



# Paediatric Nutrition

Edited by

Colin Binns and Mi Kyung Lee

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Colin Binns and Mi Kyung Lee (Eds.)

# Paediatric Nutrition



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# Editorial

## **An agenda for research into nutrients in paediatrics**

Food and nutrition has been central to human culture, philosophy and science since the beginning of civilisation. However the building blocks of food and nutrition, the nutrients, remained unknown until the late 19<sup>th</sup> century. Over the next 100 years advances in physics, chemistry and physiology led to rapid developments in our knowledge, first with development of an understanding of energy and the macronutrients, followed by the minerals and vitamins. The first vitamins to be explored scientifically were thiamine, vitamin D and C and in 1935 ascorbic acid was synthesised, beginning the 20<sup>th</sup> century rapid development of knowledge of nutrients[1].

Recommendations for intakes of nutrients were first made before chemical characterisation had begun in earnest and the long road to the establishment of formal nutrient recommendations has been described by Harper[2,3]. Recommendations on the intake of limes containing vitamin C to prevent scurvy began in the British Navy at the end of the 18<sup>th</sup> century. Since then times of crisis, mainly warfare, have stimulated further research into nutrients and improving the nutritional status (and hence fighting ability) of the population. Early recommendations for intakes were focussed on adult males, and children were simply not considered. After World War One and the onset of the Great Depression, the League of Nations established several commissions to investigate the provision of adequate nutrition for populations and the establishment of nutrient intake recommendations. In the 1930s the special needs of children and nursing mothers were considered for the first time in the recommendations of the British Committee on Nutrition [4]. This was followed by the reports of the League of Nations that included recommendations for nutrients during periods of growth that were extrapolations of adult requirements of the known macro and micronutrients[3]. In 1941 the Food and Nutrition Board of the National Academy of Sciences was established and the first edition of the Recommended Dietary Allowances was published. When commenting on this, Nutrition Reviews noted that the RDAs “emphasize once more the truth of the opinion that the dietary requirements can be met by a well-chosen diet of natural foods[5].” Since that time there have been numerous variations of the RDAs published by national and international organisations under a variety of titles. The cost of development and the

size of documentation has grown exponentially and current volumes of the US Dietary Reference Intakes occupy a whole shelf[6].

Setting nutrient requirements for children has required different approaches to setting the values for adults and it is only in the last 50 years that extra effort has been placed on establishing children's nutrient requirements. Nutrients cannot be simply extrapolated on the basis of weight from adults, but must provide for higher metabolic rates and energy expenditure, and growth and development. In the history of nutrients protein was regarded as the key to infant and child health. Low levels of protein were associated with poor growth and malnutrition (kwashiorkor) in children. Although growth is a major consideration for children, it only requires a small proportion of the total energy and protein requirements. In 1957 Hegsted concluded that "growth was a minor determinant of protein or other nutrient needs after the first months of life. The amount of new tissue protein deposited per day in growing children or during the adolescent growth spurt is very small compared with the total maintenance requirement of protein. This distinguishes humans and other primate species from common laboratory and domestic species[7]." More recently the role of early life protein intake in the laying the basis for later obesity through influencing insulin-like growth factor 1 (IGF-1) levels has been a focus of research[8,9]. There are important lessons to be learned from the exploration of protein needs of infants and children and the requirements follow a U-shaped curve. But it is even more complex as in real life there increased requirements associated with response to illness, injury and periods of rapid growth.

The establishment of iron requirements for infants and children and the development of interventions to overcome deficiencies has proved to be complex. Iron deficiency has been associated with poor growth, reduced cognitive development and ill health [10]. Yet breastmilk contains only a low level of iron, albeit in a readily bioavailable form [11]. Lactoferrin is important for transporting iron within the body, but is protective against infection by making iron unavailable to micro-organisms that require iron for growth[12]. All infant feeding guidelines recommend the introduction of complementary foods at around 6 months of age which provide increased amounts of iron. Recent attempts to increase iron supplies for children in developing countries by genetically modifying foods have not been entirely successful as they have resulted in increased rates of infection, including malaria[13-15]. This has led to a re-evaluation of how some nutrient requirements are set and deficiencies are met.

The current agenda for paediatric nutrients research will occupy nutritionists, biochemists, paediatricians and epidemiologists for many years ahead. Some of the immediate needs to be answered are to define the interactions and outcomes of nutrient levels with future health and disease beyond childhood, epigenetics and nutrients, interactions of nutrients with the human microbiome, sustainability and climate change, ethnicity gene interactions with nutrients.

The developmental origins of health and disease (DOHAD) hypothesis has added new emphasis to early life nutrition. How nutrition and growth influence later chronic disease has been the subject of many observational studies[16-18]. Future developments will probably rely on animal, perhaps primate models and the use of laboratory studies, as longer term prospective human studies are not feasible. The implications for setting of nutrient requirements may be far reaching – no longer is deficiency or short term growth the criteria, but long term life-course outcomes must be considered.

In recent years there has been considerable interest in the human microbiome and long term health outcomes following the initiative of the National Institutes of Health to sponsor research in this field[19]. It is well known that in the human body microbial cells outnumber human cells at least ten-fold. But what is not known is how they influence nutrient requirements, particularly in early life when the stable microbiome is being finalised. Malnutrition has an effect on the establishment of a stable microbiome[20]. Dysfunction of the microbiome has been linked to disorders as diverse as obesity, under-nutrition, diabetes and gastro-intestinal cancer[21-23]. It can be anticipated that nutrient- microbiome interaction will become an important area for nutrient research.

For infants the gold standard of nutrition and for nutrients is breastmilk. Many of the nutrients contained in breastmilk are in relatively low concentrations, but in highly bioavailable forms[24]. In the development of infant formula nutrients such as iron have to be included in greater concentrations than in breastmilk to adjust for the bioavailability[25]. Breastmilk is the most sustainable of infant foods in an era where resources, climate change and sustainability are paramount. A recent report of the Institute of Medicine explored current and emerging knowledge on nutrients in the light of the increasing environmental constraints on the food system[26]. This will continue to be an important area of research as climate affects different

aspects of nutrition, for example bioavailability. All of these emerging issues meant that it is likely that there will be further special issues of “Nutrients” devoted to paediatric concerns.

In this ‘Nutrients’ special collection we present a range of paediatric papers. The first section of reviews contains papers on two important topics. UNICEF estimates that more than 500000 children die every year of diarrhoeal disease, and most could be prevented or treated with relatively simple interventions[27]. The recent Global Burden of Disease study also confirms the continuing burden of mortality and morbidity from diarrhoeal disease in children[28]. The systematic review by Lamberti and colleagues confirms the value of zinc supplementation in the management of diarrhoea and endorses the current WHO recommendations.

Six of the papers relate to breastfeeding and there are several more on early infancy reflecting the importance of breastfeeding in early nutrition and in influencing life course nutrition. The paper by Imai et al joins a large number of observational studies that show an association between early nutrition and growth and later obesity. In this study infant feeding method and the early introduction of solids are associated with a higher BMI. The ethical impossibility of randomised controlled trials of breastfeeding and obesity means that we have to rely on the weight of observational studies and recognise that residual confounding may persist. Other papers document the optimal nutrients provided by breastmilk, supporting the continued promotion of breastfeeding. There are two papers that document the provision of nutrients from supplements in children, an area that will need continuing study to ensure that supplement use is appropriate. The special issue concludes with several papers on methodology in nutrient and body composition research.

The papers selected for this special collection illustrate the breadth of paediatric research, but there are still many scientific challenges remaining. There is still great potential for improvement in the health of children through nutrition and understanding of nutrient requirements, metabolism and social context is important to realising these potential health gains.

Dr. Mi Kyung Lee BSc MA PhD and Prof Colin Binns MBBS MPH PhD

*Guest Editors*

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## 1. General Review

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# Oral Zinc Supplementation for the Treatment of Acute Diarrhea in Children: A Systematic Review and Meta-Analysis

Laura M. Lamberti <sup>1</sup>, Christa L. Fischer Walker <sup>1,\*</sup>, Kit Y. Chan <sup>2,3</sup>, Wei-Yan Jian <sup>2</sup> and Robert E. Black <sup>1</sup>

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**Abstract:** Evidence supporting the impact of therapeutic zinc supplementation on the duration and severity of diarrhea among children under five is largely derived from studies conducted in South Asia. China experiences a substantial portion of the global burden of diarrhea, but the impact of zinc treatment among children under five has not been well documented by previously published systematic reviews on the topic. We therefore conducted a systematic literature review, which included an exhaustive search of the Chinese literature, in an effort to update previously published estimates of the effect of therapeutic zinc. We conducted systematic literature searches in various databases, including the China National Knowledge Infrastructure (CNKI), and abstracted relevant data from studies meeting our inclusion and exclusion criteria. We used STATA 12.0 to pool select outcomes and to generate estimates of percentage difference and relative risk comparing outcomes between zinc and control groups.

We identified 89 Chinese and 15 non-Chinese studies for the review, including studies in 10 countries from all WHO geographic regions, and analyzed a total of 18,822 diarrhea cases (9469 zinc and 9353 control). None of the included Chinese studies had previously been included in published pooled effect estimates. Chinese and non-Chinese studies reported the effect of therapeutic zinc supplementation on decreased episode duration, stool output, stool frequency, hospitalization duration and proportion of episodes lasting beyond three and seven days. Pooling Chinese and non-Chinese studies yielded an overall 26% (95% CI: 20%–32%) reduction in the estimated relative risk of diarrhea lasting beyond three days among zinc-treated children. Studies conducted in and outside China report reductions in morbidity as a result of oral therapeutic zinc supplementation for acute diarrhea among children under five years of age. The WHO recommendation for zinc treatment of diarrhea episodes should be supported in all low- and middle-income countries.

**Keywords:** zinc; children; global health; China

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## 1. Introduction

In response to mounting evidence supporting the efficacy and effectiveness of therapeutic zinc supplementation for diarrhea among children under five years of age, the World Health Organization (WHO) and the United Nation's Children Fund (UNICEF) issued a global recommendation in 2004, which advised zinc supplementation in addition to oral rehydration solution (ORS) for the treatment of all diarrhea episodes among children <5 years of age [1,2]. Systematic reviews have quantified the association between therapeutic zinc supplementation and a reduction in the duration and severity of childhood diarrhea episodes in low- and middle-income countries (LMICs) [1,3,4]. Many of the studies contributing to this body of evidence were conducted in South Asia [5–7], but literature stemming from East Asia has not been included in past reviews. In 2011, Zhang published a systematic review which identified 11 Chinese studies assessing zinc treatment for diarrhea and signified the need to update previous meta-analyses with literature published in languages other than English [8].

We sought to conduct an extensive search for studies of oral therapeutic zinc supplementation published in Chinese and any other language. We also aimed to combine evidence across regions in order to generate global estimates of the effect of oral therapeutic zinc supplementation on selected morbidity and mortality outcomes among children under five years of age.

## 2. Methods

We conducted a systematic literature search for studies published in any language between 1980 and November 2012 using the MeSH search terms “zinc” and “diarrhea” limited to “humans” in the following databases: Biosis, Cumulative Index to Nursing and Allied Health (CINAHL), Cochrane Central Register of Controlled Trials (CENTRAL), Embase, the WHO International

Clinical Trials Registry Platform (ICTRP), Global Health, Latin American and Caribbean Health Sciences Literature (LILACS), PubMed, Scopus, Web of Science, IndMed, Egyptian Universities Library Consortium, Index Medicus for the Eastern Mediterranean Region (IMEMR), China National Knowledge Infrastructure (CNKI), WanFang, and Chinese BioMedical (CBM) database.

