Association of maternal plasma total cysteine and growth among infants in Nepal: A cohort study

Nikhil Arora

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Philosophy in Global Health at the University of Bergen.

Centre for International Health

Department of Global Public Health and Primary Care

Faculty of Medicine

University of Bergen, Norway

2020

Acknowledgements

I feel very grateful to have known a great team of researchers at the Centre for Intervention Science in Maternal and Child Health and been given the opportunity to work on variables from VitaPreg project as a part for my master's thesis.

There are many people that have contributed to make this work possible, and I wish to express my deepest gratitude to all of them:

My deepest respect for, and many thanks to the participating mothers (from early gestation periods) and their infants in this extensive project and contributing with valuable information.

First and foremost I wish to express my sincere appreciation to my main supervisor, Postdoctoral fellow Catherine Schwinger. She convincingly guided and encouraged me to be professional and do the right thing even when the road got tough. She has been the source of enormous statistical knowledge with great humor and kindness to me. Without her persistent help, the goal of this study would not have been realized. I am deeply grateful for having the opportunity to work with you, Catherine. Thank you!

Furthermore, I would like to thank Professor Tor A. Strand for your wonderful co-supervision, for introducing me to the topic and for accepting me with open arms to work on your study variables. His true positive interest for the project, constructive feedback, optimism and support on the way has been of great value for me. My deepest gratitude Tor!

My deepest gratitude goes to every member of VitaPreg project based in Nepal who have made huge contribution by following up participants to collect and store the blood samples and variables.

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Important abbreviations

AA	Amino acid
AARR	Average annual rate of reduction
ANC	Antenatal care
BMI	Body mass index
CI	Confidence interval
EAA	Essential amino acid
GAM	Generalized additive model
HAZ	Height-for-age Z-score
IGF - 1	Insulin-like growth factor - 1
IQR	Inter-quartile range
IUGR	Intrauterine growth restriction
LAZ	Length-for-age Z-score
LBW	Low birth weight
NAC	N-acetyl cysteine
NEAA	Non-essential amino acid
RCT	Randomized controlled trial
SAM	Severe acute malnutrition
SD	Standard deviation
SDG	Sustainable development goal
SES	Socioeconomic status
tCys	Plasma total cysteine
UN	United Nations
UNICEF	United Nations International Children's Emergency Fund
VLBW	Very low birth weight
WHA	World Health Assembly
WHZ	Weight-for-height Z-score
WLZ	Weight-for-length Z-score

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1. Introduction

Growth and development are pivotal outcomes for children. Child growth and development reflect an image of the individual's health status [1]. Moreover, it is also recognized as an indicator for health system performance, nutritional status and health in populations [1, 2]. The periods before pregnancy, during pregnancy and early childhood are suggested to be critical for growth and development [3], having profound influence on the individual's later development [4-6]. Sub-optimal child growth is associated with an increased risk of morbidities and mortality [7, 8], thus having a huge impact on the development of society and countries as a whole. Today, malnutrition constitutes a considerable part of the burden of disease in the world, especially affecting low- and middle-income countries [9, 10].

Malnutrition is one of the common risk factors for many leading causes of deaths among the under-5 children, contributing to more than 45% of deaths among them [11]. The UN sustainable development goals (SDGs) were negotiated internationally keeping in view the interests of low- and middle-income countries at the full-term of the Millennium Development Goals in 2015 with an aim to transform the world. SDG 2 *"to end hunger, achieve food security and improved nutrition, and promote sustainable agriculture"* was adopted recognizing the importance of nutrition for optimal growth and development, with target 2.2 as *"to end all forms of malnutrition"* by 2030 [12]. Further, this emphasis was taken forward by the UN Decade of Action on Nutrition 2016-2025, adopted by UN member states with a commitment to undertake 10 years of sustained and coherent implementation of policies, programs and increased investments to achieve SDG target 2.2, everywhere, leaving no one behind [13].

This introduction includes a section on malnutrition, describing common indices used to assess its various forms along with outlining their related consequences. To complete this section, the burden of malnutrition is described and an overview over important determinants of malnutrition with help of a conceptual framework for

optimal growth is presented. In section 1.2, the importance of amino acids is summarized concentering focus on cysteine, its metabolic pathways and its role during the periods of pregnancy and child growth. In this thesis, the use of term 'child growth' and its related concepts are restricted to children under 5 years of age.

1.1 Malnutrition

Malnutrition can be defined as a physical condition in which the body does not receive adequate amount of nutrients for its proper functioning [14]. It can be result of an improper diet, or from physical incapability to assimilate or metabolize nutrients [15]. Malnutrition manifests itself in many forms. One is 'undernutrition' – which includes stunting (low height-for-age), wasting (low weight-for-height), underweight (low weight-for-age) and micronutrient deficiencies or insufficiencies. The other is overweight and obesity. Suboptimal diet is a common cause of malnutrition across all its forms [16]. In this thesis, I use the terms 'malnutrition' and 'undernutrition' interchangeably and do not consider overweight and obesity.

1.1.1 Assessment of malnutrition and its related consequences

Stunting

The indices used to describe stunting are based on length-for-age (for birth to 2 years) or height-for-age (for 2 to 5 years) [2, 17]. Children are defined as stunted if their length/height is at least two standard deviations lower than the average for their age in a reference distribution. Stunting is commonly classified as moderate when Z-score is ≤ -2 and severe when the Z-score is ≤ -3. It is described as a form of growth failure which develops over a long period of time in children when grown with limited access to food, health and care. Thus, it is also referred as 'chronic malnutrition' [9]. However, the process of stunting can start immediately after acute nutrient stress, with physical signs of stunting becoming noticeable several months later [18].

Stunting is often found to be associated with short term consequences impacting on the child's health and long term consequences such as delayed mental development, poor school performance, and reduced intellectual capacity, which in turn affects economic productivity [4-6]. Stunted mothers are at greater risk for obstetric complications because of a smaller pelvis, increasing the risk of perinatal and neonatal deaths [1]. Small statured/malnourished mothers are at greater risk of delivering infants with low birth weight, contributing to the intergenerational cycle of stunting, as infants born with low birth weight to malnourished mothers tend to be smaller/malnourished as adults who later become small statured/malnourished mothers to stunted infants [1, 19].

Wasting

The nutritional indices commonly used to describe wasting are based on weight-for-length (for children < 2 years) or weight-for-height (for children 2 to 5 years) [2, 17]. Children are defined as wasted if their weight-for-length/height is at least two standard deviations below a reference distribution. Wasting is commonly classified as moderate when Z-score is ≤ -2 and severe when the Z-score is ≤ -3. It is characterized by a rapid deterioration in nutritional status over a short period of time which can be due to acute food shortages or disease. Thus, it is also referred as 'acute malnutrition' [1, 9]. However, studies have shown that both moderate and severe wasting can last for several months/years if untreated [20, 21].

Wasting often jeopardizes the immune system performance and can trail to increased severity, duration and receptivity to infectious diseases [1]. These infections potentially can result in loss of appetite, thus confirming a cyclical relationship between infection and wasting, further increasing vulnerability to death [22].

Evidence found in the studies by Golden [18], Garenne *et al.* [20] and Isanaka *et al.* [21] do not relate wasting and stunting to the usual meaning of terms "acute" and "chronic", respectively. Moreover, the co-occurrence of wasting and stunting in the

same child is also identified in many children across the world. Thus, the terms 'acute' and 'chronic' malnutrition for wasting and stunting respectively, have recently been argued to be misleading [8].

Underweight

The index used to define underweight is based on weight-for-age [2, 17]. However, weight-for-age Z-scores are not able to differentiate between wasting and stunting [23]. Children are defined as underweight if their weight is at least two standard deviations below a reference distribution of the same age and sex. The mortality risk is evinced to be high in mildly underweight children and even higher in severely underweight children [1].

Birth weight has been another commonly used index. Low birth weight (LBW) has been defined as weight at birth < 2500 grams (5.5 pounds), regardless of gestational age. Further, birth weight < 1500 grams is categorized as very low birth weight (VLBW) and < 1000 grams as extremely low birth weight (ELBW) [24]. LBW is closely related with fetal and neonatal mortality and morbidity, suppressed cognitive growth and development, and chronic diseases in adulthood [1].

