the pathophysiology of EED that should be taken into account: 1) gut permeability, 2) microbial translocation, 3) systemic inflammation, 4) gut inflammation, 5) dysbiosis, and 6) nutrient malabsorption. The permeability of small bowel is increased in EED so the bowel becomes more permeable for large particles such as intestinal bacteria and microbial-associated macromolecules that move to the systemic circulation. The particles can activate the immune system which leads to systemic inflammation. Inflammation is present also locally in the

small bowel. Another typical feature of EED is diffuse villous atrophy. This decreases area of absorption in the small bowel which leads to nutrient malabsorption. Malabsorption and inflammation can lead to impaired growth. (24)

EED and growth failure

Consequences of EED are individual: someone can have mild malabsorption and major bacterial translocation in the gut while someone else can have severe malabsorption but only minor bacterial translocation (24). When the absorption of nutrients is deteriorated the risk of malnutrition is increased (25). Consequences of deteriorated nutrient absorption can be clinically significant in childhood when growth velocity and nutrient demand are high (8). EED causes inflammation which deteriorates linear growth and therefore can lead to stunting (24). Chronic inflammation itself may also be one reason for stunting (5). Even though EED is associated with stunting it has been difficult to prove causality between these conditions (27).

Methods and biomarkers in diagnosing EED

The nature of EED is still unclear because the condition is multifactorial and there is no simple way to diagnose EED. Some invasive methods have been used, such as endoscopies. These are not easily accessible and they cause discomfort for a patient so it has been considered that there should be a non-invasive point-of-care method for diagnosing EED. (24, 27, 28) There is currently no gold-standard method or biomarker to diagnose EED (27), making studies challenging. The requirements for it are that it should be affordable, simple and practical (27). So far, the most common non-invasive method is lactulose:mannitol test which is based on absorption of two sugars: non-absorptive disaccharide lactulose and transcellularly absorptive monosaccharide mannitol are administered orally. These sugars are secreted mostly unmetabolized and excreted by the kidneys. Thus, it is possible to measure amounts of lactulose and mannitol in the urine and compare results to the oral dose to assess the gut permeability. The result is called lactulose:mannitol ratio (L:M ratio). If the amount of lactulose in the urine is elevated it can be assumed that the small intestine permeability is increased. (24, 27, 28)

Some fecal biomarkers have been tested, for example calprotectin and lactoferrin. The problem is that the tested fecal biomarkers indicate an inflammatory process in the gastrointestinal tract but

the biomarkers are not specific for EED. (24) In addition, it has been suggested that breastfeeding might affect the concentrations of calprotectin and lactoferrin in infants. Another problem is that there are no reliable reference values for these biomarkers in children. (27)

In addition, some serum biomarkers have also been tested for EED diagnosis, eg. interleukin 6 (IL-6), C-reactive protein (CRP), lipopolysaccharide, ferritin, and IGF-1 (24). IGF-1 could be evaluated in the assessment of growth since linear growth is diminished by systemic inflammation and EED. It has been also suggested that IGF-1 could be one of the biomarkers in monitoring EED, though there was no clear correlation between L:M ratio and IGF-1 concentration. (27) Measuring IGF-1 concentrations could be used in monitoring and diagnosing EED if this method is improved and validated in the future.

Prevention of stunting caused by EED

One possible intervention to prevent stunting caused by EED is zinc supplementation. Zinc deficiency might affect villous function decreasing villous surface area and absorptive capacity in the gastrointestinal tract. In addition, zinc deficiency can reduce appetite. Thus, zinc supplementation could be one possible intervention to prevent harmful factors that play a role in the development of EED. Other possible interventions include access to clean drinking water and building and using of toilets. In addition, breastfeeding is an intervention: exclusive breastfeeding is recommended during the first six months of life, and breastmilk could be used as a part of nutrition for children who are older than six months (24).

EED and IGF-1

EED is a low-grade chronic inflammatory condition, which suppresses IGF-1 production (5). The mechanism of deteriorated growth in EED is mediated via cytokine interleukin 6 (IL-6), which decreases production of IGF-1 and inhibits its effect on growth plates accordingly. Thus, linear growth is diminished. (24) It has been reported that Zimbabwean infants with EED were stunted, had low IGF-1 concentrations, and high cytokine concentrations (25). Prendergast et al. found that low IGF-1 concentrations and stunting were associated with high inflammatory markers in blood, but no evidence about association between gut permeability and stunting was found (5).

1.4 Insulin-like growth factor 1

IGF-1 is a peptide hormone produced mainly by the liver hepatocytes (16). Other tissues, such as the brain, muscle, and bone are also able to produce IGF-1 (29). IGF-1 consists of 70 amino acids and it has four chains: A, B, C, and D. A and B chains are similar with insulin, a hormone produced by the beta cells of pancreas, but C and D chains are not present in insulin molecule (16). IGF-1 and insulin have many similar effects on growth (12). The structure of IGF-1 receptors and insulin receptors are similar but ligand affinity is different (30). IGF-1 can act as a local paracrine hormone or as a systemic hormone. Other growth factors lack this feature. (29) IGF-1 stimulates cell proliferation and differentiation and protects cell from apoptosis. Effects of IGF-1 are mediated via IGF-1 receptors which are present in all tissues. (14)

IGF-1 deficiency or dysfunction is associated with growth failure (15). IGF-1 concentration analysis can be used to evaluate hormonal growth regulation and possible problems with GH – IGF-1 axis (16). Fluctuation of GH concentration due to its pulsatile release makes it difficult to measure. IGF-1 concentrations are more stable. (12) Therefore, measuring IGF-1 concentrations is more practical than measuring GH concentrations.