Titles and abstracts were reviewed by two independent reviewers, and complete manuscripts were obtained for further review of pertinent studies. Discrepancies were resolved in consultation with a third reviewer. We restricted inclusion to individually randomized controlled trials (RCTs) of children under five years of age with acute diarrhea, including dysentery, where diarrhea was defined as the passage of at least three loose or watery stools in a 24-h period. We excluded cluster RCTs, studies that exclusively enrolled a particular subgroup of children (e.g., HIV-infected children; preterm infants), and studies of persistent diarrhea. We included RCTs assessing oral zinc supplementation of any zinc salt in comparison to a control group receiving placebo supplement. For studies conducted in China, where placebo supplements may not have been readily available, we included trials in which cases received the same supportive therapy regardless of zinc allocation. For all studies, administration of minerals (excluding iron), vitamins, and supporting therapy beyond zinc were only considered acceptable if these were received by both the intervention and control groups. Studies that used supplements that included iron, zinc-fortified ORS, or zinc-fortified foods were excluded.

Included studies were reviewed for the following outcomes: diarrhea duration; the proportion of diarrhea episodes lasting  $>3$  and  $>7$  days; duration of hospitalization; duration of fever; duration of vomiting; proportion of cases vomiting; stool frequency (number per day); stool output (mL); and death from diarrhea or any cause. Two independent reviewers entered data into structured tables, and discrepancies were resolved in consultation with a third reviewer.

We conducted independent analyses for studies assessing diarrhea due to unspecified causes and those assessing specific pathogens (e.g., rotavirus) that were laboratory confirmed prior to enrollment. All data analyses were conducted in STATA 12.0 [9]. We fit Poisson and logistic regression models to continuous and binary outcomes, respectively, weighting all outcomes by sample size. These models generated pooled estimates and 95% confidence intervals lower bound by zero for all outcomes and upper bound by one for proportions.

For continuous outcomes, we calculated the overall percentage difference between the pooled estimates for the zinc and control groups. For binary outcomes, we calculated estimates of relative risk (RR) with placebo as the reference group and conducted random effects meta-analyses to combine RRs across studies [9].

We conducted hypothesis testing to assess the equivalence of pooled outcomes and of effect estimates by placebo and non-placebo controlled trials. To compare effect estimates, we tested the difference of mean percentage differences for continuous outcomes and the ratio of relative risks (RRR) for binary outcomes [10]. We subsequently pooled placebo and non-placebo controlled trials for outcomes with no statistically significant difference in effect size.

We assessed the association between the dose of oral zinc supplement and diarrhea duration by regressing the mean percentage difference in diarrhea duration comparing the zinc and control

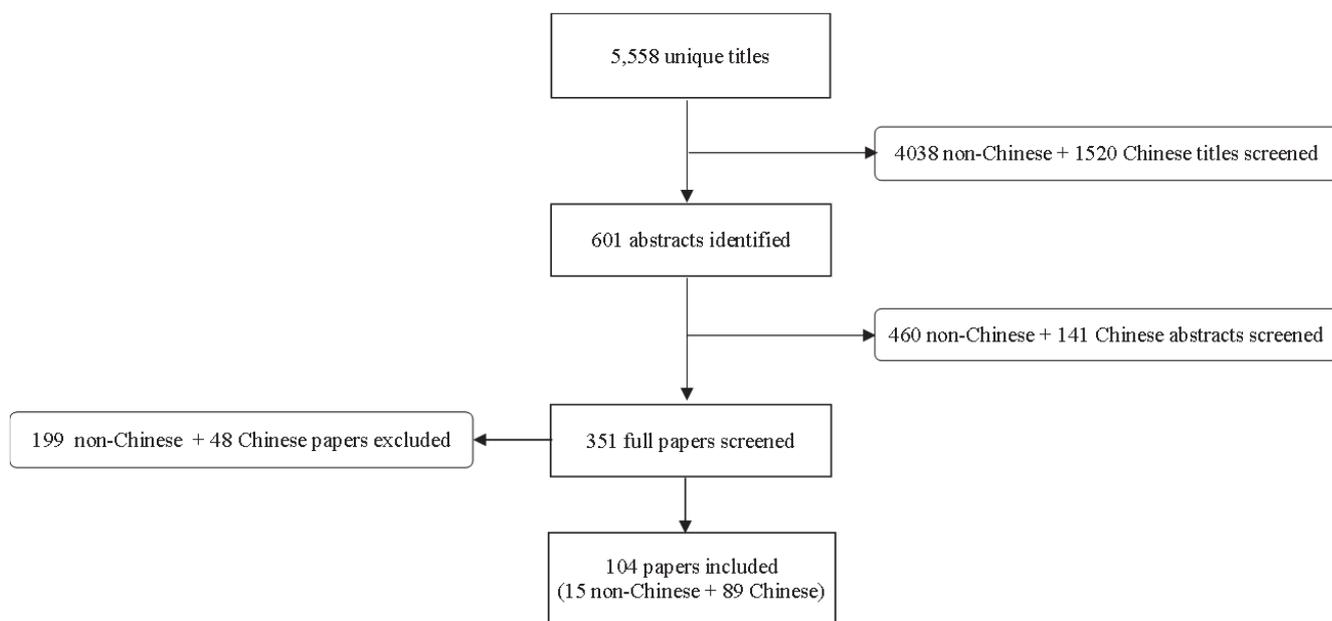
groups onto a categorical variable which indicated whether zinc dose was lower than, equal to, or greater than the WHO recommendation.

During the course of our analyses, we identified a zinc product called Licorzinc that appeared to be unique to China. To determine whether outcomes for Chinese studies were generalizable comparing Licorzinc to other better established zinc products, we conducted hypothesis testing to assess the equivalence of the mean percentage difference in episode duration between zinc and placebo. We also calculated the RRR to compare the RR of episodes lasting >3 days between studies using Licorzinc and other zinc products.

We plotted funnel plots to assess our primary outcomes for publication bias. We also employed the Child Health Epidemiology Reference Group (CHERG) grading system to assess the quality of evidence for each outcome on a four-point scale (“high”, “moderate”, “low”, “very low”) [11].

### 3. Results

The systematic literature search of the non-Chinese databases uncovered 4038 titles, and 15 were included after subsequent review of abstracts and full manuscripts for inclusion and exclusion criteria (Figure 1) [5–7,12–23]. Of the included studies, 13 were conducted in a hospital setting and two assessed episodes occurring in the community. Included studies were conducted in sites located within 10 countries: India ( $n = 6$ ); Bangladesh ( $n = 5$ ); Nepal ( $n = 1$ ); Turkey ( $n = 1$ ); Brazil ( $n = 1$ ); Pakistan ( $n = 1$ ); Ethiopia ( $n = 1$ ); Yemen ( $n = 1$ ); and Poland ( $n = 1$ ). These studies enrolled a total of 3271 zinc-allocated and 3314 placebo-allocated diarrhea cases. The systematic literature search for Chinese studies resulted in 1520 titles, of which 89 were included (Figure 1) [24–112]. All included studies were conducted in a hospital setting, and 33 studies focused on diarrhea attributable to laboratory confirmed rotavirus. None of the included studies identified through the Chinese database were placebo-controlled; for Chinese studies, zinc and control groups received a range of supportive treatments, including fluid infusion, probiotics and antivirals. The total enrolment of included Chinese studies was 6198 zinc group and 6039 control group diarrhea cases. Table 1 describes the trial setting, sample size, and zinc intervention for all included studies.

**Figure 1.** Results of systematic literature search and review.

**Table 1.** Characteristics of included studies.

Author [Reference]	Year Published	Country	Trial Setting	Specific Causative Organisms	Age Group (months)	Sample Size		Zinc Salt	Tablet or Syrup	Daily Zinc Dose	Length of Supplementation (days)
						Zinc Group	Control Group				
Al Sonboli [17]	2003	Brazil	Hospital	Unknown	3-60	37	37	Not Listed	Tablet	3-5 mos: 22.5 mg 6-60 mos: 45 mg	5
Bahl [7]	2002	India	Community	Unknown	6-35	404	401	Zinc Gluconate	Syrup	6-11 mos: 15 mg 12-35 mos: 30 mg	14
Brooks [16]	2005	Bangladesh	Hospital	Unknown	1-6	91	93	Zinc Acetate	Syrup	20 mg	Duration of episode
Brooks [16]	2005	Bangladesh	Hospital	Unknown	1-6	91	93	Zinc Acetate	Syrup	5 mg	Duration of episode
Dutta [23]	2011	India	Hospital	Unknown	6-24	44	41	Not Listed	Syrup	40 mg	14
Elneimr [21]	2007	Yemen	Hospital	Unknown	3-24	88	92	Zinc Acetate	Syrup	20 mg	14
Faruque [12]	1999	Bangladesh	Hospital	Unknown	6-24	343	341	Zinc Acetate	Syrup	14.2 mg	15
Fischer Walker [19]	2006	Pakistan	Hospital	Unknown	1-5	281	279	Zinc Sulfate	Tablet	10 mg	14
Fischer Walker [19]	2006	India	Hospital	Unknown	1-5	186	187	Zinc Sulfate	Tablet	10 mg	14
Fischer Walker [19]	2006	Ethiopia	Hospital	Unknown	1-5	87	90	Zinc Sulfate	Tablet	10 mg	14
Larson [18]	2005	Bangladesh	Hospital	Unknown	3-59	267	266	Zinc Sulfate	Tablet	20 mg	10
Patel [20]	2009	India	Hospital	Unknown	6-59	264	271	Zinc Sulfate	Syrup	20 mg	14
Patro [22]	2010	Poland	Hospital	Unknown	3-48	81	79	Zinc Sulfate	Syrup	3-5 mos: 10 mg 6-48 mos: 20 mg	10
Polat [15]	2003	Turkey	Hospital	Unknown	2-29	52	54	Zinc Sulfate	Syrup	20 mg	10
Roy [13]	1999	Bangladesh	Hospital	Unknown	3-24	32	35	Zinc Acetate	Syrup	20 mg	14
Sachdev [5]	1988	India	Hospital	Unknown	6-18	25	25	Zinc Sulfate	Tablet	40 mg	Not Listed
Sazawal [6]	1995	India	Hospital	Unknown	6-35	456	481	Zinc Gluconate	Syrup	20 mg	Not Listed
Strand [14]	2002	Nepal	Community	Unknown	6-35	442	449	Not Listed	Syrup	6-11 mos: 15 mg 12-35 mos: 30 mg	From enrolment until 7 days after episode subsided

Table 1. *Cont.*

Zhao [24]	2011	China	Hospital	Unknown	4-36	40	40	40	Licorzinc	Tablet	4-5 mos: 10.8 mg 6-12 mos: 14.4 mg 13-36 mos: 21.6 mg	Not Listed
Zhang [25]	2009	China	Hospital	Rotavirus	6-24	60	60	60	Zinc Gluconate	Not Listed	20 mg	Duration of episode
Lin [26]	2010	China	Hospital	Rotavirus	1.5-36	58	58	58	Zinc Gluconate	Syrup	1.5-5 mos: 10 mg 6-36 mos: 20 mg	Duration of episode
Zhou [27]	2010	China	Hospital	Rotavirus	6-24	42	40	40	Zinc Gluconate	Not Listed	20 mg	14
Yang [28]	2011	China	Hospital	Unknown	3-36	42	40	40	Zinc Gluconate	Tablet	3-5 mos: 10 mg 6-36 mos: 20 mg	10-14
Liu [29]	2010	China	Hospital	Unknown	5-18	40	40	40	Zinc Gluconate	Not Listed	5 mos: 10 mg 6-18 mos: 20 mg	10-14
Chen [30]	2006	China	Hospital	Rotavirus	0-24	30	30	30	Zinc gluconate	Not Listed	10 mg	Not Listed
Liu [31]	2011	China	Hospital	Unknown	6.8-22	90	90	90	Zinc Gluconate	Tablet	20 mg	Not Listed
Liu [32]	2009	China	Hospital	Unknown	6-36	112	108	108	Zinc Gluconate	Tablet	20 mg	10
Fu [33]	2010	China	Hospital	Rotavirus	2-24	98	102	102	Zinc Gluconate	Syrup	5 mg	Not Listed
Zhou [34]	2008	China	Hospital	Unknown	2-48	40	40	40	Licorzinc	Not Listed	2-5 mos: 7.5 mg 6-12 mos: 11.25 mg 13-48 mos: 18.75 mg	10-14
Chen [35]	2008	China	Hospital	Rotavirus	4-48	60	60	60	Licorzinc	Not Listed	4-5 mos: 7.2 mg 6-48 mos: 10.8 mg	Not Listed