Micronutrient deficiencies

Micronutrient deficiency is a suboptimal nutritional status, developed as a result of inadequate dietary intake or absorption, or higher physiological needs of one or more vitamins and/or minerals [9]. Micronutrient status is examined by biochemical assessment of blood/urine sample(s) in the laboratory or by clinical examination [1]. Although any individual can experience micronutrient deficiency, pregnant women and children are at high risk because pregnancy and childhood development often increase the demand for specific vitamins and/or minerals [25].

Deficiency of micronutrients may cause poor physical and mental development in children, vulnerability or exacerbation of a disease, mental retardation, blindness and

general loss in productivity and potential [25]. Keeping these human consequences in mind, communities worldwide have focused on several micronutrients including iron, zinc, vitamin A, folate and iodine, as these may be difficult to satisfy without diverse diets [26-29]. However, the reviews by Ramakrishnan *et al.* [27] and Mayo-Wilson *et al.* [29] have found small positive effects of zinc supplementation only on wasting status in children under 5 years and on linear growth in children aged 6 months to 12 years, respectively.

1.1.2 Prevalence of malnutrition

Malnutrition is a universal problem holding back development with inadmissible human consequences. According to the latest reports, 149.0 million children under 5 years are stunted (21.9% of under-5 children) and 49.5 million are wasted (7.3% of under-5 children) worldwide [10]. Moreover, there is evidence shown by Richard *et al.* [30] and Schoenbuchner *et al.* [31] that children who are wasted are more likely to become stunted and children who are stunted are more likely to become wasted. Globally, 15.95 million children under 5 years (3.62% of under-5 children) are both stunted and wasted [32]. Annually, 20.5 million babies are born low birth weight [10] and more than 2 billion people suffer from micronutrient deficiency worldwide [9].

The prevalence of stunting and wasting among children under 5 years is found to be the highest in South Asia (33.3% and 15.3%, respectively) [9, 32]. In the 1990s, Nepal had some of the highest levels of undernutrition globally, with almost two-thirds of under-5 children being stunted. The prevalence of stunting among under-5 children in Nepal has markedly decreased, from 57% in 1996 to 36% in 2016, indicating decline of 14%, 16% and 12% between the periods 2001-2006, 2006-2011 and 2011-2016, respectively [33]. However, the decline in the prevalence of wasting during the same period was minimal, with 10% of children under-5 being wasted according to Nepal Demographic and Health Surveys (NDHS) – 2016 [33].

During states of deficit in caloric intake, different physiological adjustments take place in the body by utilizing body's nutritional reserves, mainly fat and muscle stores, to ensure adequate fuel supply for survival [34]. Wasting and stunting if untreated, are associated with a decrease in muscle and fat mass, which if severe, compromise the adequate fuel supply to vital organs of the body and further lead to death [8]. Estimates have shown that among the 5.3 million under-5 deaths annually [10], over one million deaths are attributable to stunting and approx. 800 000 deaths to wasting [35, 36]. The risk of death increases as a child becomes more wasted and the same is true for stunting. Results from a meta-analysis done by McDonald *et al.* [7] showed 1.5, 2.3 and 2.5 times increased risk of mortality being stunted, wasted and underweight, respectively, compared to the group of children without deficits. Severe wasting and severe stunting carried a 12 times and 5 times higher risk of death respectively, compared to non-wasted or stunted child [36]. It also concluded that risk of mortality increases significantly even further if two or more anthropometric deficits are present in same child [7].

The World Health Assembly (WHA) Global Nutrition Targets 2025 endorsed by the world's governments, including one to reduce the number of stunted children under 5 years by 40% (i.e. to reduce the number to 100 million) by 2025 and another to reduce and maintain childhood wasting to less than 5% along with reducing number of infants born with weight lower than 2500 grams by 30% by 2025, have been enshrined within SDG 2, target 2.2 [37, 38]. Despite the efforts, there has been some progress in reducing malnutrition, but it has been too slow to meet the WHA targets set for 2025 and the SGD targets set for 2030. According to reports, the required average annual rate of reduction (AARR) of stunting is 3.9% for reaching the WHA target by 2025 [39] compared to 2.2%, the current AARR [10]. The global prevalence for wasting was 7.3% in 2018, compared to 7.9% in 2012, indicating negligible progress towards the 5% WHA target by 2025 [10]. Thus, in aggregate, the global burden of malnutrition has been unsatisfactorily high, and progress unsatisfactorily low.

1.1.3 Determinants of malnutrition

In 1991, UNICEF first published a conceptual framework summarizing the main determinants of malnutrition, which describes them on three different levels emphasizing the multi-sectoral nature of malnutrition problem. The three levels include – immediate, underlying and basic causes. The framework was adapted further in the Lancet series 2013, with addition of possible interventions at various levels [5] (Figure 1). On the immediate level, inadequate dietary intake and disease burden are recognized as the main determinants of malnutrition. These are described as results of underlying causes, which are grouped as access to food (food security), feeding and care giving resources, and access to health care and a healthy environment. Further, these underlying causes are influenced by basic causes of malnutrition, which relate to wider political, social, economic and cultural constructs [5, 40].
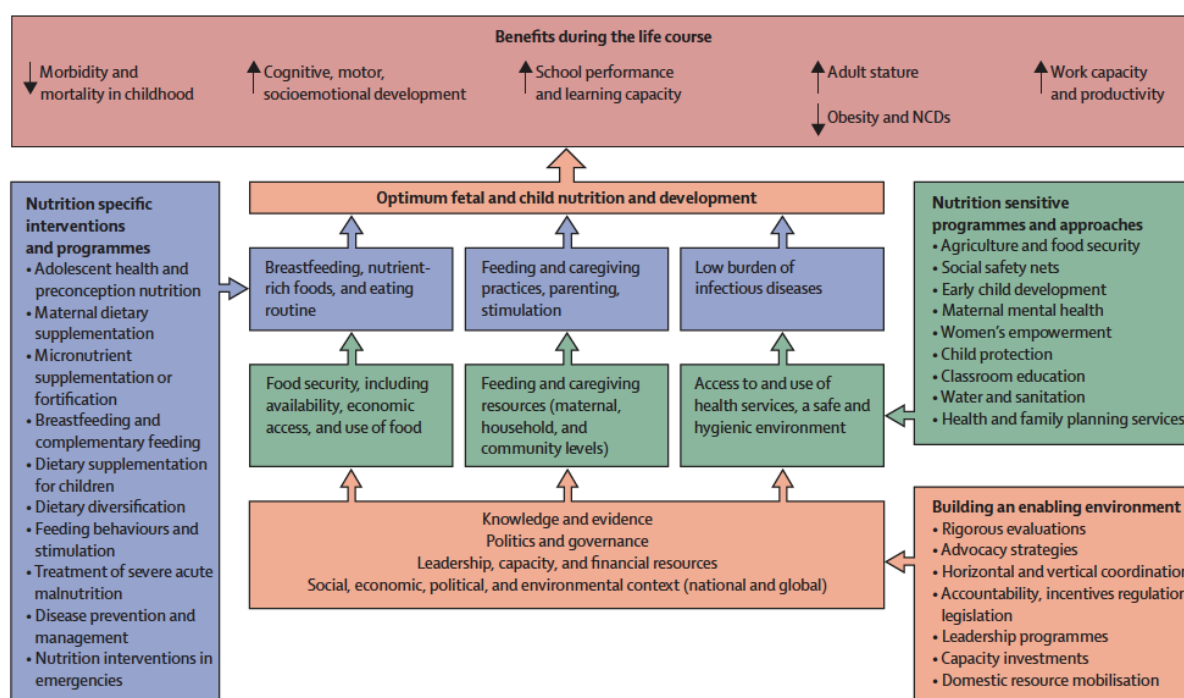

Figure 1: Conceptual framework for determinants of child malnutrition, reproduced from Black *et al.* [5] with permission from Elsevier

Factors that directly contribute towards stunted growth and development include poor maternal health and nutrition, inadequate infant and young child feeding

practices, and infections. The maternal specific factors contributing to stunting include intrauterine growth restriction (IUGR) and small stature due to maternal undernutrition along with short birth spacing and adolescent pregnancy [41-43]. Wasting is usually a direct result of a combination of infection and nutritionally inadequate diets with the main underlying causes include – poor access to appropriate, timely and affordable health care, inadequate caring and feeding practices, lack of food security both in terms of food quantity and diversity, and lack of a sanitary environment [22].