1.5 IGF binding protein 3

Insulin-like growth factor binding proteins (IGFBP) have three functions: they are carrier proteins that transport IGF-1 to target cells, affect interaction of IGF-1 and their receptors and prolong half-time of IGF-1 (11). There are 6 different binding proteins for IGF-1, from IGFBP-1 to IGFBP-6 (29). The amount of IGFBP-3 is the most significant (11). About 90 % of IGF-1 is attached to IGFBP-3 (13). IGF-1 and IGFBP-3 form a complex with an acid-labile subunit. IGF-1 is stored in the blood stream when attached to the complex: the complex can regulate the amount of biologically available IGF-1 by releasing IGF-1 molecules when needed. (29, 31) IGF-1 molecules are more tightly bound to their carrier proteins than to their receptors. When IGFBPs and IGF-1 are attached the amount of free IGF-1 is decreased. Half-time of IGF-1 alone is 10 minutes but attachment to IGFBP-3 and acid-labile subunit makes the half-time to about 12 – 15 hours. IGFBP-3 concentrations are increased when growth hormone or IGF-1 concentration is increased. Thus, lack of growth hormone decreases IGFBP-3 concentration.

Nutritional intake is the most important regulator of all IGFBPs. Concentrations of IGFBP-3 remain relatively stable during the day (11, 13, 32). Besides nutrition status, IGFBP-3 concentration also depends on age like IGF-1. They both increase with age reaching the highest values at puberty and decrease after that (31). IGFBP-3 concentration can also be used as an indicator of GH – IGF-1 – axis among infants. Evaluation of GH – IGF-1 –axis becomes more accurate if both IGFBP-3 and IGF-1 are measured. On the other hand, if it is only possible to measure one of the indicators, it has been suggested that IGFBP-3 should be measured among those who are younger than two years and IGF-1 should be used among the older children. (16)

1.6 IGF-1 and IGFBP-3 concentrations in previous studies

IGF-1 and IGFBP-3 concentrations among infants and children have been reported in some previous studies. The summary of these studies is presented in Table 1. The reference values for 0 – 6 years old Turkish children were established by Yüksel et al (31). Bidlingmaier et al. studied IGF-1 concentrations at different age groups from birth to senescence (33). Bedogni et al. used chemiluminescence assay method to establish reference values for IGF-1 concentrations among 0 – 18 years old Italian children and adolescents. (34) Löfqvist et al. used samples from 1.1 – 18.3 years old Swedish subjects to establish reference values for IGF-1 (35). Jones et al. studied acute illness and suppression of growth hormone axis in 6 weeks to 18 months Zimbabwean children (19). There were also studies from Korea (36), India (37), Burkina Faso (38) and United States (39). The IGF-1 and IGFBP-3 concentrations are shown in Table 1 for children between 0-3 years old if the data is available.

Table 1. Summary of IGF-1 and IGFBP-3 concentrations and laboratory methods used in some previous studies.

Authors (reference)	Publication year	Country of subjects	Age of subjects	Main results about IGF-1 and IGFBP-3 concentrations	IGF-1 concentrations (converted to ng/ml) among < 3 years old children*	IGFBP-3 concentrations (converted to ng/ml)	Laboratory method
Bedogni et al. (34)	2012	Italy	0 – 18 years	Increase with age. Sex affects the results less than age.	25 – 252 **		Chemiluminescence assay
Bidlingmaier et al. (33)	2014	United States, Canada, Europe (Austria, Denmark, Germany, Sweden)	0 – 94 years	Low during the first year of life, then gradually increase and reach the highest values during puberty at age of 15.	17.9 – 164.2 among females, 27.0 – 204.5 among males **		Automated chemiluminescent immunoassay (IDS-iSYS; Immunodiagnostic Systems)
Brabant et al. (40)	2003	Germany, Belgium, Sweden	1 month – 88 years	Depend on age, the highest values among 14-year-old girls and 16 year-old-boys.	13 – 159 **		Chemiluminescent immunoassay (Nichols Advantage®; Nichols Institute Diagnostics, San Clemente, Calif., USA)
Chaler et al. (41)	2009	Argentina, Italy	0 – 18 years	Increase from early childhood to puberty. Higher levels among girls compared to boys. IGFBP-3 levels increase from early childhood to puberty.	38.5 – 151.0 **	2.32 – 2.82 **	Chemiluminescent assay
Hyun et al. (36)	2012	Korea	0 -17 years	IGF-1 and IGFBP-3 concentrations increase with age among prepubertal children and decrease after that.	not available **		Immunoradiometric assay (IRMA, Immunotech)

Jones et al. (19)	2015	Zimbabwe	6 weeks – 18 months	Lower among children with recent acute illness.	around 20 – 60 (estimation)		ELISA (R&D Systems)
Kouanda et al. (38)	2008	Burkina Faso	0 – 5 years	Decrease from birth to 24 months, increase after that.	27 – 45 (from < 5 months of age to 5-year-old children) **		Filter paper, radioimmunoassay, chromatography
Löfqvist et al. (35)	2001	Sweden	1.1 – 18.3 years	Increase with age among prepubertal and early pubertal children. Difference between boys and girls during puberty.	around 0.05 – 0.1 (estimation) **		Radioimmunoassay (Mediagnost GmbH, Tübingen, Germany)
Soldin et al. (39)	2008	United States	0 – 18 years	IGF-1 and IGFBP-3 concentrations increase with age, the highest values during puberty.	30 – 174 (reference intervals) **	1300 – 3500 (reference intervals) **	Chemiluminescent immunometric methods (IMMULITE 2000®)
Wiley et al. (37)	2018	India	0 – 2 years	Positive association with height at 2 years of age and milk intake.	mean 49.4 at age of 2 years	mean 1953.8 at age of 2 years	not available
Yüksel et al. (31)	2011	Turkey	0 – 6 years	IGF-1 and IGFBP-3 concentrations increase with age.	0.408 – 1. 609 **	53.30 – 73.38 **	ELISA kits

* The results shown in this column represent the participants who were younger than 3 years old even though some studies included also older participants.