Table 1. *Cont.*

Guan [36]	2012	China	Hospital	Rotavirus	1.5-45.6	45	45	Licorzinc	Not Listed	1.5-5 mos: 7.5 mg 6-11 mos: 11.25 mg 12-45.6 mos: 18.75 mg	10-14
Wu [37]	2010	China	Hospital	Rotavirus	4-13	46	46	Licorzinc	Not Listed	4-5 mos: 10 mg 6-13 mos: 20 mg	Not Listed
Zhou [38]	2010	China	Hospital	Unknown	6-24	65	60	Licorzinc	Tablet	20 mg	Not Listed
Luo [39]	2009	China	Hospital	rotavirus	6-36	55	50	Licorzinc	Tablet	18.75 mg	Not Listed
Zhang [40]	2010	China	Hospital	Unknown	5-48	50	50	Licorzinc	Not Listed	Not Listed *	Not Listed
Ju [41]	2007	China	Hospital	Unknown	6-36	40	38	Licorzinc	Tablet	6-12 mos: 11-25 mg 13-36 mos: 15 mg	Not Listed
Wang [42]	2012	China	Hospital	Unknown	6-36	30	30	Licorzinc	Tablet	Not Listed *	3
Hong [43]	2009	China	Hospital	Rotavirus	3-60	140	120	Zinc Sulfate	Syrup	3-11 mos: 20 mg 12-36 mos: 30 mg 37-60 mos: 40 mg	Not Listed
Lin [44]	1994	China	Hospital	Unknown	0.5-24	46	58	Zinc Sulfate	Syrup	10-14 mg/kg *	Not Listed
Yan [45]	2011	China	Hospital	Unknown	5-36	70	50	Zinc Sulfate	Syrup	5 mos: 50 mg 6-36 mos: 100 mg	Not Listed
He [46]	1997	China	Hospital	Unknown	6-36	52	58	Zinc Gluconate	Not Listed	20 mg	Not Listed
Wei [47]	2011	China	Hospital	Unknown	3-36	44	42	Zinc Gluconate	Syrup	3-5 mos: 10 mg 6-36 mos: 20 mg	10-14
Yang [48]	2012	China	Hospital	Unknown	0-36	80	80	Zinc Gluconate	Tablet	0-5 mos: 10 mg 6-36 mos: 20 mg	10

Table 1. *Cont.*

Pu [49]	2010	China	Hospital	Rotavirus	0-24	38	34	Zinc Gluconate	Not Listed	0-5 mos: 10 mg 6-24 mos: 20 mg	Not Listed
Zhang [50]	2011	China	Hospital	Rotavirus	3-36	53	53	Zinc Gluconate	Not Listed	3-5 mos: 10 mg 6-36 mos: 20 mg	10
Sun [51]	2008	China	Hospital	Unknown	1.5-36	45	45	Zinc Gluconate	Syrup	1.5-5 mos: 10 mg 6-36 mos: 20 mg	Not Listed
Zhang [52]	2011	China	Hospital	Unknown	3-36	90	90	Zinc Gluconate	Syrup	3-5 mos: 10 mg 6-36 mos: 20 mg	Not Listed
Lin [53]	2010	China	Hospital	Rotavirus	6-54	28	20	Zinc Gluconate	Tablet	6-54 mos: 20 mg	14
Liu [54]	2009	China	Hospital	Unknown	3-36	95	91	Zinc Gluconate	Not Listed	3-5 mos: 10 mg 6-36 mos: 20 mg	10-14
Qiao [55]	2011	China	Hospital	Unknown	6-36	73	72	Zinc Gluconate	Tablet	6-36 mos: 20 mg	14
Zhang [56]	2007	China	Hospital	Unknown	0-24	85	90	Zinc Gluconate	Not Listed	0-5 mos: 10 mg 6-24 mos: 20 mg	10
Zhao [57]	2012	China	Hospital	Unknown	0-24	70	70	Zinc Gluconate	Syrup	0-5 mos: 10 mg 6-24 mos: 20 mg	10-14
Cai [58]	2011	China	Hospital	Unknown	0-24	88	84	Zinc Gluconate	Not Listed	0-5 mos: 10 mg 6-24 mos: 20 mg	14
Zhang [59]	2012	China	Hospital	Rotavirus	6-17	120	120	Zinc Gluconate	Tablet	20 mg	10-14
Qiao [60]	2012	China	Hospital	Unknown	0-24	85	85	Zinc Gluconate	Not Listed	0-5 mos: 10 mg 6-24 mos: 20 mg	10
Zhong [61]	2012	China	Hospital	Rotavirus	3-48	50	50	Zinc Gluconate	Tablet	3-5 mos: 10 mg 6-48 mos: 20 mg	10
Wang [62]	2011	China	Hospital	Rotavirus	0-24	60	60	Zinc Gluconate	Not Listed	0-5 mos: 10 mg 6-24 mos: 20 mg	10
Yang [63]	2008	China	Hospital	Rotavirus	0-36	164	168	Zinc Gluconate	Not Listed	0-5 mos: 10 mg 6-36 mos: 20 mg	10

Table 1. *Cont.*

Zhao [64]	2012	China	Hospital	Rotavirus	6-36	60	60	Zinc Gluconate	Syrup	35 mg	10
Ma [65]	2012	China	Hospital	Rotavirus	4-42	41	41	Zinc Gluconate	Not Listed	20 mg	Not Listed
Chen [66]	2012	China	Hospital	Rotavirus	0-36	93	93	Zinc Gluconate	Not Listed	0-5 mos: 10 mg 6-36 mos: 20 mg	10
Hu [67]	2009	China	Hospital	Rotavirus	4-36	60	60	Zinc Gluconate	Tablet	4-5 mos: 10 mg 6-36 mos: 20 mg	10
Yuan [68]	2011	China	Hospital	Unknown	1-36	100	100	Zinc Gluconate	Tablet	1-12 mos: 70 mg 13-36 mos: 140 mg	14
Tan [69]	2011	China	Hospital	Unknown	3-36	50	35	Zinc Gluconate	Tablet	3-5 mos: 10 mg 6-36 mos: 20 mg	10-14
Liu [70]	2010	China	Hospital	Unknown	0-36	89	77	Zinc Gluconate	Syrup	0-5 mos: 10 mg 6-36 mos: 20 mg	10
Hu [71]	2011	China	Hospital	Unknown	3-60	108	100	Zinc Gluconate	Tablet	3-5 mos: 10 mg 6-60 mos: 20 mg	14
Li [72]	2008	China	Hospital	Unknown	6-36	40	38	Zinc Gluconate	Tablet	6-12 mos: 7.5 mg 13-36 mos: 15 mg	3
Gao [73]	2012	China	Hospital	Unknown	3-36	74	74	Zinc Gluconate	Not Listed	3-5 mos: 10 mg 6-36 mos: 20 mg	14
Wu [74]	2011	China	Hospital	Unknown	3-60	20	20	Zinc Sulfate	Syrup	10 mg	10
Wu [74]	2011	China	Hospital	Unknown	3-60	20	20	Zinc Sulfate	Not Listed	10 mg	10
Liu [75]	2011	China	Hospital	Unknown	3-60	54	53	Zinc Gluconate	Tablet	3-5 mos: 10 mg 6-60 mos: 20 mg	3-5

Table 1. *Cont.*

Chen [76]	2010	China	Hospital	Unknown	5-36	42	20	Zinc Gluconate	Not Listed	5 mos: 10 mg 6-36 mos: 20 mg	10-14
Ma [77]	2012	China	Hospital	Unknown	2-36	63	63	Zinc Gluconate	Not Listed	2-5 mos: 70 mg 6-36 mos: 140 mg	10-14
Lu [78]	2012	China	Hospital	Unknown	6-18	120	140	Zinc Gluconate	Not Listed	140 mg	10-14
Ma [79]	2012	China	Hospital	Unknown	6-36	58	52	Zinc Gluconate	Syrup	6-36 mos: 20 mg	10
Ao [80]	2012	China	Hospital	Rotavirus	0-24	87	80	Zinc Gluconate	Syrup	0-5 mos: 10 mg 6-24 mos: 20 mg	Not Listed
Gu [81]	2011	China	Hospital	Unknown	3-60	56	60	Zinc Gluconate	Syrup	3-5 mos: 10 mg 6-60 mos: 20 mg	10
Wen [82]	2006	China	Hospital	Unknown	0-24	30	29	Zinc Gluconate	Not Listed	20 mg	10-14
Wang [83]	2011	China	Hospital	Unknown	3-36	60	60	Licorzinc	Not Listed	10-20 mg *	Duration of episode
Liu [84]	2012	China	Hospital	Rotavirus	8-30	90	90	Licorzinc	Not Listed	8-30 mos: 20 mg	Not Listed
Liu [85]	2012	China	Hospital	Unknown	3-60	100	100	Licorzinc	Tablet	3-5 mos: 10 mg 6-60 mos: 20 mg	Not Listed
Tong [86]	2011	China	Hospital	Unknown	2-36	98	98	Licorzinc	Not Listed	2-5 mos: 10 mg 6-36 mos: 20 mg	Not Listed
Qiu [87]	2010	China	Hospital	Rotavirus	1-24	53	52	Licorzinc	Tablet	1-5 mos: 10 mg 6-24 mos: 20 mg	14
Kong [88]	2011	China	Hospital	Unknown	3-30	35	35	Zinc Gluconate	Tablet	3-5 mos: 10 mg 6-11 mos: 15 mg 12-30 mos: 20 mg	14
He [89]	2007	China	Hospital	Rotavirus	5-22	60	63	Zinc Gluconate	Not Listed	20 mg	Not Listed

Table 1. *Cont.*

Kang [90]	2010	China	Hospital	Rotavirus	6–36	92	80	Zinc Gluconate	Tablet	20 mg	14
Su [91]	2012	China	Hospital	Rotavirus	6–36	97	97	Zinc Gluconate	Not Listed	20 mg	Not Listed
Huang [92]	2010	China	Hospital	Rotavirus	2–36	100	100	Not Listed	Tablet	2–5 mos: 10 mg 6–36 mos: 20 mg	Not Listed
Zhang [93]	2006	China	Hospital	Unknown	0–36	83	63	Licorzinc	Syrup	0–5 mos: 10 mg 6–36 mos: 20 mg	10–14
Wang [94]	2012	China	Hospital	Unknown	4–30	60	60	Zinc Gluconate	Syrup	10 mg	Not Listed
Lin [95]	2008	China	Hospital	Unknown	0.5–34	60	60	Zinc Gluconate	Tablet	0.5–5 mos: 140 mg 6–34 mos: 280 mg	10–14
Yan [96]	2011	China	Hospital	Unknown	6–60	57	57	Zinc Gluconate	Tablet	20 mg	10
Yu [97]	2012	China	Hospital	Unknown	0–36	40	40	Zinc Gluconate	Tablet	0–5 mos: 10 mg 6–36 mos: 20 mg	10–14
Zhang [98]	2011	China	Hospital	Rotavirus	4–36	128	128	Zinc Gluconate	Syrup	4–5 mos: 10 mg 6–36 mos: 20 mg	14
Xu [99]	2010	China	Hospital	Rotavirus	2–36	84	83	Zinc Gluconate	Not Listed	2–5 mos: 10 mg 6–36 mos: 20 mg	14
Tan [100]	2010	China	Hospital	Unknown	3.5–60	55	55	Zinc Gluconate	Syrup	3.5–5 mos: 10 mg 6–60 mos: 20 mg	10–14
Shen [101]	2012	China	Hospital	Rotavirus	2.5–40	46	42	Zinc Gluconate	Not Listed	2.5–5 mos: 10 mg 6–40 mos: 20 mg	Duration of episode
Wang [102]	2010	China	Hospital	Unknown	6–48	52	51	Zinc Gluconate	Tablet	20 mg	Not Listed