It is noted that the process underlying wasting and stunting involves multiple common risk factors and the interactions between these risk factors can change over time [44]. Many of the determinants of wasting and stunting are the same [45], but they may vary in combination and magnitude, leading to different trends [44]. In addition, findings showed that periods of being wasted, or having fluctuating weight, increase the risk of becoming stunted later [30, 31]. Other findings indicate that during a period of being treated for severe wasting, child growth in height slows down until their weight has recovered [46]. This pattern was also described in relation to seasonality [44]. However, causal pathways for a direct link between wasting and stunting have not yet been fully understood [8].

An analysis examining the drivers of maternal and child nutrition success from the mid-1990s to 2010s in Nepal by Cunningham *et al.* [47], showed that the success is result of various nutrition-specific and nutrition-sensitive interventions. The improvements in length-for-age Z-score (LAZ) among children under 2 years in Nepal have been sustained over the entire 1996-2011 period and were associated with 4 or more antenatal care (ANC) visits, child born in health facility and child being vaccinated, followed by an asset index, maternal education and toilet use. The improvements of weight-for-height Z-score (WHZ) for children under 5 years were not rapid during 1996-2011, where toilet use was a much more important factor,

followed by 4 or more ANC visits, child born in health facility, child being vaccinated and the asset index [47].

Evidence from several studies has led to the agreement that most stunting happens during the first 1000 days - starting from conception up to the 2nd birthday of the child [3, 5], although stunting can still happen after the first two years of life [48]. Moreover, a significant proportion (20-30%) of wasting and stunting is found to originate in utero, emphasizing the importance of maternal health before and during pregnancy for the prevention of wasting and stunting [44]. This accentuates that the nutritional status of pregnant women is not only relevant for her own health, but also has important consequences for the growth and development of her fetus/child.

1.2 Amino acids

A diverse diet is a key to meet the requirements for all necessary nutrients, especially among vulnerable populations such as growing children and pregnant women [25]. Macronutrients, which include carbohydrates, proteins and fats, are consumed in large but varied proportions across different populations. Since cereal, rice and cassava (poor in protein content) are less expensive than food commodities rich in proteins (like pulses, beans, meat, fish, dairy, nuts), poorer households tend to usually have a more monotonous cereal and/or rice based diet lacking dietary diversity required to meet adequate requirements [49, 50]. Research has been carried out to explore the role of macronutrients and its precursors in child growth. High quality protein intake has been shown to promote child growth with a suggested pathway via insulin-like growth factor - 1 (IGF - 1) production [51] and stunting has been reported as a manifestation of protein deficiency [52(p.512), 53].

Amino acids (AAs) are the building blocks of proteins. During the anabolic process of growth, there is a net deposition of proteins, despite an increased rate of both protein synthesis and breakdown. Large quantities of broken-down proteins get reused for its own synthesis. This process is not completely efficient, which makes AAs in diet

crucial [54]. Moreover, deficiency of AAs has found to suppress cell and organismal growth via the mechanistic target of rapamycin complex 1 (mTORC1) sensing pathway [55].

AAs have traditionally been categorized as nutritionally essential AAs (EAAs) or non-essential AAs (NEAAs) in humans. EAAs are defined as either those AAs whose carbon skeletons cannot be synthesized *de novo* in humans or those that normally are insufficiently synthesized *de novo* relative to its various needs for growth in humans. In contrast, NEAAs are those AAs which are produced *de novo* in adequate amounts to meet the various requirements in humans and which do not need to be provided in the diet [56]. Some NEAAs are considered conditionally essential in specific situations like in preterm infants, during periods of rapid growth [57], and in states of catabolic stress [58-60], thus considering them as semi-EAAs.

With the categorization based on nitrogen balance and protein synthesis, listed EAAs are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. NEAAs are listed as alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine, taurine, and tyrosine [56, 61]. It is reported that cysteine [57, 62, 63], tyrosine [63], arginine and taurine [54, 64] are semi-EAAs in infants. It is also found that infants while being in the period of rapid growth require greater amounts of EAAs than healthy adults [65].

Wu *et al.* [66] argued that AAs should be classified as EAAs or NEAAs not only based on the nitrogen balance, but that functional needs for AAs should be included as a major criterion. Until lately, the concept of 'nutritional non-essentiality' and an incomplete understanding of AA biochemistry, nutrition, and physiology has led to the ignorance of the importance of NEAAs in the practice of nutrition [56]. This argument by Wu *et al.* [66] found support from evidence shown in healthy adults by Meléndez-Hevia *et al.* [67] and in preterm infants by Sevastiadou *et al.* [68], proving that endogenous synthesis of NEAAs was insufficient to meet physiological needs.

This new nutritional concept of functional AAs, which is defined as those AAs that participate in and regulate key metabolic pathways in humans [69], has led to the recognition of dietary essentiality of nutritional NEAAs [70].

1.2.1 Cysteine

Cysteine is a proteinogenic sulfur-containing AA. The presence of sulfur makes cysteine to form disulfide linkages, which in turn control protein structure and stability [71]. Free (non-protein bound) cysteine in plasma often exists in homogeneous (cystine) or mixed disulfide (homocysteine-cysteine) forms. Plasma cysteine measures are often reported as total cysteine which refers to all circulating forms including free, disulfide, and albumin-bound cysteine [72]. The cysteine pool is a function of dietary intake, protein turnover, and endogenous synthesis. Like other AAs, cysteine is found in foods having high protein content i.e. beef, fish, poultry, lentils, dairy and nuts. The recommended daily intake of cysteine in adults is 4.1 mg/kg body weight [73].

Cysteine, although classified as NEAA as it is synthesized by recycling from methionine and serine, is considered to be conditionally EAA for infants [57, 59, 60]. Cysteine is synthesized by transsulfuration from homocysteine, a product of the essential sulfur AA, methionine. The first transsulfuration reaction is catalyzed by *cystathionine β-synthase* where homocysteine condenses with serine to form cystathionine, which is further cleaved by *cystathionase* (or *cystathionine γ-lyase*), releasing cysteine (Figure 2) [56, 71]. Because of the biochemical immaturity of the enzyme *cystathionase* in fetal liver tissues [57, 62], cysteine is considered conditionally EAA in infants. The activity of *cystathionase* is found to increase postnatally [74].

Cysteine plays several important functions in the body. Besides its involvement in protein synthesis, it is a component of glutathione (having antioxidant properties) and a precursor of taurine and sulfate. Because of the antioxidative, anti-inflammatory, mucolytic and anti-apoptotic effects, N-acetyl cysteine (NAC) - a supplement of cysteine, is being used for the treatment of polycystic ovary syndrome (PCOS), chronic