** The study includes also participants older than 3 years old.

Estimation: The numerical values shown here are estimated from graphs.

2. MATERIALS AND METHODS

2.1 Objectives

The purpose of this study was to analyze distribution of plasma IGF-1 and IGFBP-3 concentrations among 6- to 30-month-old rural Malawian children. The results were compared to previous studies of IGF-1 and IGFBP-3 concentrations carried out by other research groups with different laboratory methods and children with different ages and ethnicity. The distribution of IGF-1 and IGFBP-3 concentrations were analyzed in three different age groups (6, 18 and 30 months) to see how concentrations vary within age. The study also assessed if maternal/child characteristics and environmental factors affect IGF-1 and IGFBP-3 concentrations. Maternal/child characteristics included maternal age, height, BMI, CRP, AGP and child gender. Environmental factors included household wealth, season, site of sample collection, source of drinking water and sanitation facility.

2.2 Study site and study population

This study is a sub-study using data and plasma samples that were collected from a randomized, partially blinded, parallel group controlled trial iLiNS-DYAD (International Lipid-Based Nutrient Supplements, mother-child dyads) in rural Malawi. 1391 pregnant women were enrolled. The purpose of the project was to determine how stunting among children could be prevented by using lipid-based nutrient supplements for a mother during pregnancy and lactation and for a baby during infancy. (42)

Enrollment of pregnant women happened at three public antenatal clinics before 20th week of pregnancy. Attendance percentage of antenatal clinic visits in rural Malawi is very high so it can be assumed that the study population of children represents typical rural Malawian children well. (43) The study site consists of the catchment areas of three health centres in Lungwena, Malindi and Mangochi, Mangochi district, Southern Malawi (44).

781 live-born Malawian children participated a follow-up from birth to 2.5 years of age. The blood samples were collected from children during three separate follow-up visits at the clinics. The samples were not available at each visit from every participant. (43) Valid IGF-1 concentration results were available from 1705 samples and valid IGFBP-3 concentration results were available from 1638 samples.

2.3 Data collection

The whole blood samples were collected at the study clinics and centrifuged at 3,000 RPM for 15 minutes and separated plasma was aliquoted into storage cryovials. The samples were first put at -20 °C freezers within 1 – 4 hours from sample collection. They were transported to -80 °C freezers within 48 hours. The samples were shipped with dry ice to Finland where laboratory analyses of IGF-1 and IGFBP-3 were performed. The samples were thawed and centrifuged prior to laboratory analyses. (43)

Information of maternal characteristics and environmental factors were collected with data collection forms during maternal enrollment and follow-up visits at the study clinics by trained professionals. Maternal height and weight were measured and biological samples were taken at the clinics.

2.4 Laboratory analyses

IGF-1 concentrations of the stored plasma samples were analyzed with MILLIPLEX® MAP HIGF-1, II Magnetic Bead Panel Kit. IGFBP-3 concentrations were analyzed with MILLIPLEX® MAP Human IGF Binding Magnetic Bead Panel kit. These are commercial kits by EMD Millipore (Merck Life Science Oy). The analysis was based on the LUMINEX xMAP® technology. (45)

Laboratory analyses of IGF-1

Frozen plasma samples were thawed completely at room temperature, then followed by vortexing and centrifuge to remove particulates. To separate IGF-1 from their binding proteins, the samples were extracted by combining 25 µl plasma and 100 µl Activation Buffer. They were mixed well, left at room temperature for 30 minutes and centrifuged for 5 minutes. 25 µl of supernatant was transferred into a microfuge tube and 15 µl of Neutralization Buffer was added and mixed well. Then 260 µl of Assay Buffer was added to the neutralized sample for a final volume of 300 µl. The samples were diluted to 1:60.

Reagents for the immunoassay were prepared according to the manufacture's manual. Antibody-immobilized beads were prepared by adding 150 µl of each antibody-bead vial and then assay buffer was added to reach the final volume of 3.0 ml. Quality control 1 and 2 were reconstituted

with 250 ml of deionized water. Wash buffer was prepared by mixing 60 ml of 10X Wash Buffer and 540 ml of deionized water. Then IGF-1 standard was prepared: at first Standard #7 and 250 µl of deionized water were mixed. Then Standard #6 was prepared by mixing 200 µl of Assay Buffer and 100 µl of Standard #7. The Standard #5 was prepared similarly but previously prepared Standard #6 was used in this case. The procedure continued with the similar logic to prepare all the Standards from #7 to #1. Then the Working Standards were ready. Assay Buffer was used as a background (the 0 pg/ml standard). (45)