Table 1. *Cont.*

Chen [103]	2011	China	Hospital	Unknown	1–36	50	50	50	Zinc Gluconate	Tablet	1–5 mos: 5 mg 6–36 mos: 10 mg	Not Listed
Meng [104]	2012	China	Hospital	Unknown	0–24	90	90	90	Zinc Gluconate	Tablet	0–5 mos: 2.5 mg 6–12 mos: 5 mg 13–24 mos: 10 mg	Not Listed
Zhong [105]	2010	China	Hospital	Unknown	1–24	60	60	60	Zinc Gluconate	Tablet	1–5 mos: 2.5 mg 6–12 mos: 5 mg 13–24 mos: 7.5 mg	5–7
Xie [106]	2010	China	Hospital	Rotavirus	6–36	128	124	124	Zinc Gluconate	Tablet	20 mg	Not Listed
Fan [107]	2012	China	Hospital	Unknown	0–36	163	121	121	Not Listed	Not Listed	0–5 mos: 10 mg 6–36 mos: 20 mg	10
Zhou [108]	2012	China	Hospital	Rotavirus	6–24	75	75	75	Zinc Gluconate	Syrup	20 mg	10–14
Zhao [109]	2008	China	Hospital	Unknown	0–36	44	43	43	Zinc Gluconate	Tablet	0–5 mos: 10 mg 6–24 mos: 20 mg	Not Listed
Wan [110]	2006	China	Hospital	Unknown	6–36	26	24	24	Not Listed	Not Listed	Not Listed	Not Listed
Yang [111]	2012	China	Hospital	Unknown	6–60	60	60	60	Not Listed	Not Listed	20 mg	Not Listed
Luo [112]	2012	China	Hospital	Unknown	0–36	168	196	196	Not Listed	Not Listed	0–5 mos: 10 mg 6–36 mos: 20 mg	Not Listed

\* Study not included in dose analyses.

The results of the studies identified through non-Chinese databases are summarized in Tables 2 and 3. Acute episodes were 4% (95% CI: 1%–8%) shorter in duration among children treated with zinc compared to those receiving placebo (Table 2). Among children hospitalized for diarrhea, the duration of hospitalization was reduced by 37% (95% CI: 21%–53%) comparing the zinc and control groups (Table 2). Stool frequency was decreased by 6% (95% CI: 2%–10%) among zinc-treated children. Zinc-treated children had a reduced relative risk (RR) of acute diarrhea lasting beyond three and seven days and an increased risk of vomiting (RR: 1.83; 95% CI: 1.40–2.39) (Table 3).

**Table 2.** Pooled means of select outcomes for non-Chinese studies.

Outcome	Study Sites <sup>1</sup>	Pooled Mean (95% CI) <sup>2</sup>		Percent Difference <sup>3</sup> (%)
	<i>N</i>	Zinc Group	Control Group	
Duration of Episode (days)	13	3.51 (3.43–3.60)	3.67 (3.59–3.76)	–4.4 (–7.8, –1.0)
Duration of Hospitalization (days)	1	2.00 (1.99–2.01)	3.17 (2.38–3.96)	–36.9 (–52.6, –21.2)
Stool Output (mL)	2	391.2 (388.5–393.8)	388.8 (386.2–391.5)	0.6 (–0.3, 1.6)
Stool Frequency (Number per day)	6	5.04 (4.88–5.19)	5.36 (5.20–5.52)	–6.0 (–9.9, –2.0)

<sup>1</sup> Individual studies may contribute more than one study site (*N*) to each estimate; <sup>2</sup> Estimates for  $\geq 2$  study sites generated by Poisson regression model weighted by sample size; <sup>3</sup> Percent difference calculated by:  $100 \times ((\text{Pooled Zinc Estimate} - \text{Pooled Control Estimate}) / \text{Pooled Control Estimate})$ ; 95% CI calculated by:  $\text{Percent Difference} \pm 1.96 \times \{ |(\text{mean}_{\text{zinc}} / \text{mean}_{\text{control}})| \times \sqrt{[(\text{std error}_{\text{zinc}})^2 / (\text{mean}_{\text{zinc}})^2 + (\text{std error}_{\text{control}})^2 / (\text{mean}_{\text{control}})^2]} \} \times 100$ .

**Table 3.** Pooled relative risk of select outcomes for non-Chinese studies.

Outcome	Study Sites <sup>1</sup>	Pooled Estimate Percentage (95% CI) <sup>2</sup>		Pooled Relative Risk <sup>3</sup> RR (95% CI)
	<i>N</i>	Zinc Group	Control Group	
Episodes > 3 days (%)	3	29.7 (26.7–32.7)	39.5 (36.3–42.7)	0.78 (0.67–0.90)
Episodes > 7 days (%)	6	10.3 (8.9–11.7)	14.9 (13.2–16.5)	0.74 (0.55–0.99)
Vomiting (%)	3	18.8 (16.0–21.6)	9.4 (7.3–11.4)	1.83 (1.40–2.39)

<sup>1</sup> Individual studies may contribute more than one study site (*N*) to each estimate; <sup>2</sup> Estimates for  $\geq 2$  study sites generated by logistic regression model weighted by sample size; <sup>3</sup> Estimates for  $\geq 2$  studies generated by random effects meta-analysis.

Outcomes pooled across studies conducted in China showed reductions in the duration of diarrhea, hospitalization, fever, vomiting, stool output and stool frequency among zinc-treated children with acute diarrhea attributable to rotavirus and to non-specific causes (Table 4). The reduction in the duration of diarrhea was 37% (95% CI: 35%–39%) among non-specific episodes and 31% (95% CI: 29%–34%) among rotavirus episodes (Table 4). The RR of diarrhea lasting beyond three days was reduced among zinc-treated patients with non-specific (RR: 0.73; 95% CI: 0.66–0.79) and rotavirus (RR: 0.70; 95% CI: 0.63–0.78) diarrhea (Table 5; Figures 2 and 3).

**Table 4.** Pooled means of select outcomes for Chinese studies.

Outcome	Specific Causative Pathogens	Study Sites <sup>1</sup> <i>N</i>	Pooled Mean (95% CI) <sup>2</sup>		Percent Difference <sup>3</sup> (%)
			Zinc Group	Control Group	
Duration of Episode (days)	Unknown	40	2.96 (2.90–3.03)	4.68 (4.60–4.77)	–36.8 (–38.7, –34.8)
Duration of Hospitalization (days)	Rotavirus	24	3.45 (3.36–3.54)	5.01 (4.89–5.12)	–31.1 (–33.5, –28.8)
Duration of Fever (days)	Unknown	10	4.65 (4.50–4.80)	6.43 (6.25–6.61)	–27.7 (–30.8, –24.6)
Duration of Vomiting (days)	Rotavirus	2	4.15 (3.79–4.51)	6.1 (5.66–6.54)	–32.0 (–39.6, –24.3)
Stool Output (mL)	Unknown	13	1.90 (1.80–1.99)	2.81 (2.70–2.92)	–32.4 (–36.5, –28.2)
Stool Frequency (Number per day)	Rotavirus	4	1.96 (1.78–2.14)	3.18 (2.95–3.41)	–38.4 (–45.6, –31.2)
	Unknown	6	1.15 (1.05–1.25)	1.53 (1.41–1.64)	–24.8 (–33.3, –16.4)
	Rotavirus	3	1.84 (1.64–2.04)	2.49 (2.26–2.72)	–26.1 (–36.6, –15.6)
	Unknown	1	40 (38.1–41.9)	70 (68.0–72.0)	–42.9 (–46.0, –39.7)
	Rotavirus	1	278.4 (256.8–300.0)	425.4 (382.1–468.7)	–34.6 (–42.9, –26.2)
	Unknown	1	4 (3.8–4.2)	8 (7.6–8.4)	–50.0 (–53.5, –46.5)
	Rotavirus	2	3.74 (3.30–4.18)	4.27 (3.77–4.77)	–12.4 (–27.0, 2.1)

<sup>1</sup> Individual studies may contribute more than one study site (*N*) to each estimate; <sup>2</sup> Estimates for  $\geq 2$  study sites generated by Poisson regression model weighted by sample size; <sup>3</sup> Percent difference calculated by:  $100 \times ((\text{Pooled Zinc Estimate} - \text{Pooled Control Estimate}) / \text{Pooled Control Estimate})$ ; 95% CI calculated by:  $\text{Percent Difference} \pm 1.96 \times \{ |(\text{mean}_{\text{zinc}} / \text{mean}_{\text{control}})| \times \sqrt{[(\text{std error}_{\text{zinc}})^2 / (\text{mean}_{\text{zinc}})^2 + (\text{std error}_{\text{control}})^2 / (\text{mean}_{\text{control}})^2]} \} \times 100$ .

**Table 5.** Pooled relative risk of select outcomes for Chinese studies.

Outcome	Specific Causative Pathogens	Study Sites <sup>1</sup> <i>N</i>	Pooled Estimate Percentage (95% CI) <sup>2</sup>		Relative Risk <sup>3</sup> RR (95% CI)
			Zinc Group	Control Group	
Episodes > 3 days (%)	Unknown	44	31.4 (29.4–33.5)	49.2 (46.6–51.8)	0.73 (0.66–0.79)
Episodes > 7 days (%)	Rotavirus	29	31.8 (29.5–34.1)	50.3 (47.4–53.3)	0.70 (0.63–0.78)
	Unknown	1	26.9 (-)	39.2 (-)	0.75 (0.42–1.37)

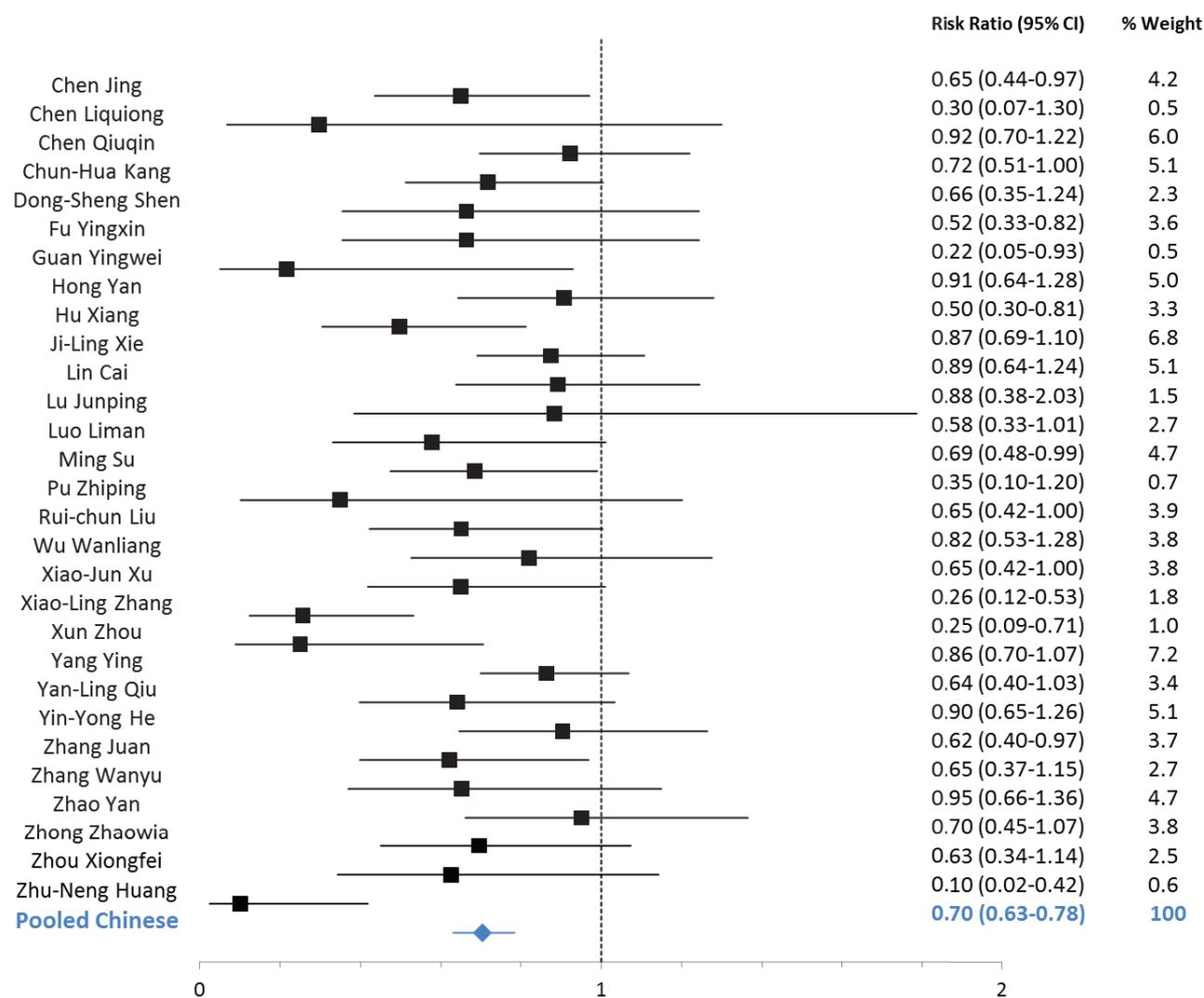
<sup>1</sup> Individual studies may contribute more than one study site (*N*) to each estimate; <sup>2</sup> Estimates for  $\geq 2$  study sites generated by Poisson regression model weighted by sample size; <sup>3</sup> Estimates for  $\geq 2$  studies generated by random effects meta-analysis.