bronchitis, ulcerative colitis, asthma, neurodegenerative disorders (Alzheimer and Parkinson disease), and as a prophylactic to prevent premature birth and recurrent pregnancy loss [75]. NAC evinced to improve placental functions in various animal studies by upregulating placental antioxidant activity and placental growth factors, thus preventing placental oxidative stress [76-78]. NAC supplementation showcased to ameliorate IUGR in guinea pigs [76] and cadmium-induced fetal growth restriction in mice [77]. Also, a recent study demonstrated longitudinal bone growth in mice through upregulation of IGF – 1 after supplementing cysteine [79].
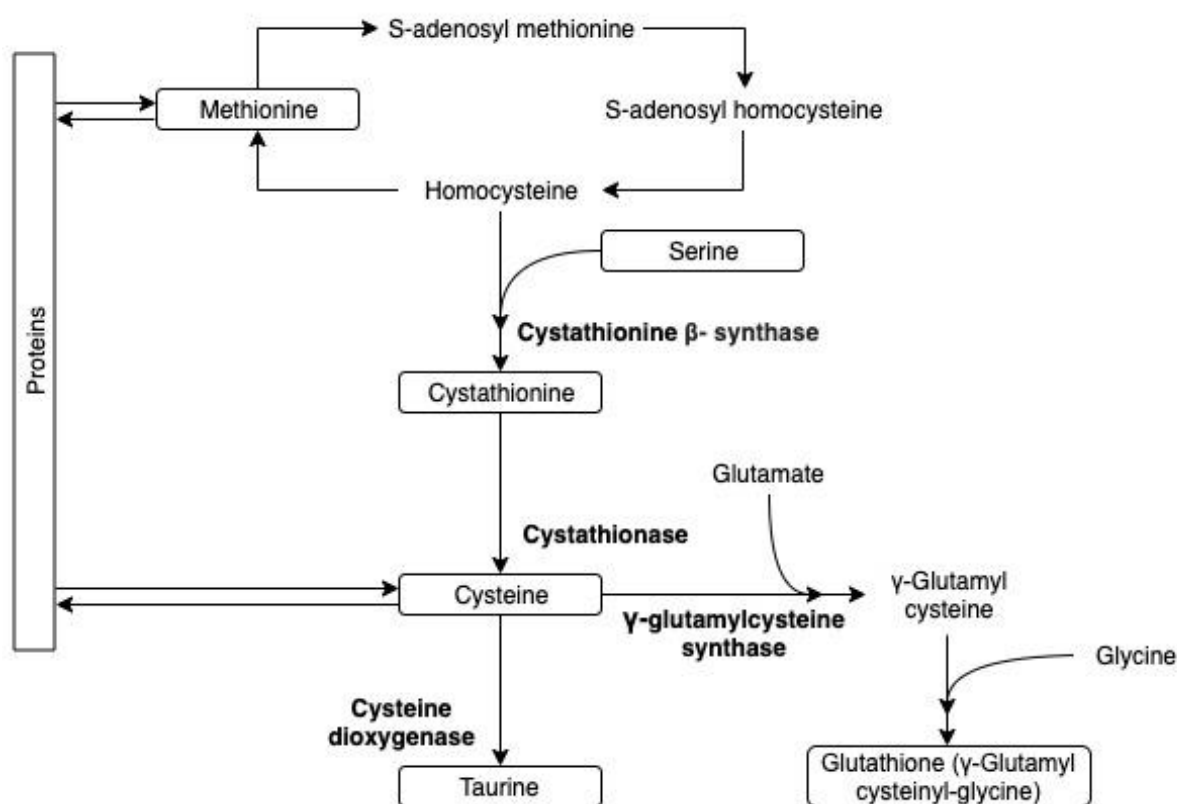
Figure 2: Cysteine - metabolic pathways[1]

Since a long time, there has been a controversy regarding the use of cysteine as a supplement for premature newborn infants. Snyderman [80] showed that

[1] Adapted from:

1. Brosnan, J.T.; Brosnan, M.E. Sulfur-Containing Amino Acids: An Overview. *The Journal of Nutrition* **2006**, 136, 1636S-1640S, doi:10.1093/jn/136.6.1636S.

2. Elshorbagy, A.K.; Smith, A.D.; Kozich, V.; Refsum, H. Cysteine and Obesity. *Obesity* **2012**, 20, 473, doi:10.1038/oby.2011.93.

supplementing cysteine enterally has positive effects on nitrogen retention and weight gain in preterm infants. Pohlandt [81] argued for adding cysteine as a supplement in preterm infants receiving total parenteral nutrition by showing that plasma half-cysteine concentrations did not increase when adequate methionine was provided, whereas Zlotkin and team casted a doubt on the essential nature of cysteine by observing that infants who received cysteine supplemented to total parenteral nutrition failed to retain nitrogen and to gain weight better than those who had not received it [82]. Some studies challenged the notion of cysteine being non-essential, and supported the findings of Snyderman [80] and Pohlandt [81], indicating very limited endogenous synthesis of cysteine from methionine, showing five times lower level of plasma cysteine concentrations [83] and higher plasma cystathionine concentrations [84] in preterm infants than in term infants.

On the contrary, a study by Malloy *et al.* [85] supported the results shown by Zlotkin and team. Later, some studies also came up with the same findings as Zlotkin and team, using different techniques to determine the requirements of cysteine in LBW preterm infants older than 32 weeks of gestation [86, 87]. Also, a study assessing the cysteine synthesis in VLBW neonates [88], concluded that cysteine is probably not a conditionally EAA in these infants providing their methionine intake is adequate. Studies have also highlighted the presence of *cystathionase* activity in extrahepatic tissues (kidneys and adrenals) [74], whose activity by the second trimester is reported to reach two-thirds the levels of the mature controls [89]. This strengthens even further the evidence provided by Zlotkin and team and suggests that the activity of *cystathionase* is sufficient to produce cysteine even in preterm infants, if provided with adequate methionine [82].

In addition, a cysteine kinetics study showed a reduced cysteine production because of decreased protein breakdown in children with severe childhood undernutrition [59], and greater dietary cysteine requirements of children with severe acute

malnutrition (SAM), which thus commenced the argument to give cysteine as a supplement in children with SAM [60].

Küster *et al.* [90] observed low cysteine levels in mothers for infants born preterm, and demonstrated strong correlation between maternal cysteine concentration and cysteine (and glutathione) concentration in the offspring. Furthermore, because of structural similarity and metabolic linkage to homocysteine, cysteine is suspected to cause endothelial dysfunction and thus a risk factor for vascular diseases [91]. Plasma cysteine levels were found to strongly associate with cerebrovascular and peripheral vascular disease in a U-shaped manner, whereas there was a weak positive association of cysteine levels with coronary heart disease [91]. The potential endothelial dysfunction of cysteine is further speculated to provoke placental vascular dysfunction and thus causing pregnancy-related complications [92]. The findings by El-Khairy *et al.* [93] showcased that high maternal plasma cysteine levels were strongly associated with higher risks of pre-eclampsia, premature delivery and LBW. However, in this study plasma cysteine levels were measured years after the outcome assessment (i.e. pregnancy-related complications), making it difficult to rule out other factors affecting this association. Despite the dependence of growing fetus on placental circulation for its nutritional needs, the potential transgenerational influence of cysteine on growth in infants has not been studied considerably.

2. Rationale

The purpose of this research is to gain a better understanding of the growth of infants in relation to the maternal plasma levels of cysteine, which has not been studied extensively.

Stunting, a manifestation of chronic undernutrition, and wasting, a consequence of acute undernutrition, have a multidimensional origin and have an impact at the individual, community, and national levels in both short- and long-term [6, 94]. The recent success of tremendous decrease in the prevalence of stunting among children under 2 years in Nepal is the outcome of upturn in access to health services (particularly during pregnancy), coverage and use of toilets, levels of education (particularly among mothers) and wealth accumulation. However, the improvements in terms of adequate dietary intake are still not up to the mark [47].

Cunningham *et al.* [47] emphasized on the scope of improvements in infant and young child feeding practices in Nepal. There are still many gaps in knowledge regarding the causes of undernutrition, and understanding these gaps are important. The causes affecting nutritional status of women which are fundamental for her own health, but also closely related to child growth and development have not been geared on comprehensively.

Findings from recent trials on mice [77, 79], guinea pigs [76] and those from El-Khairy *et al.* [93], have raised speculations that transgenerational influence of cysteine can prove to be an important component answering some questions related to child malnutrition. Moreover, our piece of research is fortunate to have access to data where the maternal plasma levels of cysteine (exposure) is measured before the outcome assessment (i.e. LBW), which can address one of the limitation of the study by El-Khairy *et al.* [93].

Leroy *et al.* [48] found that 70% of the growth deficit among the under-5 children in low- and middle-income countries can be attributed to stunting occurring in the first 1000 days of life. Practices of poor mother's nutrition before and during the gestational period are still prevalent in many low- and middle-income countries and these have not given importance to the extent needed. Similarly, the importance of the maternal plasma levels of cysteine and its relation to growth in infants is not taken into consideration until now. Today, a substantial increase in efforts will be required to break the global status of inertia in terms of the prevalence of stunting and wasting, and to lower the rate in the direction of WHA targets by 2025 [9, 10]. Thus, studying the association between the maternal plasma levels of cysteine and growth in infants can prove to be suggestive of an important relationship between them, which if taken into consideration, can help the low- and middle-income countries overcome the prevailing burden related to stunting and wasting, and its associated consequences.