A 96-well plate was used. 200 µl of Assay Buffer was added into each well of the plate. The plate was sealed and mixed for 10 minutes on a plate shaker at the room temperature (20 – 25 °C). Assay Buffer was decanted and removed from all the wells by inverting the plate and tapping it onto absorbent paper towels. 25 µl of Standard or Control were added to appropriate wells according to the well map. 25 µl of Assay Buffer was added to background and sample wells. Then 25 µl of Assay Buffer was added to background, standards, and control wells. 25 µl of extracted and diluted plasma samples were added to appropriate wells. 25 µl of beads were added to each well. The plate was sealed, wrapped with foil and incubated with agitation for 17 hours at 4 °C. After the incubation the plate was set on a magnetic plate washer for 60 seconds so that magnetic beads were able to settle. The well contents were removed by inverting and tapping the plate. The magnetic plate was removed, 200 µl of Wash Buffer was added to each well and the plate was shaken for 30 seconds. The magnet plate was attached again. After 60 seconds the well contents were removed like during the first wash. The plate washing was repeated 3 times. Then 50 µl of Detection Antibodies were added into each well and the plate was sealed, covered with foil and incubated with agitation for 1 hour at room temperature, then followed by adding 50 µl of the reporter molecule Streptavidin-Phycoerythrin to each well. The plate was sealed, covered with foil and incubated with agitation for 30 minutes at room temperature. The well plate was set on a magnetic plate and the plate was washed 3 times as described earlier. 100 µl of Sheath Fluid was added to all wells. The beads were resuspended on a plate shaker for 5 minutes. Then the plate was run on Bioplex-200 instrument with Bio-Plex Manager software (Bio-Rad). The Median Fluorescent Intensity data using a 5-parameter logistic method for calculating IGF-1 concentration in samples was saved and analyzed. The final concentrations were multiplied by the dilution factor. (45)

Laboratory analyses of IGFBP-3

Laboratory analyses of IGFBP-3 were conducted with a similar procedure according to the instructions provided by the manufacturer (45, 46). The test kit included necessary fluids that were used in the analyses. Standard 7 was prepared by using the Human IGFBP-3 standard. (46) Other names of the reagents were similar compared to IGF-1 test kit (45, 46).

Laboratory analyses of maternal CRP and AGP

Maternal CRP and AGP at enrollment were analyzed from plasma samples by immunoturbidimetry on the Cobas Integra 400 system autoanalyzer (F. Hoffmann-La Roche Ltd, Basel, Switzerland) in US.

2.5 Statistical analyses

The data was analyzed using IBM SPSS Statistics version 25. Spearman's correlation was used for numerical variables which were not normally distributed (IGF-1, IGFBP-3, CRP, AGP, height, BMI, maternal age, household wealth). Correlation was significant at the 0,05 level (2-tailed) unless otherwise specified in further sections. The distributions of IGF-1 and IGFBP-3 concentrations were analyzed among different age groups by using independent samples Kruskal-Wallis test. Mann-Whitney U-test was used when comparing the results between girls and boys at different age groups and when comparing the results between dry and rainy season. Kruskal-Wallis test was used when association between child plasma IGF-1 concentration and a categorical variable (the site of sample collection, source of drinking water, sanitation facility) was analyzed. Similar method was used in analyses of plasma IGFBP-3 concentrations and a categorical variable.

2.6 Ethical aspects

The data and plasma samples were collected as a part of iLiNS-DYAD project. Ethical aspects have been considered in TEKES research plan: "The controlled trial was performed according to Good Clinical Practice guidelines (ICH-GCP) and it adhered to the principles of Helsinki declaration and regulatory guidelines in Malawi. The trial protocol was discussed and approved prior to the onset of data collection by ethics committees in Malawi (College of Medicine) and Finland (Pirkanmaa

Hospital District). All participants were given appropriate information on the studies and they all signed an informed consent form before enrolment. Individual identifiers have been removed from the study databases and hence the suggested secondary analyses pose no major ethical questions." (43)

3. RESULTS

3.1 Study subjects, maternal characteristics and environmental factors

Mean child ages at sample collection were 6.39 (SD 1.19, n = 519) months, 18.14 (SD 0.34, n = 605) months and 30.07 (SD 0.20, n = 581) months. Maternal data at time of enrollment were used in the analyses of this study. The maternal characteristics are represented in Table 2. The wealth of household was evaluated with an asset index which is based on asset ownership of certain things and environmental factors (for example sanitation facilities) (47). Thus, evaluation of household wealth was not based on household income. Distributions of the sites of sample collection, sources of drinking water and sanitation facilities are represented in Tables 3, 4 and 5, respectively.

Table 2. Maternal characteristics and household wealth at enrollment.

	N	Mean ± SD
Maternal age (years)	686	25.1 ± 6.0
Maternal height (cm)	686	156 ± 5.6
Maternal BMI (kg/m^2)	685	21.9 ± 2.7
Household wealth	685	-0.075 ± 0.95
Maternal CRP (mg/l)	674	8.29 ± 17.0
Maternal AGP (g/l)	674	0.71 ± 0.25

N: total number, SD: standard deviation, BMI: body mass index, CRP: C-reactive protein, AGP: alpha-1-acid glycoprotein

Table 3. The distributions of the sites of sample collection.

	N	%
Lungwena	357	51.4
Malindi	127	18.3
Mangochi	204	29.4
Missing	7	1.0

N: total number, %: percentage of the study population

Table 4. The distributions of the source of drinking water among the households of the study population.

	N	%
Piped water	100	14.4
Borehole	518	74.6
Protected well	15	2.2
Unprotected well	22	3.2
Lake	35	5.0
River, pond	3	0.4
Not specified / missing	1	0.1

N: total number, %: percent of the study population

Table 5. The distributions of the sanitation facility among the households of the study population.

	N	%
None	32	4.6
Regular pit latrine	597	86.0
Ventilated improved pit latrine	54	7.8
Water closet	10	1.4
Not specified	1	0.1

N: total number, %: percentage of the study population

3.2 IGF-1 and IGFBP-3 concentrations

Plasma IGF-1 concentrations at ages of 6, 18, and 30 months are shown in Table 6. The distribution of IGF-1 concentration was the same across categories of child age ($p = 0.097$).

Table 6. Plasma concentrations of IGF-1 at 6-, 18- and 30-month-old Malawian children.