We did not identify any studies reporting diarrhea-specific or all-cause mortality for inclusion in this review. Nor did we identify non-Chinese studies reporting duration of fever or vomiting, or Chinese studies reporting the proportion of children vomiting.

The mean episode duration and proportion of episodes lasting >3 days were not statistically significantly different comparing zinc-treated children in Chinese and non-Chinese studies. There was no statistically significant difference between the estimated relative risk of an episode lasting >3 days (RRR: 1.07; 95% CI: 0.90–1.27) comparing Chinese and non-Chinese studies; therefore, we pooled this outcome across regions (RR: 0.74; 95% CI: 0.68–0.80) (Figure 3). The percentage difference between the mean episode duration of zinc-treated and control group children was statistically significantly larger for Chinese compared to non-Chinese studies ( $p < 0.05$ ), so this

outcome was not pooled across regions. We did not have sufficient power to compare other commonly reported outcomes by region.

**Figure 2.** Forest plot for the effect of therapeutic zinc supplementation on Rotavirus diarrhea episodes >3 days.

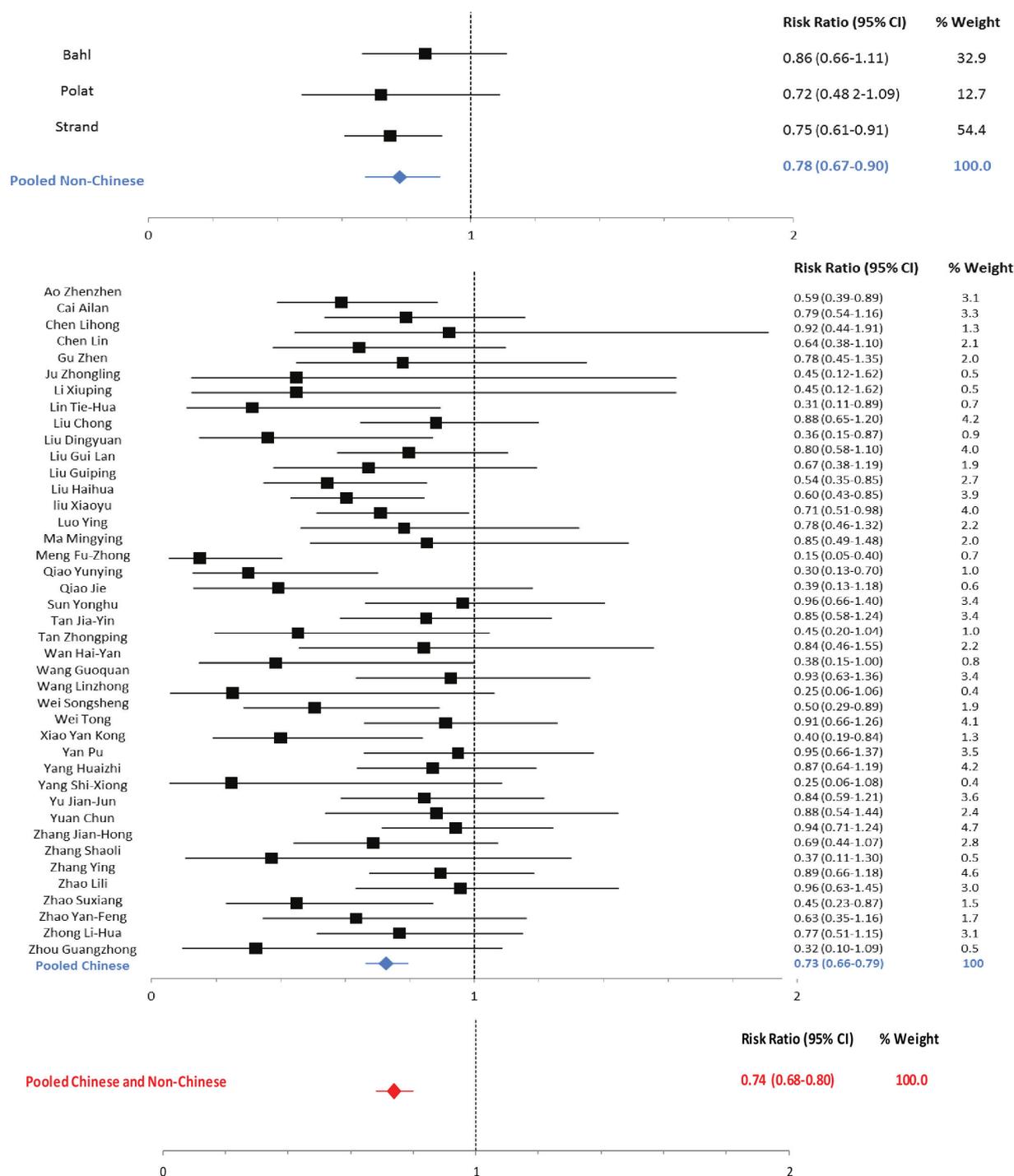


Zinc dose was not associated with the mean percent difference in diarrhea duration comparing zinc and control children for non-Chinese ( $p = 0.50$ ) or Chinese ( $p = 0.12$ ) studies. Comparing Chinese studies that used Licorzinc to those that used other zinc supplements, there were no statistically significant differences in the mean percent difference in the duration of rotavirus episodes ( $p = 0.56$ ), the RR of non-specific episodes lasting >3 days (RRR: 0.99; 95% CI: 0.72–1.35), or the RR of rotavirus episodes lasting >3 days (RRR: 0.93; 95% CI: 0.68–1.26). The percentage difference in the mean duration of non-specific episodes comparing zinc and control group children was statistically significantly higher for Licorzinc compared to “other zinc” studies ( $p = 0.01$ ).

Our assessment of publication bias yielded largely symmetrical funnel plots for all outcomes.

Under the CHERG grading system, the studies included in this review were of moderate quality (Table 6) [11]. Effect estimates were largely consistent in directionality for all outcomes.

**Figure 3.** Forest plot for the effect of therapeutic zinc supplementation on non-specific diarrhea episodes lasting >3 days.



**Table 6.** Quality assessment of studies measuring the association between therapeutic zinc supplementation and selected outcomes.

Number of Studies	Design	Limitations	Consistency	Directness	
				Generalizability to Population of Interest	Generalizability to Intervention of Interest
<b><i>Diarrhea Duration (mean): Moderate outcome-specific quality</i><sup>1</sup></b>					
53 non-specific 24 Rotavirus	RCT	Chinese studies not placebo-controlled (-0.5)	All but 4 studies showing decreased mean duration of diarrhea among zinc-treated children (+1)	Mostly South Asia and China (-0.5)	Generalizable
<b><i>Diarrhea Duration (&gt;3 days): Moderate outcome-specific quality</i><sup>1</sup></b>					
47 non-specific 29 Rotavirus	RCT	Chinese studies not placebo-controlled (-0.5)	All studies showing decreased risk of diarrhea duration >3 days among zinc-treated children (+1)	Mostly South Asia and China (-0.5)	Generalizable
<b><i>Diarrhea Duration (&gt;7 days): Moderate outcome-specific quality</i><sup>1</sup></b>					
7 non-specific	RCT	Chinese studies not placebo-controlled (-0.5)	All but one study showing decreased risk of diarrhea duration >7 days among zinc-treated children (+1)	Mostly South Asia and China (-0.5)	Generalizable
<b><i>Hospitalizations Duration: Moderate outcome-specific quality</i><sup>1</sup></b>					
11 non-specific 2 Rotavirus	RCT	Chinese studies not placebo-controlled (-0.5)	All studies showing decreased mean duration of hospitalization among zinc-treated children (+1)	Only one non-Chinese study (-0.5)	Generalizable
<b><i>Stool Output: Moderate outcome-specific quality</i><sup>1</sup></b>					
3 non-specific 1 Rotavirus	RCT	Chinese studies not placebo-controlled (-0.5)	All but one study showing decreased stool output among zinc-treated children (+1)	Only South Asia and China (-0.5)	Generalizable
<b><i>Stool Frequency: Moderate outcome-specific quality</i><sup>1</sup></b>					
7 non-specific 2 Rotavirus	RCT	Chinese studies not placebo-controlled (-0.5)	All but three studies showing decreased stool frequency among zinc-treated children (+1)	Mostly South Asia and China (-0.5)	Generalizable

Table 6. Cont.

<b><i>Vomiting: Moderate outcome-specific quality</i></b> <sup>1</sup>			
3 non-specific	RCT	None	All studies showing increased vomiting among zinc-treated children (+1) No Chinese studies (-0.5) Generalizable
<b><i>Vomiting Duration: Moderate outcome-specific quality</i></b> <sup>1</sup>			
6 non-specific 3 Rotavirus	RCT	Chinese studies not placebo-controlled (-0.5)	All but one study showing decreased duration of vomiting among zinc-treated children (+1) No non-Chinese studies (-0.5) Generalizable
<b><i>Fever Duration: Moderate outcome-specific quality</i></b> <sup>1</sup>			
13 non-specific 4 Rotavirus	RCT	Chinese studies not placebo-controlled (-0.5)	All studies showing decreased duration of fever among zinc-treated children (+1) No non-Chinese studies (-0.5) Generalizable

<sup>1</sup> Quality assessment scoring based on previously published criteria [11].

#### 4. Discussion

The findings of our systematic review confirm and highlight the benefits of therapeutic zinc supplementation for diarrhea among children under five years of age in low- and middle-income countries. The effects of zinc treatment, which include reductions in episode duration, stool output, stool frequency and length of hospitalization, were consistent across Chinese and non-Chinese studies and non-specific and rotavirus diarrhea. These results suggest that zinc therapy of diarrhea is largely beneficial and important in both low- and middle-income settings.

The results of the large number of Chinese trials in rotavirus diarrhea are a substantial addition to the global evidence base because there have been no non-Chinese trials. One study in India based on a post-hoc subgroup analysis suggested that zinc treatment was not beneficial for rotavirus diarrhea [113]; however, the evidence from China demonstrates that therapeutic zinc supplementation reduces the duration and severity of rotavirus episodes. As rotavirus is the predominant cause of severe acute diarrhea worldwide and most likely the leading cause of diarrhea mortality [114], zinc treatment of rotavirus diarrhea could potentially yield large reductions in hospitalizations and deaths.

In comparison to non-Chinese studies, the difference between the mean episode duration of zinc-treated and control group children was statistically significantly higher for Chinese studies ( $p < 0.05$ ). It is possible that this difference resulted from lack of placebo-controlled groups and blinding among Chinese studies. However, estimates of the effects of therapeutic zinc supplementation on other outcomes were largely consistent across study locations and we were able to generate a pooled global effect size for the proportion of episodes  $>3$  days. The consistency of effect estimates between studies conducted in and outside China suggests that the lack of placebo-controlled groups in Chinese studies did not greatly bias the results.