3. Aim and objectives

3.1 Aim

To gain a better understanding of the growth of infants in relation to maternal plasma levels of cysteine.

3.2 Specific objectives

a) To study the association between plasma total cysteine (tCys) concentration in pregnant women and birth weight;

b) To study the association between tCys concentration in pregnant women and post-natal linear growth (i.e. length-for-age Z-score) in children 6 months' postpartum; and

c) To study the association between tCys concentration in pregnant women and ponderal growth (i.e. weight-for-length Z-score) in children 6 months' postpartum.

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2   Association of maternal plasma total cysteine and
3   growth among infants in Nepal: A cohort study

4   **Nikhil Arora [1], Tor A. Strand [2,3], Ram K. Chandyo [4], Amany Elshorbagy [5,6], Laxman Shrestha [4], Per**
5   **M. Ueland [7], Manjeswori Ulak [2,4] and Catherine Schwinger [2,*]**

6   [1] Centre for International Health, Department of Global Public Health and Primary Care, University of
7   Bergen, 5020 Bergen, Norway; docnikhilarora@gmail.com
8   [2] Centre for Intervention Science in Maternal and Child Health, Centre for International Health, Department
9   of Global Public Health and Primary Care, University of Bergen, 5020 Bergen, Norway; tor.strand@uib.no
10   (T.A.S.); manjeswori@gmail.com (M.U.)
11   [3] Department of Research, Innlandet Hospital Trust, 2629 Lillehammer, Norway
12   [4] Department of Child Health, Institute of Medicine, Tribhuvan University, Kathmandu 44600, Nepal;
13   rkchandyo1@gmail.com (R.K.C.); laxmanshree12@gmail.com (L.S.)
14   [5] Department of Physiology, Faculty of Medicine, University of Alexandria, Alexandria 21131, Egypt;
15   amany.elshorbagy@alexmed.edu.eg
16   [6] Department of Pharmacology, University of Oxford, Oxford OX13QT, United Kingdom;
17   amany.elshorbagy@pharm.ox.ac.uk
18   [7] Department of Clinical Science, University of Bergen, 5020 Bergen, Norway; per.ueland@ikb.uib.no
19   * Correspondence: c.schwinger@uib.no

21   **Abstract:** Cysteine is a conditionally essential amino acid that has been positively associated with
22   growth in children. However, transgenerational effects remain unclear. The aim of this analysis was
23   to assess whether maternal plasma total cysteine (tCys) concentration is associated with various
24   growth indicators in infants living in peri-urban settings in Bhaktapur, Nepal. We used data from
25   the 561 mothers enrolled in an ongoing randomized controlled trial. We built linear regression
26   models to evaluate the association between maternal tCys and birth weight, length-for-age Z-scores
27   (LAZ) and weight-for-length Z-scores (WLZ) at birth and 6 months of age. Maternal tCys was
28   inversely associated with birth weight among boys after adjusting for confounders ($p < 0.05$). There
29   was a negative association between maternal tCys and LAZ at birth among boys ($p < 0.01$). No
30   associations between maternal tCys and WLZ at birth or WLZ/LAZ at 6 months of age were found
31   significant, although there was a trend for maternal tCys to be associated positively with WLZ at
32   birth among girls ($p < 0.10$). This is a first study evaluating transgenerational relation of tCys on
33   growth in infants. Further, larger and more comprehensive studies are needed to determine if and
34   how maternal tCys alters child growth.

37

38   1. Introduction

39   Malnutrition is a wide-spread health problem leading to profound short- and long-term
40   consequences for child growth and development [1-3], as well as for survival [4]. It is estimated that
41   more than 45% of all deaths globally among children under the age of 5 years have malnutrition as
42   an underlying risk factor, and it is a leading cause of the burden of disease in the world [5].
43   Evidence from several studies on fetal and child growth has shown the importance of the first
44   1000 days – starting from conception up to the 2nd year after birth [2, 6]. A significant proportion (20-
45   30%) of wasting and stunting is found to originate in utero [7]. These observations reinstated the fact

46 that nutritional status of pregnant women is not only relevant for her own health but also affects her
47 growing fetus/child.
48   Although there are many risk factors associated with malnutrition [2, 8-10], a diverse diet, rich
49 in all necessary nutrients is very important especially among vulnerable populations such as growing
50 children and pregnant women [11]. High quality protein intake has been shown to promote child
51 growth [12], possibly via enhancing insulin-like growth factor – 1 (IGF - 1) production [13].
52   Amino acids are the building blocks of proteins. Deficiency of amino acids was found to
53 suppress cell and organismal growth via the mechanistic target of rapamycin complex 1 (mTORC1)
54 sensing pathway [14]. Cysteine, a sulfur containing proteinogenic amino acid, controls structure and
55 stability of proteins [15] and is also the limiting precursor of the major intracellular antioxidant
56 glutathione (GSH) [16]. Cysteine is considered conditionally essential for newborns because of an
57 immaturity of the enzyme *cystathionase* (or *cystathionine γ-lyase*) which is required for the final step of
58 transsulfuration pathway in the recycling of methionine and serine to cysteine [17, 18].
59   N-acetyl cysteine (NAC) is a supplement of cysteine shown to improve placental functions in
60 various animal studies by upregulating placental antioxidant activity and placental growth factors,
61 thus preventing placental oxidative stress [19-21]. NAC supplementation ameliorated intrauterine
62 growth restriction in a study on guinea pigs [19] and cadmium-induced fetal growth restriction in a
63 study on mice [20]. The anti-oxidative properties of NAC has led to its use as prophylaxis to prevent
64 premature birth and recurrent pregnancy loss in pregnant women [22].
65   Plasma total cysteine (tCys) was associated positively with anthropometric status in a study
66 among 6-30 months old Indian children [23]. Küster *et al.* [24] demonstrated a strong correlation
67 between maternal cysteine concentration and cysteine (and GSH) concentration in the offspring. To
68 the best of our knowledge, there has been very little emphasis on transgenerational influence of tCys
69 on growth in infants. With this piece of research, we aim to assess how maternal tCys concentration
70 is associated with postnatal anthropometric status in infants in Bhaktapur, Nepal.

71 **2. Materials and Methods**

72 *2.1 Original study*

73   This is a secondary analysis of data from an ongoing randomized controlled trial (RCT)
74 registered at www.clinicaltrials.gov with ID NCT03071666, which is taking place in Bhaktapur,
75 Nepal. The trial aims to measure the effect of vitamin B_{12} supplementation during pregnancy and
76 postpartum on growth and neurodevelopment in early childhood. Details on the original study
77 procedures have already been published [25]. In brief, the trial will enroll 800 pregnant Nepalese
78 women (not later than 15 weeks of pregnancy) aged 20-40 years old and residing in Bhaktapur
79 municipality and surrounding areas. Exclusion criteria includes no informed consent, taking dietary
80 or multi-vitamin supplements containing vitamin B_{12}, known cases of any chronic disease under
81 treatment (such as tuberculosis, diabetes, hypertension, hypo or hyperthyroidism, pernicious anemia
82 and Crohn's disease) or current users of anticonvulsant drugs, severe anemia (hemoglobin
83 concentration < 7 g/dL), suffering from any condition that requires treatment with vitamin B_{12}, and
84 strict vegans. In addition to vitamin B_{12} (50 µg) or placebo, all pregnant women were also given folic
85 acid (0.4 mg) for the first 2 months of pregnancy followed by iron (60 mg elemental iron) and calcium
86 supplements (500 mg) until 45 days after delivery according to WHO guidelines.
87   Ethical approval for the trial was obtained from Nepal Health Research Council (NHRC;
88 registered number 253/2016) and the Regional Committee for Medical and Health Research Ethics of
89 Western Norway (REK vest; reference number 2016/1620). This study was conducted in accordance
90 with the Declaration of Helsinki.