Child age (months)	N	Mean ± SD (ng/ml)	95 % CI
6	519	13.10 ± 8.23	12.40 - 13.80
18	605	12.47 ± 7.62	11.87 - 13.07
30	581	14.54 ± 10.8	13.67 - 15.41

IGF-1 : insulin-like growth factor 1, N: total number, SD: standard deviation, CI: confidence interval

Plasma IGFBP-3 concentrations at ages of 6, 18, and 30 months are shown in Table 7. The distribution of IGFBP-3 concentration was the same across categories of child age ($p = 0.347$).

Table 7. Plasma concentrations of IGFBP-3 at 6-, 18- and 30-month-old Malawian children.

Child age (months)	N	Mean ± SD (ng/ml)	95 % CI
6	474	2643 ± 808.0	2570 - 2717
18	584	2676 ± 719.5	2618 - 2734
30	580	2652 ± 747.1	2592 - 2712

IGFBP-3: insulin-like growth factor binding protein 3, N: total number, SD: standard deviation, CI: confidence interval

IGF-1 and IGFBP-3 concentrations among boys and girls

Mean IGF-1 and IGFBP-3 concentrations among 6-, 18- and 30-month-old boys and girls are shown in Table 8. The difference in IGF-1 concentrations between 6-month-old boys and girls was not significant ($p = 0.574$). However, the differences were significant between boys and girls at age of 18 months ($p \leq 0.001$) and at age of 30 months ($p \leq 0.001$). IGF-1 concentrations were higher among girls than boys at ages of 18 and 30 months though the trend was also similar at age of 6 months.

IGFBP-3 concentrations were higher among girls than boys and the difference was statistically significant in all age groups ($p = 0.03$, $p \leq 0.001$ and $p \leq 0.001$ at ages of 6, 18, and 30 months, respectively).

Table 8. Plasma concentrations of IGF-1 and IGFBP-3 among boys and girls at 6-, 18- and 30-month-old Malawian children.

		IGF-1		IGFBP-3	
Child age (months)	Sex of child	Mean ± SD (ng/ml)	95 % CI	Mean ± SD (ng/ml)	95 % CI
6	Boy	12.97 ± 8.67	11.88 - 14.05	2542 ± 748	2441 - 2642
	Girl	13.10 ± 7.65	12.20 - 14.00	2731 ± 853	2624 - 2837
18	Boy	11.29 ± 7.19	10.48 - 12.11	2513 ± 646	2437 - 2588
	Girl	13.52 ± 7.90	12.64 - 14.39	2834 ± 754	2749 - 2919
30	Boy	12.55 ± 10.16	11.37 - 13.73	2470 ± 688	2391 - 2550
	Girl	16.36 ± 11.03	15.12 - 17.60	2821 ± 761	2736 - 2907

IGF-1: insulin-like growth factor 1, IGFBP-3: insulin-like growth factor binding protein 3, SD: standard deviation, CI: confidence interval

3.3 Correlations between maternal characteristics, environmental factors and IGF-1 concentrations

Correlations between IGF-1 concentrations and maternal characteristics are shown in Table 9. Maternal height and IGF-1 concentrations were associated in all age groups. Maternal body mass index (BMI) and maternal AGP concentrations were associated with IGF-1 concentrations at age of 6 months, wealth of household with IGF-1 at age of 30 months. Maternal age and CRP concentrations were not associated with IGF-1 concentrations in any age group.

Table 9. Correlation coefficients between maternal characteristics and IGF-1 concentrations at 6-, 18- and 30-month-old Malawian children.

	6 months		18 months		30 months	
	Coefficient	P value	Coefficient	P value	Coefficient	P value
Maternal age	0.41	0.345	0.009	0.826	0.040	0.330
Maternal height	0.150	0.001	0.112	0.005	0.119	0.004
Maternal BMI	0.123	0.005	0.011	0.787	-0.011	0.784
Household wealth	0.044	0.316	0.062	0.122	0.134	0.001
Maternal CRP	0.047	0.284	0.041	0.310	0.038	0.359
Marernal AGP	-0.124	0.005	0.076	0.062	0.032	0.437

IGF-1: insulin-like growth factor 1, BMI: body mass index, CRP: C-reactive protein, AGP: alpha-1-acid glycoprotein

IGF-1 concentrations during dry season (May to October) and rainy season (November to April) are shown in Table 10. IGF-1 concentrations were higher among the samples collected during dry season compared to the samples collected during rainy season at ages of 6 and 18 months ($p \leq 0.001$ and $p = 0.003$, respectively). Though the trend was similar, the differences in IGF-1 concentrations were not statistically significant between the samples collected during dry season and rainy season at age of 30 months ($p = 0.062$).

Table 10. Plasma concentrations of IGF-1 among 6-, 18- and 30-month-old Malawian children whose samples were collected during dry and rainy season.

Child age (months)	Season	Mean ± SD (ng/ml)	95 % CI
6	Dry	14.72 ± 8.81	13.61 - 15.84
	Rainy	11.71 ± 7.45	10.85 - 12.58
18	Dry	13.40 ± 8.13	12.49 - 14.31
	Rainy	11.56 ± 6.99	10.79 - 12.34
30	Dry	15.53 ± 11.55	14.20 - 16.86
	Rainy	13.59 ± 9.96	12.46 - 14.72

IGF-1: insulin-like growth factor 1, SD: standard deviation, CI: confidence interval

IGF-1 concentrations were similar across the site of sample collection and the source of drinking water at ages of 6, 18 and 30 months ($p = 0.746$, $p = 0.855$ and $p = 0.074$ for the site of sample collection; $p = 0.492$, $p = 0.116$ and $p = 0.055$ for the source of drinking water, respectively).