Zinc dose did not affect the estimate of the effect of zinc supplementation on the duration of diarrhea for non-Chinese or Chinese studies. Although Licorzinc was associated with slightly greater reductions in the mean duration of non-specific diarrhea than other zinc products, zinc effect sizes were generally comparable across Chinese studies regardless of type of zinc preparation.

There is a dearth of literature meeting our inclusion criteria that assessed diarrhea-specific and all-cause mortality. Although a previous review published mortality effect estimates [4], the sole study reporting diarrhea-specific deaths was cluster-randomized and thus violated our inclusion criteria [115]. In addition, three studies of all-cause mortality were also excluded from our review; one was on persistent diarrhea [116], and two others were review papers [3,117].

Using previously published scoring criteria, the studies included in our review yielded pooled estimates of overall moderate quality [11]. The majority of studies contributing to this review were conducted in China and South Asia; however, studies conducted outside Asia were consistent in the directionality of effect estimates. The consistency and quality of all outcomes bolsters the evidence in support of oral zinc supplementation for the treatment of acute diarrhea among children under five in low- and middle-income countries.

## 5. Conclusions

Oral therapeutic zinc supplementation reduces the morbidity of acute diarrhea among children under five in and outside China. Global efforts should be made to support scale-up of the WHO recommended regimen of therapeutic zinc in all regions.

## Authors' Contributions

LML conducted the systematic review of non-Chinese studies, analysis and led the initial manuscript preparation. CLFW assisted with the analysis and the manuscript preparation. KC and WYJ conducted the systematic review of Chinese studies. REB conceptualized the idea and assisted with the interpretation of the analysis and the final manuscript preparation.

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## Conflicts of Interest

The authors declare no conflict of interest.

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## Introducing Solid Foods to Infants in the Asia Pacific Region

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**Abstract:** For infants' optimal growth and development, the introduction of nutritionally suitable solid foods at the appropriate time is essential. However, less attention has been paid to this stage of infant life when compared with studies on breastfeeding initiation and duration. The practice of introducing solid foods, including the types of foods given to infants, in the Asia Pacific region was reviewed. In total nine studies using the same questionnaire on infant feeding practices were analysed to gain a better understanding of trends in the introduction of solid foods in this region. All studies showed less than optimal duration of exclusive breastfeeding indicating an earlier time of introduction of solid foods than recommended by the WHO. Most mothers commonly used rice or rice products as the first feed. In many studies, the timing of introducing solid foods was associated with breastfeeding duration. Compared with the Recommended Nutrient Intakes for infants aged above six months, rice/rice products are of lower energy density and have insufficient micronutrients unless they have been fortified. Although the timing of introducing solid foods to infants is important in terms of preventing later health problems, the quality of the foods should also be considered. Recommendations to improve the introduction of solid foods include measures to discourage prelacteal feeding, facilitating breastfeeding education and providing better information on healthier food choices for infants.

**Keywords:** complementary foods; infants; Asia pacific region; infant feeding practices

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## 1. Introduction

Appropriate nutrient intake, in quantity, bioavailability, and timing in infancy are essential for optimal growth and development. Exclusive breastfeeding for six months and then the introduction of nutritious complementary or solid foods, while breastfeeding continues, contributes to the prevention of acute and chronic diseases in early and later life [1]. Most reviews have concluded that “exclusive breastfeeding” for the first six months of life provides sufficient nutrients for infants for around six months, and then appropriate “complementary foods” should be introduced with continued breastfeeding, preferably until around two years of age or longer [2–4]. Both breastfed and infant formula fed infants should be introduced to safe and nutritious “complementary foods” at around six months to prevent retardation of growth and to minimize the risk of nutrient deficiencies [5].

“Complementary foods” are defined as foods other than breastmilk, infant formula or follow-on formula given to infants and these can be liquids, semi-liquids, and solids [6]. When these foods, particularly solid foods, are introduced to infants, textures should be changed as appropriate to the age of infants to give a variety of textural experiences. It is widely believed that foods with a pureed texture should be the first solid foods introduced [7]. “Solid foods” can be defined as non-drinkable food made by the food industry or by the family [8]. Complementary foods must also be nutritionally adequate and provide the bioavailable nutrients required, in combination with breastmilk, to meet all needs for growth and optimal health [4]. Since the nutrients in breastmilk are generally more bioavailable than from other sources, breastmilk remains an important component of nutrition after the introduction of solids. The term, “weaning” is often used to describe the infants who start taking solid foods [9] but “weaning” usually indicates a transition period or process from breastmilk or infant formula to solid foods [7]. It often refers to different events in different cultures and so it will not be used in this paper.

The timing of the introduction of solid foods is important because the early introduction of solid foods to infants by definition results in a shorter duration of “exclusive breastfeeding”, which in turn increases the risk of morbidity. Several studies have found that the early introduction of solid foods before six months of age has been associated with an increased risk of diarrheal disease or gastro-intestinal infection in infancy [10,11], food allergies [12], and overweight or higher Body Mass Index (BMI) in childhood [13]. The early introduction of solid foods may also change the composition of gastro-intestinal bacteria, the microbiome, which has implications for health [14–16]. In contrast, the late introduction of solid foods (after six months of age) predisposes to micronutrient deficiencies including iron and zinc status, which affect cognitive and neurological development [17,18] and may lead to other problems, including feeding difficulties [19].

In the most recent guidelines for infant feeding launched by the WHO/UNICEF in 2003, the introduction of solid foods to infants is recommended at six months of age (180 days) [20] but other international organizations recommendations may differ slightly from the WHO recommendation. The nutrition committee of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), recommended that the introduction of solid foods should not be commenced before 17 weeks of age and not later than 26 weeks of age. [21]. The American Academy of Paediatrics (AAP) stated that solid foods should not be commenced before six months of age [22]. Despite the WHO recommendations, many mothers have tended to introduce solid foods to their

infants before six months. A cohort study of 401 mothers in Ireland found that the median age of introducing solid foods to infants was 16 weeks (interquartile range = 14–17.7), while 22.6% of the mothers introduced solid foods to their infants before 12 weeks postpartum [7]. In a British study ( $n = 604$ ), the median age of introducing solid foods reported by the mothers was 15 weeks (interquartile range = 13–16), despite the national government recommendation to start solid foods for infants from six months of age [11].

In the Asia Pacific region, the recommended timing for the introduction of solid foods for infants varies between countries. For instance, in China, the Ministry of Health formerly recommended the introduction of solid foods to infants after four months of age (16 weeks), but more recently they have changed to six months of age [23]. In Australia, the National Health and Medical Research Council Infant Feeding Guidelines state that “solid foods” should be commenced at around six months of age [4]. The introduction of solid foods to infants is influenced by many cultural factors and traditional beliefs. For example, in Japan, a traditional ceremony is usually held at 100 days after birth and this ceremony was the time when mothers started to introduce additional liquid foods, including fruit juice and vegetable soup. Although the recent guidelines state that it is not necessary to provide any liquids other than breastmilk before six months, the ceremony still remains as a traditional custom in Japan. While changes in duration and exclusivity of breastfeeding have been extensively researched, less is known about how the patterns of infant feeding, including introducing solid foods, are changing and how these changes relate to differences in cultural practices between countries. The aim of this study was to review the timing of the introduction of solid foods in the Asia Pacific region by comparing and contrasting previous studies that used the same questionnaires and to describe the types of foods that are introduced.

## 2. Methods

### 2.1. Study Details and Descriptions of Sample Recruitments

Nine studies (five countries) undertaken by Curtin University, School of Public Health, of infant feeding practices conducted in the Asia Pacific region were reviewed including data on the first introduction of solid foods to infants. The recruitment process of the samples in each study has been published in detail elsewhere [24–30]. In Australia, the Perth Infant Feeding Study I (PIFS I) was undertaken to obtain information about infant feeding practices and provide information to assist in developing the Infant Feeding Guidelines and was repeated a decade later as the Perth Infant Feeding Study II (PIFS II). The questionnaires and methodology developed for these initial studies were then used in a similar way in other countries allowing for comparisons to be made. All of the Australian mothers in these studies were recruited in hospitals after birth. The Vietnamese mothers were recruited after giving birth in hospital, in community health centres, or at their home. In the two Chinese studies, all mothers were recruited at either hospitals or health centres as birthing at home is uncommon in this country. The mothers in the Zhejiang study were recruited from a city (Hangzhou), suburban, and rural areas. The Maldivian mothers were recruited at clinics associated with hospitals, the Japanese mothers were recruited at community health centres when they came for health examinations of their infants at 18 months of age and one study of mothers who have migrated to Australia used community samples

recruited using “snowball” techniques. In the seven cohort studies the mothers were asked to complete an initial questionnaire on their infant feeding practices while in hospital and were then followed up at regular intervals for six months. Similar questionnaires were used in all of the studies and the three cross-sectional studies have been included in this review. The mothers in the Maldives study are representative of a conservative Islamic society, while the other countries are generally representative of Asia Pacific cultures.

## 2.2. Study Questionnaire

All reviewed studies used the same questionnaires on infant feeding practices and demographic details. Where necessary for cultural, translation, and ethical reasons several questions were modified. Specific areas of the questionnaire include:

- Demographics: maternal age, occupation, marital status, method of birth, parity, family income, husband’s/partner’s occupation, maternal smoking status, alcohol intake during lactation, infants’ birth weight
- Infant feeding practices: timing of feeding changes, intention to breastfeed, expressing breastmilk, breastfeeding problems, the reasons for ceasing breastfeeding, starting time of solid food and infant formula, and types of the first solid food given to infants.

## 2.3. Ethical Consideration

All of the studies were approved by the Human Research Ethics Committees of Curtin University and other relevant authorities. These included the local health authorities in Vietnam and Japan, and participating hospitals in China, Australia, and the Maldives. Confidentiality was assured and mothers were advised that their participation was voluntary and that they could withdraw at any time without prejudice.

## 3. Results

The details of each study including the study year, country, sample size, response rate, and methodology are shown in Table 1. Table 2 lists the sample characteristics reported by mothers in each study. In all studies, the majority of mothers were married and mothers in industrialized countries, particularly in cities, had more years of education than those in developing countries, but aboriginal mothers had the lowest education levels. In Australia, there are similar sample characteristics in maternal age in the PIFS I and II studies, while the Aboriginal mothers in PABS are younger. In both Chinese studies (Xinjiang, a remote area that is located in the Northwest, and Zhejiang, an industrialized province in Eastern China) most mothers were primiparous (75.8% and 88.6%, respectively) and there were higher rates of caesarian section (44.1% and 67.0%, respectively), than in the other studies. The Himeji study that was undertaken in the central part of Japan had the highest rate of low birth weight (8.4%) and unemployment in mothers (71.4%) among the studies.

**Table 1.** Details of the infant feeding studies in Asia Pacific region.

* Authors	Data collection periods	Country	Study name	Sample size	Response rate (%)	Study method
1 Binns <i>et al.</i>	1992/93	Australia	Perth Infant Feeding Study I (PIFS I)	556	77	cohort
2 Binns <i>et al.</i>	2001/02	Australia	Perth Aboriginal Breastfeeding Study (PABS)	425	93	cohort
3 Duong <i>et al.</i>	2002	Viet Nam	Rural Viet Nam Infant Feeding Study	463	96	cohort
4 Binns <i>et al.</i>	2002/03	Australia	Perth Infant Feeding Study Mark II (PIFS II)	587	68	cohort
5 Xu <i>et al.</i>	2002/03	China	Xinjiang Infant Feeding Study	1219	97	cohort
6 Abdurraheem <i>et al.</i>	2004	Maldives	Maldives Infant Feeding Study	251	81	cross-sectional
7 Qiu <i>et al.</i>	2004/05	China	Zhejiang (Hangzhou) Infant Feeding Study	1520	96	cohort
8 Li <i>et al.</i>	2002	Australia	Chinese Infant Feeding Study living in Perth	506	95	cross-sectional
9 Inoue <i>et al.</i>	2007	Japan	Himeji Infant Feeding Study	1612	69	cross-sectional

\* Sources: Study1: [31]; 2: [32]; 3: [29]; 4: [27]; 5: [28]; 6: [33]; 7: [34]; 8: [26]; 9: [25].