91 *2.2 Laboratory assessment and anthropometric measurements*

92   Maternal blood samples (3 mL) were collected into vials containing ethylenediaminetetraacetic
93 acid (EDTA) as anticoagulant, at the time of enrollment into the trial. The plasma was centrifuged at
94 approximately 700 *g* at room temperature for 10 minutes, separated and transferred into storage vials,

95 and stored at -70° before analyses. Plasma total cysteine (tCys) concentration was measured using a
96 modified gas chromatography-mass spectrometry method based on ethyl chloroformate
97 derivatization [26]. Concentrations of plasma cobalamin (or vitamin B12) and plasma folate were
98 estimated by microbiological assays using a chloramphenicol-resistant strain of Lactobacillus casei
99 and colistin sulfate-resistant strain of Lactobacillus leichmannii, respectively [27, 28].

100 Birth weight of infants were measured by hospital staff and records were gathered.
101 Anthropometric measurements of the infants were taken in their homes at birth (or immediately after
102 birth) and at 6 months of age by the study staff. Length was measured according to standard
103 guidelines using portable board (Seca). Weight was measured with portable electronic scale (Seca)
104 that measures to the nearest 0.01 kg.

105 The study staff had received training before initiation of the trial and supervisors monitored all
106 fieldwork activities. The supervisors monitored 5% of measurements taken by field workers. Data
107 was double entered into a database and checked for consistency by a supervisor.

108 *2.3 Data management and analysis*

109 Statistical analyses were done using Stata 16.0 (Stata Corp. 2019, College Station, TX) and R
110 version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria). All analyses in the present
111 study were restricted to the group of 561 mother-infant dyads where maternal tCys concentration
112 and anthropometric measurements of their children at birth and 6 months of age were available.

113 Birth weights gathered from hospital records were in grams and used as such in our analyses.
114 Z-scores for length-for-age (LAZ) and weight-for-length (WLZ) for infants were calculated according
115 to WHO Child Growth Standards [29]. A WAMI-index was calculated to represent household
116 socioeconomic status (SES) using the indicators: water and sanitation access, household wealth
117 (assets), maternal education, and income. Calculations were adapted from Psaki *et al.* [30] with each
118 of the indicators equally contributing to the index. The WAMI-index is between 0 and 1 with a higher
119 index indicating a higher SES. Mean (SD; standard deviation) or median (IQR; inter-quartile range)
120 were calculated for continuous variables and proportions for categorical variables.

121 We built linear regression models in order to understand the association between maternal tCys
122 concentration (predictor variable) and birth weight or anthropometric status (LAZ and WLZ scores)
123 in infants at birth and at 6 months of age (predicted variables). Birth weight, LAZ, WLZ and maternal
124 tCys concentration were used as continuous variables. As there is no established cut-off for tCys, we
125 additionally categorized maternal tCys into < 25th percentile, 25th-75th percentile and > 75th percentile
126 and used this variable in separate models. We performed purposeful selection of covariates for
127 adjustment into our models [31]. The covariates that we checked for as our potential confounders in
128 each model include maternal age, BMI, parity, years of education, household SES, infant's gender,
129 maternal plasma cobalamin (or vitamin B12) and folate concentrations. First, univariate analyses of
130 each potential covariate were done, using the significance level of 0.25 as a screening criterion for
131 initial variable selection. Second, a multivariable analysis with the selected covariates for each
132 predicted variable was done. All covariates not significant at a level of 0.05 were taken out one by
133 one from the multivariable model and removed if the coefficient for predictor variable did not change
134 by ≥ 15%. Third, each of the covariates that had been screened out in step 1 were added back to the
135 models one by one and retained if it changed the coefficient of the predictor variable by ≥ 15%. Lastly,
136 each of the final models were checked for its interaction by gender. A p-value ≤ 0.05 was considered
137 statistically significant.

138 In addition, generalized additive model (GAM) analyses were performed to explore any non-
139 linear associations between maternal tCys and birth weight or anthropometric indices (LAZ and
140 WLZ) at birth and at 6 months of age. All confounders identified in the linear regression models were
141 adjusted for and values < 2.5th percentile and ≥ 97.5th percentile for maternal tCys were excluded to
142 avoid overfitting at the extremes.

143 **3. Results**

144   *3.1 Population characteristics*

145   Maternal characteristics were available for 561 enrolled mothers, and birth weight and
146   anthropometric measurements at birth (or immediately after birth) were available for 521 infants. The
147   data for the current analyses was used from the ongoing study, therefore anthropometric
148   measurements at 6 months were available for 376 infants (Figure S1). Demographic, nutritional and
149   socio-economic indices of the available mother-infant dyads are summarized in Table 1. On average,
150   mothers were 27.5 years old at enrollment, with 48.0% (269) mothers being nulliparous. The mean (±
151   SD) maternal tCys concentration was 207.3 (± 24.6) μmol/L. The median age (IQR) of infants at two
152   anthropometric assessments by study staff was 3 (2-5) days and 182 (181-184) days. For 18
153   observations, WLZ score at birth was outside the reference range of WHO Child Growth Standards.
154   There was improvement in mean LAZ and WLZ scores for infants at 6 months of age relative to
155   assessment done at birth.

156   Table 1: Characteristics of study population

Maternal characteristics (n = 561)	
Mean age (SD), *years*	27.5 (3.8)
Mean gestational age (SD) at enrollment by LMP[1], *weeks*	10.2 (3.0)
Mean weight (SD), *kgs*	55.3 (7.7)
Mean height (SD), *cms*	152.8 (5.3)
Mean BMI (SD), *kgs/m²*	23.7 (3.0)
Parity, *% (n)*	
0	48.0 (269)
≥1	52.0 (292)
Mean education (SD), *years*	11.0 (3.5)
Mean plasma cysteine levels (SD), *μmol/L*	207.3 (24.6)
Median plasma folate concentration (IQR), *nmol/L*	57.3 (33.0 - 76.4)
Mean plasma cobalamin concentration (SD), *pmol/L*	204.5 (78.5)
Mean WAMI-index score (SD)[2]	0.65 (0.14)
Infant characteristics (n = 521)[3]	
Gender, *% (n)*	
Male	53.6 (279)
Mean birth weight (SD) measured at hospital, *grams*	3009 (428)
Median age (IQR) for assessment at birth, *days*	3 (2 - 5)
Median age (IQR) for assessment at 6 months[4], *days*	182 (181 - 184)
Mean LAZ score (SD) at birth	- 0.85 (1.07)
Mean LAZ score (SD) at 6 months[4]	- 0.56 (0.90)
Mean WLZ score (SD) at birth[5]	- 0.80 (1.11)
Mean WLZ score (SD) at 6 months[4]	0.26 (1.03)

[1] Missing value of 1 observation thus n = 560

[2] Missing values of 7 observations for variables used in WAMI-index score calculations thus n = 554

[3] Infants reported as dropped-out = 40

[4] Out of 521 infants, variables at 6 months of age were available for 376 infants

[5] WLZ score at birth (or immediately after birth) for 18 observations were outside the reference range of WHO Child Growth Standards (n = 503)

BMI: Body mass index; IQR: Inter-quartile range; LAZ score: Length-for-age Z-score; LMP: Last menstrual period; SD: Standard deviation; WLZ score: Weight-for-length Z-score

157   *3.2 Maternal cysteine and infant growth*

158   Table 2 shows the association between maternal tCys concentration and the selected growth
159   indicators. For the birth weight model, the interaction between maternal tCys and gender was
160   significant (results not shown here). On sub-grouping by gender, maternal tCys concentration was

161 negatively associated with birth weight among boys [ß = -2.611, 95% CI: -4.547, -0.676] after adjusting
162 for relevant confounders and the interaction term (p < 0.05). The birth weight decreased on average
163 by approx. 105 grams among boys across the highest quartile compared to middle half of maternal
164 tCys concentration (Table S1). Maternal tCys concentration was also inversely associated with LAZ
165 score at birth [ß = -0.005, 95% CI: -0.009, -0.001] (p < 0.01). However, stratified analyses revealed that
166 latter association was significant among boys only (Table S2).