IGF-1 concentrations were similar across the sanitation facilities at ages of 6 and 30 months ($p = 0.559$ and $p = 0.066$, respectively) but the difference was statistically significant at age of 18 months ($p = 0.007$). Statistically significant results of pairwise comparison are shown in Table 11. IGF-1 concentrations were higher among those who have access to water closet compared to those who use regular pit latrine, ventilated improved pit latrine or have no sanitation facilities.

Table 11. The results of selected comparisons and adjusted significances. IGF-1 concentrations were compared across the sanitation facilities among 18-month-old Malawian children.

Compared sanitation facilities	Adjusted significance*
Regular pit latrine - water closet	0.004
None - water closet	0.031
Vent. impr. pit latrine - water closet	0.024

*Significance values have been adjusted by the Bonferroni correction for multiple tests. Vent. impr. pit latrine: ventilated improved pit latrine

3.4 Correlations between maternal characteristics, environmental factors and IGFBP-3 concentrations

Correlations between IGFBP-3 concentrations and maternal characteristics are shown in Table 12. There were no association either between IGFBP-3 concentrations and maternal BMI. Maternal height was associated with IGFBP-3 concentrations in all age groups. Wealth of household was associated with IGFBP-3 concentrations at ages of 6 and 30 months, and maternal AGP was negatively associated with IGFBP-3 concentrations at ages of 6 and 18 months. Maternal age and CRP were not associated with IGFBP-3 concentrations in any age group.

Table 12. Correlations between maternal characteristics and IGFBP-3 concentrations at 6-, 18- and 30-month-old Malawian children.

	6 months		18 months		30 months	
	Coefficient	**P value**	**Coefficient**	**P value**	**Coefficient**	**P value**
Maternal age	0.058	0.210	-0.008	0.848	0.036	0.386
Maternal height	0.119	0.010	0.104	0.011	0.118	0.004
Maternal BMI	0.037	0.426	0.033	0.418	-0.029	0.479
Household wealth	0.127	0.006	0.052	0.207	0.154	≤ 0.001
Maternal CRP	-0.047	0.312	-0.014	0.734	-0.004	0.916
Maternal AGP	-0.160	0.001	-0.090	0.030	-0.076	0.069

IGFBP-3: insulin-like growth factor binding protein 3, BMI: body mass index, CRP: C-reactive protein, AGP: alpha-1-acid glycoprotein

IGFBP-3 concentrations during dry season and rainy season are shown in Table 13. IGFBP-3 concentrations at ages of 18 and 30 months were higher among the samples collected during dry season compared to rainy season ($p \leq 0.001$ and $p = 0.023$, respectively), but not at age of 6 months ($p = 0.175$).

Table 13. Plasma concentrations of IGFBP-3 among 6-, 18- and 30-month-old Malawian children whose samples were collected during dry and rainy season.

Child age (months)	Season	Mean ± SD (ng/ml)	95 % CI
6	Dry	2619 ± 587	2538 - 2700
	Rainy	2663 ± 946	2547 - 2778
18	Dry	2739 ± 630	2667 - 2811
	Rainy	2615 ± 793	2526 - 2705
30	Dry	2685 ± 673	2608 - 2763
	Rainy	2620 ± 813	2528 - 2712

IGFBP-3: insulin-like growth factor binding protein 3, SD: standard deviation, CI: confidence interval

The differences in IGFBP-3 concentrations across the site of sample collection were statistically significant at ages of 6 and 30 months ($p = 0.042$ and $p = 0.017$, respectively) but not at age of 18 months ($p = 0.536$). Adjusted significances at ages of 6 and 30 months are shown in Table 14. IGFBP-3 concentrations were higher in the samples collected in Mangochi compared to Lungwena at ages of 6 and 30 months.

Table 14. The results of comparisons and adjusted significances. IGFBP-3 concentrations were compared across the sites of sample collection among 6- and 30-month-old Malawian children.

Compared sites	Child age (months)	Adjusted significance*
Lungwena - Mangochi	6	0.042
	30	0.014
Lungwena - Malindi	6	0.519
	30	1.000
Malindi - Mangochi	6	1.000
	30	0.265

*Significance values have been adjusted by the Bonferroni correction for multiple tests.

The differences in IGFBP-3 concentration across the source of drinking water were statistically significant at ages of 6 and 30 months ($p = 0.018$ and $p = 0.005$, respectively) but not at age of 18 months ($p = 0.373$). In pairwise comparison the adjusted significances were not statistically significant at age of 6 months. The only statistically significant difference was that IGF-1 concentrations were higher among those who have access to piped water compared to those who have access to borehole at age of 30 months. Adjusted significances are shown in Table 15. If adjusted significance was 1.000 at both ages (6 and 30 months), those pairs are not presented.

Table 15. The results of selected comparisons and adjusted significances. IGFBP-3 concentrations were compared across the sources of drinking water among 6- and 30-month-old Malawian children.

Compared sources of drinking water	Child age (months)	Adjusted significance*
Protected well - piped water	6	0.403
	30	0.074
Protected well - lake	6	0.489
	30	1.000
Borehole - piped water	6	0.158
	30	0.004
Unprotected well - piped water	6	1.000
	30	0.377

*Significance values have been adjusted by the Bonferroni correction for multiple tests.

The distribution of IGFBP-3 concentration was the same across the sanitation facilities at ages of 6, 18 and 30 months (p = 0.922, p = 0.612 and p = 0.925, respectively).

3.5 Correlations between IGF-1 and IGFBP-3 concentrations

The correlations between IGF-1 and IGFBP-3 concentrations at 6-, 18- and 30-month-old Malawian children are shown in Table 16. Child plasma IGF-1 were associated with IGFBP-3 concentrations in all age groups.

Table 16. Correlation coefficients between plasma IGF-1 and IGFBP-3 concentrations at 6-, 18- and 30-month-old Malawian children.