**Table 2.** The sample characteristics reported by mothers in each study.

Variables	Study 1* n (%)	2 n (%)	3 n (%)	4 n (%)	5 n (%)	6 n (%)	7 n (%)	8 n (%)	9 n (%)
Age (year)									
<25	163 (29.3)	300 (70.6)		154 (26.2)	184 (15.1)	198 (78.8)	358 (23.6)	33 (6.5)	67 (4.2)
25–29	193 (34.7)	80 (18.8)	26.4 (4.97)	170 (29.0)	544 (44.6)	(below 30)	800 (52.6)	(below 30)	170 (29.0)
30–34	135 (24.3)	41 (9.6)	(mean ±	178 (30.3)	307 (25.2)	53 (20.4) (above	338 (22.2)	473 (93.5)	722 (44.8)
35≤	60 (10.8)	(above 30)	SD **)	84 (14.3)	66 (5.4)	30)		(above 30)	411 (25.5)
No response	6 (1.0)	4 (0.9)		1 (0.2)	118 (9.7)	0 (0.0)	24 (1.6)	0 (0.0)	61 (3.8)
Marital status									
Married/Defacto		370 (87.1)		540 (92.0)		239 (95.2)	1518 (99.9)	498 (98.2)	1535 (95.2)
Others	N/A	52 (12.2)	N/A	47 (8.0)	N/A	12 (3.2)	2 (0.1)	8 (1.8)	46 (3.1)
No response		53 (0.7)		0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)	28 (1.7)
<12	292 (52.5)	385 (90.8)	(85.5)	249 (43.2)	781 (64.1)	222 (88.5)	915 (60.5)	5 (1.0)	
≥12	248 (44.6)	34 (8.0)	(14.5)	328 (56.8)	355 (29.1)	29 (10.4)	599 (39.6)	501 (99.0)	N/A ***
No response	16 (2.9)	6 (1.2)	0 (0.0)	0 (0.0)	83 (6.8)	0 (0.0)	2 (0.1)	0 (0.0)	



Table 3 presents the median age of introducing solid foods to infants in the Asia Pacific region. Zhejiang is the earliest at 3.8 months, while Maldives and Japan were 5.5 months of age. In Vietnam, some mothers (4.8%) introduced solid foods to their infants as early as one week postpartum while the median age was approximately 4 months. Japanese mothers residing in Perth introduced solid foods earlier than those who are living in Japan. For Australian mothers the timing of introducing solid food to their infants changed over the decade between PIFS I and PIFS II with an increase in the mean age from 4.0 to 4.4 months. The most common first solid foods given to infants are rice or rice products in Asia Pacific region (Table 4) except in the Maldives where their traditional food which is made with wheat flour and fish, and Chinese migrants to Australia (egg-yolk). It is also interesting to note that over 40% of Vietnamese mothers used monosodium glutamate in the preparation of solid foods for infants [8].

**Table 3.** The median age of the first introducing solid foods (in months) by the studies.

*	Study name	Median age (SD) **
1	Perth Infant Feeding Study I (PIFS I)	4.0
2	Perth Aboriginal Breastfeeding Study (PABS)	4.7
3	Rural Viet Nam Infant Feeding Study	4.0
4	Perth Infant Feeding Study Mark II (PIFS II)	4.4
5	Xinjiang Infant Feeding Study	4.0
6	Maldives Infant Feeding Study	5.5 (2.0)
7	Zhejiang (Hangzhou) Infant Feeding Study	3.8
8	Chinese Infant Feeding Study living in Perth	N/A #
9	Himeji Infant Feeding Study	5.5 (1.1)

\* Study numbers are the same as Table 1; \*\* SD = Standard Deviation; # N/A = Not available.

**Table 4.** The type of solid foods given to infants in the Asia Pacific region.

Study number *	Study location	The most popular food	The second popular food	The third popular food
1	Australia, 1992	Rice cereal (commercial)	Fruit gels, puree	Milk Custards Yoghurt
2	Australia (Aboriginal mothers)	Milk Custards Yoghurt	Rice cereal (commercial)	Commercial foods with meat
3	Viet Nam	Rice porridge	Rice-floured porridge	Meat and egg
4	Australia, 2002	Rice cereal	Fresh/processed fruits and vegetables	N/A
5	China (Xinjiang)	Rice paste	Rice porridge	Vegetable paste
6	Maldives	Maldivian food made with wheat flour and fish	Rice porridge	Processed food
7	China (Hangzhou)	Rice cereal	Rice porridge	Mashed egg, fish
8	Australia (Chinese migrants)	Egg-Yolk	Commercial infant food	Fruit
9	Japan #	Rice gruel	Japanese noodles	Puree vegetables

N/A = Not Applicable; # Reference [35].

Associations between the timing of introducing solid foods and breastfeeding duration were explored in each study. In Australia, in the PIFS II study, mothers who introduced solids at or after 17 weeks had 11 weeks longer duration of breastfeeding than those who introduced solids before 17 weeks ( $p < 0.001$ ). The Japanese study also found that the timing of the introduction of solid foods was associated with the duration of “any breastfeeding” until six months of age (OR = 1.21, 95% CI = 1.10–1.33). Among Chinese migrants to Australia, mothers introduced solid foods to their infants at similar times to other Australian infants, but this was delayed when compared with mothers in home countries. In Viet Nam, significant factors associated with delayed introduction of solid food at 24 weeks were “if mother was a farmer” (OR = 0.52, 95% CI = 0.18–0.95) and “completed secondary school” (OR = 0.28, 95% CI = 0.10–0.54), whose “husband was satisfied with the infant’s gender” (OR = 0.30, 95% CI = 0.17–0.53), her “mother-in-law preferred exclusive breastfeeding” (OR = 0.18, 95% CI = 0.04–0.75), or her ‘friends practised exclusive breastfeeding’ (OR = 0.41, 95% CI = 0.16–1.10).

#### 4. Discussion

While the timing of introducing solid foods varies between countries, most infants in the Asia Pacific region were introduced to solids earlier than recommended by the WHO. The mean age of introducing solid foods to infants in China (Hangzhou) was 3.8 months, the earliest in these studies, while Japan and Maldives were 5.6 months, closest to the WHO recommended age. Moreover, some studies showed that the timing of the introduction of solid foods was related to not only breastfeeding duration but also maternal occupation, education background, surrounding environments including preferences of family or friends on infant feeding methods. While the timing of solid food introduction is important in reducing problems related to infant health and development, the WHO has also emphasized the importance of the quality of the foods. Solid foods given to infants are often of high volume, with low energy and nutrient density together with a low meal frequency [36]. Our review found that many countries in the Asia Pacific region used rice porridge/cereal (See Table 4) for infants’ first foods since rice is culturally believed to help with digestion. Although some countries, including Japan, excluded this question for ethical reasons, other reports still described that the most common first solid foods was rice gruel [35,37]. These rice products are often of low energy and micronutrient density, including iron, zinc and calcium. In a report by Dewey and Brown [36], the WHO/UNICEF documented that energy requirements from solid foods for infants aged 6–8 months should be 269 kcal per day (1125.5 kJ) and the infants would be able to obtain sufficient energy if they were fed at least three meals with a minimum energy density of 1.0 kcal (4.2 kJ)/g. However, rice porridge has only 37.8 kcal (158 kJ) per 100 g (0.378 kcal/g), a low energy food (See Table 5) [38]. While the WHO report recommended that infants aged 6–8 months, 9–11 months, and 12–24 months should be fed at least 2–3 times, 3–4 times, and 3–4 times per day respectively, this is only applicable when energy and nutrient density is appropriate for the infants age [39]. For infants who are fed rice porridge to meet their energy requirements following the WHO recommendations, they would have to be fed approximately seven times per day. Similarly, the supply of micronutrient composition in rice products is less than the recommended nutrient intakes (Table 6). Several studies have shown that breastfed infants have better absorption of micronutrients, including iron. However, after six months of

age, the quantities of micronutrients in breastmilk become inadequate over time, particularly for iron [40,41]. As this happens to both breast and bottle fed infants, the quality and timing of introduction of solid foods is important in providing adequate micronutrient intakes. In both developed and less developed countries, poor choices of solid foods may lead to nutritional deficiencies.

**Table 5.** Nutritional composition of rice porridge, rice cereal and egg yolk (value per 100 g).

Main nutrients	Rice porridge	Rice cereal *	Hard-boiled egg yolk
Energy, including dietary fibre (kJ)	158	1537	1450
Protein (g)	0.7	6.8	16.1
Fat (g)	0.1	1.1	31.7
Calcium (mg)	2	6	115
Iodine (ug)	0.8	3.7	127.7
Iron (mg)	0.08	15.5	4.8
Zinc (mg)	0.12	7.8	2.7
Riboflavin (B2) (mg)	0.002	1.9	0.42
Pyridoxine (B6) (mg)	0.01	0	0.33
Vitamin C (mg)	0	33	0
Folate, natural (µg)	1	70	177

Source: [38]. \* Note = this products was added vitamins B1, B2, B3, C, folate, iron and zinc.

**Table 6.** Recommended nutrient intakes for infants aged 7–12months and 12–24 months.

Main nutrients	7–12 months	12–24 months
Protein(g/day)	NA	NA
Calcium (mg/day)	400	500
Iodine (µg/day)	90	90
Iron (mg/day)	0.93 #	0.58 #
Zinc (mg/day)	4.1 *	4.1 *
Riboflavin (B <sub>2</sub> ) (mg/day)	0.4	0.5
Pyridoxine (B <sub>6</sub> ) (mg/day)	0.3	0.5
Vitamin C (mg/day)	30	30
Folate, natural (µg/day)	80	150

Source: [42]; Note: \* = Moderate bioavailability; # = 95th percentile absolute requirements.

In developing countries, the inappropriate introduction of solid foods at an early age may be reflected in the proportion of stunting and/or wasting in young children [43]. Breastfeeding and nutritious solid foods play key roles in promoting appropriate nutrition for their growth and development and thus the quality of solid foods need to be focused to reduce the prevalence of under nutrition or malnutrition.

A meta-analysis on the impact of nutritional interventions on infant survival, disease prevention, and stunting concluded that child stunting could be reduced by approximately one third, if nutritional interventions were provided to infants before 36 months of age [44]. It is important to emphasize appropriate nutritious solid foods given to infants at the appropriate time. Golden [45] estimated the Recommended Nutrient Intakes (RNIs) for children who are moderately malnourished, and suggested the importance of a balance in nutrients between the macro- and micro-nutrients. This study also

recognised the importance of nutrient density in the developing world, as many of the earliest foods introduced to infants are high volume with a low nutrient density.

Although our study showed that Maldives almost reached the WHO recommended age of introducing solid foods (5.5 months), the stunting rate under five years old was still 19% between 2006 and 2010 [46]. A more recent study in the Maldives found that within the first seven days after birth approximately 39% and 16% of infants ( $n = 458$ ) were fed honey and dates, respectively, suggesting that the earlier study may have underreported prelacteal and early life feeds [47]. These prelacteal and early infancy feeds were related to specific cultural beliefs, but may also have had detrimental effects on infant health and the incidence of stunting.

In Japan, the mean age for the introduction of solid foods is approximately 5.5 months, and prelacteal feeds are still common, in contrast to the WHO recommendations for exclusive breastfeeding. The first priority for mothers is to continue exclusive breastfeeding for the first six months of life and then introduce nutritious complementary foods, appropriate nutrition during the 6–24 months period is also critical for infants' nutrition and development [2]. Parents should be provided with more detailed information about introducing solid foods, including the quantity, timing, and quality of the foods through breastfeeding education since nutritional status during the first two years of life is critical in terms of their lifelong physical growth and mental development [5].