167 Table 2: Multivariate linear regression models for anthropometric measurements and maternal
168 plasma total cysteine (tCys) concentration (in μmol/L)

	n	Crude tCys Estimate (95% CI)	Adjusted tCys Estimate (95% CI)
Birth weight[1], *grams*	521	-1.072 (-2.579, 0.434)	-
Boys		-	-2.611 (-4.547, -0.676)*
Girls		-	0.502 (-1.792, 2.796)
LAZ score at birth	521	-0.005 (-0.009, -0.001)**	-0.005 (-0.009, -0.001)**
WLZ score at birth[2]	503	0.003 (-0.001, 0.007)	0.003 (-0.002, 0.007)
LAZ score at 6 months[3]	376	-0.002 (-0.006, 0.001)	-0.001 (-0.005, 0.003)
WLZ score at 6 months[4]	376	0.002 (-0.002, 0.006)	0.001 (-0.003, 0.005)

[1] Adjusted for maternal BMI, infant's gender and interaction between tCys and infant's gender

[2] Adjusted for maternal BMI, education and parity

[3] Adjusted for maternal BMI, parity, WAMI, plasma cobalamin and folate concentrations (n = 371 for adjusted tCys because of missing WAMI-index values)

[4] Adjusted for maternal BMI, parity and plasma cobalamin concentration

* p-value < 0.05

** p-value < 0.01

BMI: Body mass index; CI: Confidence interval; LAZ score: Length-for-age Z-score; WLZ score: Weight-for-length Z-score

169
170 Multivariable linear regression models did not show any significant associations between
171 maternal tCys concentration and WLZ score at birth [ß = 0.003, 95% CI: -0.002, 0.007] or LAZ score at
172 6 months of age [ß = -0.001, 95% CI: -0.005, 0.003] or WLZ score at 6 months of age [ß = 0.001, 95% CI:
173 -0.003, 0.005]. However, there was an indication for maternal tCys concentration to be positively
174 related with WLZ at birth among girls (p < 0.10) (Table S2). Results from multivariable linear
175 regression models using maternal tCys as categorical variable can be found in the supplementary
176 material (Table S1).
177 Figure 3 shows GAM plots for the relation between maternal tCys and various growth indicators
178 after restricting values < 2.5th percentile and ≥ 97.5th percentile for maternal tCys. The GAM plots
179 between maternal tCys and birth weight were sub-grouped among boys and girls, and the
180 associations appeared close to linear. The associations between maternal tCys and LAZ at birth or
181 WLZ at birth or WLZ at 6 months of age also appeared close to linear. An inverted U-shaped
182 relationship was suspected between maternal tCys and LAZ at 6 months of age that appeared to be
183 distributed symmetrically around the mean maternal tCys concentration.

184 **4. Discussion**

185 The current analyses were undertaken to elucidate whether maternal tCys can have an impact
186 on growth in infants until 6 months of age. In the past, there has been much debate regarding use of
187 cysteine in premature infants, but the effects of cysteine supplementation have been found to be
188 inconclusive [32-34]. A recent study done on 2102 children aged 6 – 30 months in New Delhi, India
189 showed that tCys was positively associated with height-for-age Z-score (HAZ) and weight-for-height
190 Z-score (WHZ) [23]. Although a correlation between maternal tCys concentration and cysteine

191   concentration in the offspring has been shown [24], it is not clear whether maternal tCys has any
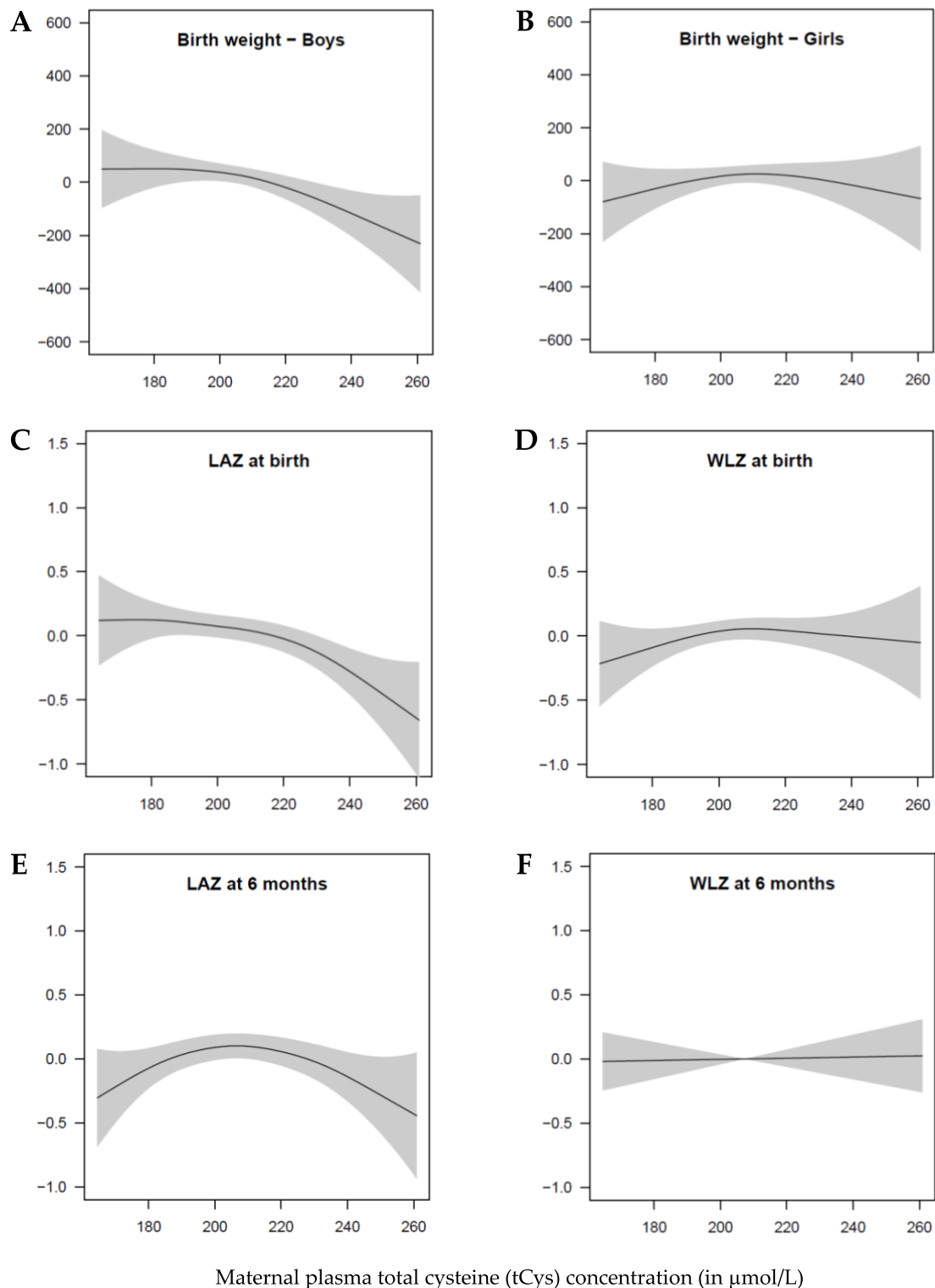192   influence on growth in infants.

Maternal plasma total cysteine (tCys) concentration (in µmol/L)

Figure 3: Generalized additive model (GAM) plots showing the relation of maternal plasma total cysteine (tCys) concentration (in µmol/L) with birth weight (in grams) sub-grouped among boys (A) and girls (B), length-for-age Z-score (LAZ) at birth (C), weight-for-length Z-score (WLZ) at birth (D), LAZ score at 6 months of age (E) and WLZ score at 6 months of age (F) after restricting values < 2.5th percentile and ≥ 97.5th percentile for maternal tCys. Values for all the growth indicators are centered around their respective median.

193 Our study indicated that maternal tCys was inversely associated with birth weight among boys
194 and LAZ at birth where each unit increase in maternal tCys (in μmol/L) the birth weight and LAZ at
195 birth decreased by 2.6 grams and 0.005 Z-scores, respectively. Also, across the highest quartile of
196 maternal tCys concentration, the birth weight decreased on average by > 100 grams among boys
197 compared to middle two quartiles. On stratification by gender, the latter association was found
198 significant among boys only. We did not find any statistically significant associations between
199 maternal tCys and WLZ at birth or LAZ at 6 months of age or WLZ at 6 months of age.