	6 months		18 months		30 months	
	Coefficient	**P value**	**Coefficient**	**P value**	**Coefficient**	**P value**
Correlation	0.537	≤ 0,001	0.570	≤ 0,001	0.588	≤ 0,001

IGF-1: insulin-like growth factor 1, IGFBP-3: IGF binding protein 3

4. DISCUSSION

4.1 Main findings

This study investigated the distribution of IGF-1 and IGFBP-3 concentrations among 6-, 18- and 30-month-old Malawian children. The study also assessed if maternal/child characteristics and environmental factors affect IGF-1 and IGFBP-3 concentrations. There were no significant differences in IGF-1 and IGFBP-3 concentrations among 6-, 18- and 30-month-old Malawian children. However, we found that IGF-1 and IGFBP-3 concentrations were higher among girls compared to boys among almost all age groups except IGF-1 at 6-month-old infants. Higher maternal height was associated with higher child IGF-1 and IGFBP-3 concentrations in all age groups. The higher the household wealth, the higher the IGF-1 concentrations at child age of 30 months and the higher IGFBP-3 concentrations at child ages 6 and 30 months. The higher the mother's AGP concentration the lower IGF-1 concentration at age of 6 months. IGF-1 and IGFBP-3 concentrations were higher among the samples collected during dry season compared to the samples collected during rainy season. IGF-1 and IGFBP-3 concentrations were strongly correlated with each other among all age groups. IGF-1 concentrations at age of 18 months were higher among those who have access to water closet compared to those who have access to some other sanitation facility. IGFBP-3 concentrations at age of 30 months were higher among those who have access to piped water compared to those who have access to borehole. IGFBP-3 concentrations were different between the samples collected in Mangochi and Lungwena among 6- and 30-month-old children.

4.2 Limitations

IGF-1 and IGFBP-3 concentrations were measured only three times and the follow-up time was relatively short. Thus, it is not possible to get accurate information about IGF-1 and IGFBP-3 concentrations among other age groups. It should be also noticed that there are many factors that affect IGF-1 and IGFBP-3 concentrations and only some of them were included in this study. Environmental factors were relatively similar in the study population. Thus, statistically significant differences among environmental factors were found in a few groups.

4.3 Distribution of IGF-1 concentrations

Previous studies have found that IGF-1 concentrations increase during childhood (31, 34-36, 39). Some other studies have found that IGF-1 concentrations are relatively high at birth, decrease during the first months of life and then increase gradually during childhood (33, 38). The distribution of IGF-1 concentration was the same across categories of child age in this study. Thus, increase in IGF-1 concentrations was not statistically significant and increase in IGF-1 concentrations during childhood was not observed in our study. It is possible that the study population suffers from malnutrition which negatively affects growth and prevents normal increase in IGF-1 concentrations.

Exclusive breastfeeding is recommended during the first 6 months of life (48). If mothers have adhered to the recommendation it can be assumed that at 6 months of age children get majority of daily energy demand from breast milk and the role of other nutrition is less significant. This situation is different at ages of 18 and 30 months: nutritional status depends more on dairy products, amount of carbohydrates and quality of fatty acids (13). In addition, important time for linear growth is from 6 to 24 months of age. Energy demand is high during that time which increases risk of stunting. (8) The study population is from low-income settings where amount and quality of nutrition might be suboptimal. Since nutritional status affects IGF-1 concentrations (16, 49), poor nutritional status combined with important linear growth phase might prevent increase in IGF-1 concentrations between ages 6 and 30 months.

EED might also explain why IGF-1 concentrations did not increase significantly from 6 to 30 months. EED is more common in low- and middle-income countries (25), such as in Malawi (50). EED deteriorates nutrient absorption which might lead to energy deficiency and cause stunting (25). Among undernourished children IGF-1 concentrations are decreased (51). In addition, stunting and undernutrition are common in sub-Saharan Africa (4) which might be one explanation for IGF-1 concentrations in this study.

Mean IGF-1 concentrations in our study were lower than in those studies carried out in higher-income countries (33-35, 39, 40) where likelihoods of stunting and EED are lower (8) than in Malawi. Kouanda et al. had study population from Burkina Faso. In their study IGF-1 concentrations decreased from birth to 24 months of age and increased after that. (38) Burkina Faso and Malawi are both low-recourse settings which may play a role for stunting, nutrition, EED

and IGF-1 concentrations. On the other hand, IGF-1 concentrations among the study population in Burkina Faso were higher than IGF-1 concentrations in our study even though economic and environmental factors might have similarities between those countries. Also ethnicity affects IGF-1 concentration (41), possibly even when comparing the results of two African countries.

It should be also taken into account that different laboratory methods were used in the studies listed in Table 1. For example chemiluminescent assay was used in some studies (33, 34, 40) but none of the studies used EMD Millipore Human IGF-1 laboratory test kit. ELISA method was also used in a couple of studies (19, 31). Kouanda et al. used filter paper, radioimmunoassay and chromatography (38). Thus, comparing IGF-1 concentrations among different studies is not straightforward. To get comparable results the same method or similar test kits should be used.

IGF-1 concentrations were higher among the girls compared to boys. Our results are consistent with previous studies (31, 41). The difference between sexes is more significant during puberty (35, 40) but should be taken into account in infancy and childhood if IGF-1 results are used for example in some diagnostic procedures.

IGF-1 concentrations were higher among the samples collected during dry season compared to rainy season among all age groups. The difference was significant at ages of 6 and 18 months. It might be possible that amount and quality of nutrition is different during dry and rainy season which affects nutritional status and IGF-1 concentrations. Another possible reason is that children are more prone to have infections during rainy season which decreases IGF-1 concentrations.