200 There is no established reference range for tCys concentrations in adults [35]. Although studies
201 in the past used different techniques to evaluate tCys [35-37], they all found cysteine to be the most
202 abundant aminothiol in healthy subjects, with total concentration approximately 250 μmol/L [38]. A
203 cross-sectional study involving 8585 healthy women and 7591 healthy men from three age groups in
204 the Hordaland county of western Norway has shown the mean (2.5-97.5 percentile) tCys
205 concentration to be 253.1 (202.1-317.1) μmol/L for women and 273.1 (218.6-338.4) μmol/L for men aged
206 40-42 years; 275.8 (215.4-347.2) μmol/L for women and 279.5 (225.6-332.8) μmol/L for men aged 43-64
207 years; and 296.3 (233.5-360.5) μmol/L for women and 296.4 (232.9-362.2) μmol/L for men aged 65-67
208 years [39]. In our study, we observed mean (± SD) tCys concentration in mothers enrolled to be 207.3
209 (± 24.6) μmol/L. This relatively lower tCys concentration might be due to higher utilization of cysteine
210 [40], hemodilution effect during pregnancy [41], potentially younger age of our enrolled population
211 relative to other studies [39] or low intake of animal products commonly seen among women of
212 reproductive age in Nepal [42].

213 Our study used data from an ongoing RCT where women in the intervention arm were given 50
214 μg of vitamin B_{12} [25]. The maternal blood samples for tCys evaluation in our analysis were taken at
215 enrollment of mothers into the original study and prior to supplementation with vitamin B_{12} or
216 placebo. Moreover, no human study so far has established evidence on the effect of vitamin B_{12}
217 supplementation during pregnancy or postpartum on growth outcomes in early childhood.
218 However, cobalamin (or vitamin B_{12}) and folate are important cofactors involved in the remethylation
219 of homocysteine to methionine, and their deficiencies are associated with elevated plasma
220 homocysteine levels and reduced transsulfuration [15]. Thus, both maternal plasma cobalamin and
221 folate concentrations were examined as potential confounders in our models.

222 *4.1 Maternal cysteine and birth weight*

223 In the study on newborns by Küster *et al.* [24], the mothers to infants born preterm were found
224 to have low cysteine levels, possibly because of a higher requirement of cysteine due to oxidative
225 stress associated with prematurity. In animal studies, supplementing NAC during pregnancy
226 prevented pregnancy related complications probably through its placental anti-oxidative effect [19-
227 21]. This is also one of the reasons for its use as a prophylaxis to prevent premature birth and recurrent
228 pregnancy loss in pregnant women [22]. Nonetheless, a large study by El-Khairy *et al.* [43] assessed
229 the outcomes of 14492 pregnancies among 5883 women in Norway and showed that high maternal
230 tCys concentration (tCys ≥ 304 μmol/L) was associated with higher risks of preeclampsia, premature
231 delivery, and very low birth weight, even though the maternal tCys was measured years after the
232 outcomes had occurred. Indeed, cysteine is speculated to provoke placental vascular dysfunction due
233 to its effect on endothelial function, which in turn causes various pregnancy related complications
234 [44]. Our findings predicted a significant association between maternal tCys and birth weight among
235 boys where with each unit of maternal tCys (in μmol/L) the birth weight decreased by 2.6 grams
236 despite low overall maternal tCys concentration in our study population. This difference in gender
237 has not been observed before and mechanism remains unclear.

238 *4.2 Maternal cysteine and linear growth*

239 Cysteine supplementation in mice resulted in increased growth plate thickness through
240 upregulation of IGF – 1 [45], thus making a probable effect of cysteine on linear growth convincing.
241 Despite the evidence from this animal study, the role of cysteine on linear growth in humans has not

242 been investigated. A positive association shown by Schwinger *et al.* [23] between tCys and HAZ in
243 children at 6 – 30 months of age did not take into account the possible influence of maternal tCys on
244 linear growth in infants at birth and thereafter. Ours is the first study to evaluate the association
245 between maternal tCys concentration and linear growth during infancy. Surprisingly, we found a
246 negative association between maternal tCys and linear growth during early infancy (i.e. at birth or
247 immediately after birth) among boys only. The mechanism underlying this association is not known,
248 and to our knowledge, no other studies have been published in infants.

249 *4.3 Maternal cysteine and ponderal growth*

250 Studies on cysteine in children with severe acute malnutrition suggested reduced cysteine
251 production [46] and greater dietary cysteine requirements [47] to combat for oxidative stress, poor
252 immune response and impaired gut function associated with severe acute malnutrition. Indeed, a
253 study in preterm infants showed positive effects of supplementing cysteine enterally on weight gain
254 [32]. A recent study by Schwinger *et al.* [23] found a positive association between tCys and WHZ in
255 children at 6 – 30 months of age. Moreover, human [48, 49] and rodent [50] studies exhibited that
256 tCys concentration was positively and independently associated with body fat mass and obesity.
257 Elevated cysteine was suggested to be a cause rather than a consequence of obesity, by promoting
258 lipogenesis, inhibiting lipolysis, decreasing energy expenditure and decreasing insulin sensitivity,
259 thus favouring lipid storage via unspecified pathways [51]. However, tCys in children aged 4 - 19
260 years was significantly associated with body fat only within an overweight/obese subgroup but not
261 in normal weight children [52]. None of these studies have taken into account the possible
262 transgenerational influence of tCys on ponderal growth in infants at birth and thereafter. Although
263 our study population sustains the concept of approximately linear relationship between maternal
264 tCys and WLZ at birth or 6 months of age, our findings fail to show any significant associations
265 between them.

266 *4.4 Limitations and strength*

267 Our analyses were based on the sample size of 521 and 376 infants at birth and 6 months of age,
268 respectively. These moderate sample size might have limited our statistical power to detect an
269 association. This piece of research is a prospective cohort study. Data comes from an RCT which
270 might limit the generalizability of our findings. Although we adjusted our models for several relevant
271 confounders, we still cannot exclude residual confounding. According to the recent reports by Nepal
272 Demographic and Health Surveys (NDHS) – 2016 , 13.5% and 15.2% of infants < 6 months were
273 stunted and wasted, respectively compared to 17.6% and 21.3%, respectively for infants 6-8 months
274 of age [53]. In our study population, 12.3% and 12.5% of infants were stunted and wasted,
275 respectively at birth (or immediately after birth) compared to only 5.3% and 1.9%, respectively at 6
276 months of age. This relatively lower prevalence of stunting and wasting in our population might be
277 because of peri-urban setting of our study. However, we do not expect the lower stunting and
278 wasting prevalence would have affected our findings.
279 All the pregnant women enrolled were given iron, folic acid and calcium supplements as per
280 WHO guidelines. We cannot exclude if these co-interventions would have affected our findings. In
281 addition, we limited our research by focusing on measures of maternal total plasma cysteine
282 concentration, and we did not take into account maternal dietary intakes into these analyses.
283 Compared to other studies mostly done in high income countries [35-37, 43], the tCys concentration
284 in our study population appeared to be on the low side. We cannot exclude that low tCys
285 concentration is an indicator of poor nutrition in general. The use of high-quality data collected under
286 supervision by trained field workers for our analyses, was one of the strengths of our study.

287 **5. Conclusions**

288 In conclusion, the Nepalese women with a high plasma total cysteine concentration had an
289 increased tendency of giving birth to low birth weight and short statured boys. The mechanism

behind these associations and difference based on gender remain undecipherable. Further, larger and more comprehensive studies with those involving detailed dietary nutrient intake evaluation are required to verify these findings in Nepalese and other populations.

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Annexes

1. Supplementary material

2. Original (VitaPreg) study protocol

3. Ethical clearance for original study (a, b)

4. Permission from Elsevier (for Figure 1)

5. Submission guidelines: *Nutrients* – Instructions for Authors

6. *Nutrients* Microsoft Word template file