4.4 Distribution of IGFBP-3 concentrations

The differences in mean IGFBP-3 concentrations were not statistically significant among three different age groups in this study. In some previous studies it has been noticed that IGFBP-3 levels increase from early childhood to puberty (36, 41). The increase in IGFBP-3 concentrations was not linear in this study. The differences in mean IGFBP-3 concentration were statistically significant between girls and boys among all age groups. Our results are inconsistent with a previous study conducted in Turkey. Yüksel et al. found no significant differences in IGFBP-3 concentrations between girls and boys. (31) Ethnicity affects IGFBP-3 concentrations (33) which might explain the inconsistency. Chaler et al. suggest that different reference values of IGF-1 and IGFBP-3 should be used for people with different ethnic background. In their study it was observed that numerical

values of IGF-1 and IGFBP-3 concentrations were different even when compared to another study with similar laboratory method but different ethnic study group. (41)

IGFBP-3 concentrations at ages of 18 and 30 months were higher among the samples collected during dry season compared to rainy season. It could be assumed that seasonal variations among IGF-1 and IGFBP-3 concentrations are dependent on each other. Thus, similar factors such as nutrition and infections might affect both biomarkers.

4.5 Correlations between IGF-1 and IGFBP-3

IGF-1 and IGFBP-3 concentrations were associated with each other in this study population. The finding can be explained by the biological functions: IGFBP-3 is the carrier protein of IGF-1 (11). On the other hand, association is moderate but not high. There might be several reasons for that: affinity of IGF-1 and IGFBP-3, other binding proteins of IGF-1 and inaccuracies of laboratory analyses might affect the concentrations. Yüksel et al. found weak correlations between IGF-1 and IGFBP-3 concentrations in some age groups among 0 – 6 years old Turkish children (31) but specific results about correlations were not defined in the paper.

4.6 Association between maternal characteristics, environmental factors and IGF-1, IGFBP-3 concentrations

It has been suggested that maternal inflammatory process could be the link between prenatal and antenatal stunting (8). Thus, maternal inflammatory process might affect child IGF-1 concentration. Our study did not find association between maternal CRP and child plasma IGF-1 concentration in any age group. Maternal CRP was measured at enrollment during pregnancy. CRP is an acute phase protein which reacts relatively fast (5) so the concentrations might vary depending on the day of sample collection. Measuring maternal CRP might not be a good indicator of long-term inflammatory processes which might have more significant and longer-lasting effects on child growth and IGF-1 concentrations. However, weak negative association was found between maternal AGP and child IGF-1 at age of 6 months. AGP concentrations change slowly compared to CRP (5) which might indicate that maternal inflammatory process has been more persistent and negatively affected child growth and IGF-1 concentrations.

The association between maternal height and child plasma IGF-1 concentration was noticed in this study. If maternal height is above the average it might be possible that also child's height is above

the average. Since IGF-1 is an important regulator of child growth (9) and IGF-1 concentrations are higher among the children whose height is above the average (52), the association between maternal height and child IGF-1 concentration can be understood. The association between maternal BMI and child plasma IGF-1 concentration was not significant. It should be taken into account that average BMI was 21.9 (SD 2.7) which is normal. The results might have been different if there were more underweight women whose children might be in higher risk of stunting. IGF-1 concentrations would be lower in that case.

Wealth of household might lead to better chances to get adequate nutrition for children. The educational level is usually higher among people living in wealthier households (8) which might be associated with better knowledge about nutrition and hygiene. Thus, advanced environmental factors such as clean drinking water and proper sanitation facilities might be associated with better child growth and higher IGF-1 concentrations. In this study population IGF-1 concentrations were associated with the wealth of household at age of 30 months. If nutrition and hygiene are better among wealthier households that might be seen in IGF-1 concentrations only when children are old enough and they have been exposed to environmental factors long enough.

The site of sample collection and the source of drinking water did not affect IGF-1 concentrations but these environmental factors affected IGFBP-3 concentrations in a few groups. IGFBP-3 concentrations from the samples collected in Mangochi were higher compared to Lungwena when the concentrations were compared at ages of 6 and 30 months. It is not clear why the results were different between these two sites and two age groups. All three sites are rural so it could be assumed that the living environments are similar. It is also assumed that maternal enrollment in the study was similar among the three sites.

The source of drinking water affected the distribution of IGFBP-3 concentration at age of 30 months when borehole and piped water were compared. Some differences were also seen when piped water and protected or unprotected well were compared but in these cases the differences were not statistically significant. It might be possible that access to piped water is associated with better hygiene, fewer infections, better growth and higher IGFBP-3 concentrations compared to those who do not have access to piped water.

The difference in the distribution of IGF-1 concentration across the sanitation facilities was statistically significant at age of 18 months. In pairwise comparison the other sanitation facility was water closet when the difference was statistically significant. Thus, it could be assumed that access

to water closet has similar effects as access to piped water. These factors might explain higher IGF-1 concentrations among those who have access to water closet. On the other hand, access to water closet did not affect IGFBP-3 concentrations in any age group.

In conclusion, IGF-1 and IGFBP-3 concentrations have some similar patterns among rural Malawian children compared to previous studies but numerical values of concentrations vary between different study groups and laboratory methods. Comparing the results of different research groups is not straightforward when the methods used are different. It should be also noticed that ethnic backgrounds of the populations are probably different which may play a role in the results of IGF-1 and IGFBP-3 concentrations. Thus, the reference values should be measured among similar population and with similar laboratory method to get comparable results which could be used for example in some diagnostic procedures. In the future, IGF-1 could be one marker in monitoring child's risk of stunting and EED in low- and middle-income countries. The method of measuring IGF-1 should be practical and feasible in low-recourse settings. The role of maternal and environmental factors should be taken into account when evaluating the results.

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