There are several limitations to consider when drawing conclusions from this study. Although these studies used almost the same questionnaire on infant feeding practices and included WHO standard infant feeding definitions, the sample selection and sizes used mean that the results may not be representative of the whole of the country. Nevertheless, similar methodology used in each study means that the main conclusions of this review can be used for nutrition education. The principal finding of the review is that most countries do not achieve the WHO goal for the timing of the introduction of solid foods. Increased promotion of optimum infant feeding guidelines is needed, including guidance for the appropriate time and manner in which solid foods are introduced. This is an important public health message for infant nutrition in the Asia Pacific region.

## 5. Conclusions

The review of previous observational studies using the same questionnaire on infant feeding practices in the Asia Pacific region has shown that many countries need further improvement in the timing and the quality of first feeds with solid foods. This should be in conjunction with promoting the optimal duration of exclusive breastfeeding. Rice and rice products are commonly used as the first foods in this region and are of low energy density. Without fortification they provide insufficient quantities of micronutrients. Education of not only the mothers, but also other family members, health professionals and the community should be provided in order to facilitate understanding about the importance of breastfeeding and the appropriate introduction of solid foods. Several strategies including a general prohibition of prelacteal feeding in hospitals (except in specific medical circumstances), a ban on distribution of free gifts of infant formula to mothers, and an expansion of the roles of midwives should be explored. Further studies on this topic are required for a better understanding and evaluation of growth and development, and will be able to contribute to the development of more effective strategies in pediatric nutrition in this region.

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## Conflicts of Interest

The authors declare no conflict of interest.

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## 2. Breastfeeding

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### Iron Stores of Breastfed Infants during the First Year of Life

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**Abstract:** The birth iron endowment provides iron for growth in the first months of life. We describe the iron endowment under conditions of low dietary iron supply. Subjects were infants participating in a trial of Vitamin D supplementation from 1 to 9 months. Infants were exclusively breastfed at enrollment but could receive complementary foods from 4 months but not formula. Plasma ferritin (PF) and transferrin receptor (TfR) were determined at 1, 2, 4, 5.5, 7.5, 9 and 12 months. At 1 month PF ranged from 38 to 752 µg/L and was only weakly related to maternal PF. PF declined subsequently and flattened out at 5.5 months. PF of females was significantly higher than PF of males except at 12 months. TfR increased with age and was inversely correlated with PF. PF and TfR tracked strongly until 9 months. Iron deficiency (PF < 10 µg/L) began to appear at 4 months and increased in frequency until 9 months. Infants with ID were born with low iron endowment. We concluded that the birth iron endowment is highly variable in size and a small endowment places infants at risk of iron deficiency before 6 months. Boys have smaller iron endowments and are at greater risk of iron deficiency than girls.

**Keywords:** iron endowment; breastfed infant; iron stores; iron deficiency

#### 1. Introduction

At birth, the body iron content of the infant is high (94 mg/kg fat-free mass) [1] due to a high hemoglobin mass and a sizable amount of storage iron [2]. This birth iron endowment renders the

infant independent of exogenous iron during the early months of life. Immediately after birth, the hemoglobin mass begins to shrink and its iron is transferred to the storage compartment [3]. The latter therefore represents the entire birth iron endowment. Plasma ferritin (PF) concentration is proportional to storage iron and thus provides a measure to follow the fate of the iron endowment. At birth, the size of the endowment varies greatly, as we [4–6] and others [7–18] have shown. Although severe maternal iron deficiency [11] and certain pregnancy complications [19] are known to reduce the size of the iron endowment, under most circumstances iron status of the mother is not a determinant of the size of the iron endowment. The cause(s) of the variation in its size remain largely unknown.

The iron endowment provides iron for growth and protects the breastfed infant against iron deficiency in the first 4–6 months of life. It follows that the size of the endowment should determine the degree of protection afforded the infant. Indeed, there is evidence that diminished size of the storage iron compartment shortens the protection and places the infant at risk of iron deficiency [4–6,20]. Essentially, all breastfed infants in our studies [4–6] who developed iron deficiency by 6 months were born with diminished iron endowment.

As the iron endowment is used up for growth, PF concentration declines, but the rate of decline is modified by exogenous iron [4–6]. Few data exist regarding the unmodified iron endowment. Such data may be valuable for examining longitudinal tracking of iron stores as well as defining gender-related differences of iron stores. Only one cohort of infants in our earlier studies (control group in [5]) came close to having an unmodified iron endowment, but as even these infants could receive supplemental formula, their iron endowment could have been modified to a significant extent. Therefore, when in a recently completed study [21] it was deemed necessary to prohibit the consumption of supplemental formula until 9 months of age, the opportunity to evaluate the size of the iron endowment minimally modified by dietary iron presented itself. The present report concerns data on the iron endowment of these infants who, besides breast milk, received no source of iron until 4 months and thereafter received iron only from complementary foods until 9 months. For this large group of breastfed infants, iron status of the mothers soon after birth was known and detailed dietary information was available.

We define iron deficiency (ID) as a state of exhausted iron stores indicated by  $PF < 10 \mu\text{g/L}$ . When there is, in addition, evidence of impaired hemoglobin synthesis, iron deficiency is considered to be severe. Iron-deficiency anemia (IDA) carries a significant risk of impaired neurocognitive development [22,23].

## 2. Experimental Section

Clinical Trial Registration: Registered at [clinicaltrials.gov](https://clinicaltrials.gov) NCT00494104.

Briefly, the parent study [21] was a randomized double-blind trial of breastfed infants who received one of four doses of Vitamin D supplementation (200 IU/day, 400 IU/day, 600 IU/day and 800 IU/day) from 1 to 9 months. Plasma 25(OH)D concentration was the primary endpoint. In order to minimize dietary Vitamin D intake, parents were asked not to feed supplemental formula.

Study design and intervals: The study was a prospective, randomized, double-blind study. Infants were enrolled and randomized at 1 month (=within 4 days of 28 days). They visited the study center every 28 days until 9 months ( $280 \pm 4$  days) and made a final visit at 12 months ( $364 \pm 4$  days). Infants received the study Vitamin D drops from 1 to 9 months. The study protocol was reviewed and

approved by the University of Iowa Institutional Review Board and parents provided written consent. The trial was registered with ClinicalTrials.gov under NCT00494104.

Subjects were full-term infants considered normal by their parents and their physicians. Infants were exclusively breastfed at enrollment. Starting at 4 months, they could receive complementary foods but no formula until 9 months. Vitamin or mineral (iron) supplements were not permitted. Infants were born between August 2006 and September 2010. Enrollment was limited to infants born between June and November so they would be between 5.5 and 9 months old at the end of winter (March to mid-May) when the primary assessment of Vitamin D status took place. Parents were asked not to give any iron or Vitamin D supplements.

Procedures: Infants visited the study center every 28 days and had weight and length measured. Parents completed an interim health and feeding questionnaire. Code-labeled Vitamin D supplements were dispensed during visits and empty and half-empty containers were collected and weighed. Infants had blood drawn at 1, 4, 5.5, 7.5, 9 and 12 months by heel prick using a disposable spring-loaded device (Tenderfoot, International Technidyne) into heparin-coated tubes. Plasma ferritin (PF) was determined using an immunoradiometric procedure (Ramco catalog no. F-11) with interassay coefficient of variation of 6.5%. Soluble transferrin receptor (TfR) was measured by enzyme immuno assay (Ramco catalog no TF-94).

Data analysis: Iron deficiency was defined as plasma ferritin  $<10 \mu\text{g/L}$  [4–6] and anemia as hemoglobin  $<105 \text{ g/L}$  before 9 months and  $<100 \text{ g/L}$  at 9 and 12 months [24]. Body iron was calculated as  $\text{Body iron (mg/kg)} = (\log(\text{TfR/PF}) - 2.8229)/0.1207$ , where TfR and PF are both in  $\mu\text{g/L}$  [25]. Gender-related differences of PF, TfR and body iron assessed by t-tests and ANOVA procedures. Tracking was determined by linear correlations between successive values. Associations of PF and TfR were determined cross-sectionally on an age-specific basis by linear correlation analysis. Percentiles of PF were determined by SAS univariate procedure. Statistical analyses were performed using SAS version 9.1.3 (SAS Institute, Cary, NC, USA).

### 3. Results

Of 213 infants enrolled at 1 month and assigned to one of the Vitamin D supplement doses, 128 completed the intervention at 9 months and 120 were evaluated at 12 months. The main reason why infants left the study was the parents' desire to use supplemental formula. At enrollment, 177 mothers donated a venous blood sample. Table 1 summarizes feeding data as reported by the parents and shows that parents adhered to feeding rules to a remarkable degree. Only one infant received cereal at 2 and 3 months and 2 infants received formula at 3 months, but the amounts were in each case small ( $\leq 1$  feeding/week) and infants continued in the study. Many infants did not receive complementary foods until late. For example, at 5.5 months 63 infants (of 153) received no complementary foods at all. Also, cereals, a rich source of iron, were received by only one-half of infants at 5.5 months.

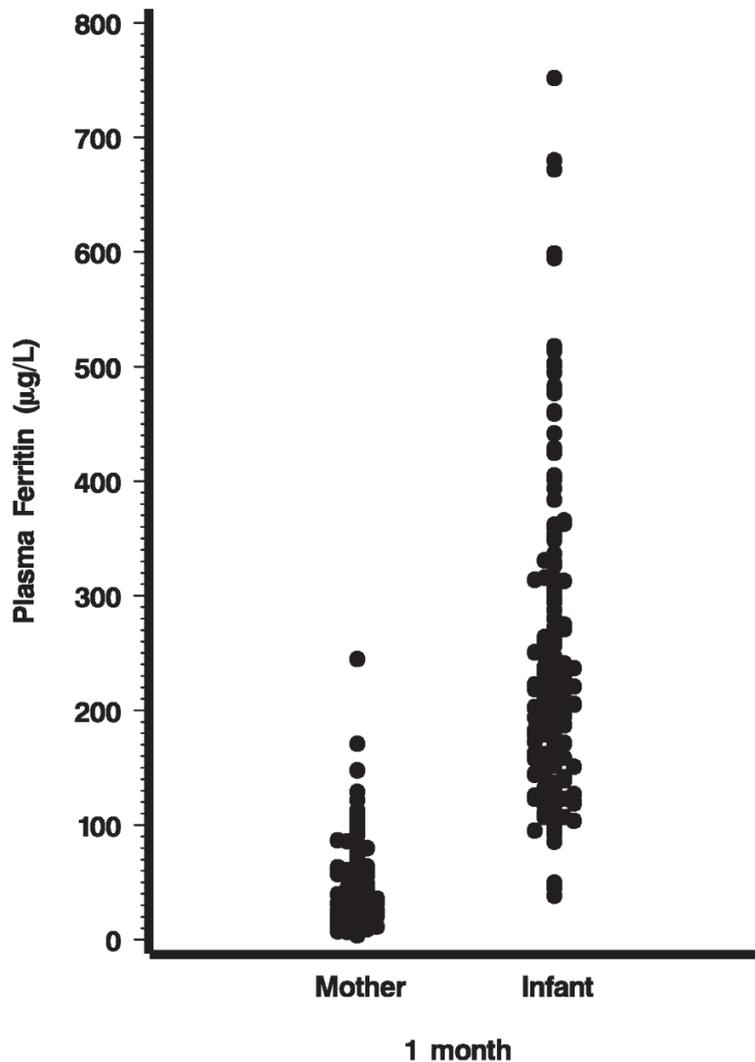
At 1 month, PF averaged  $242 \mu\text{g/L}$  with a range from 38 to  $752 \mu\text{g/L}$  (Figure 1, Table 2). Maternal PF obtained at the same time averaged  $42 \mu\text{g/L}$  (SD  $34 \mu\text{g/L}$ ) (Figure 1). It is evident that a fair proportion of mothers were in less than optimal iron nutritional status, with 12 mothers having  $\text{PF} < 10 \mu\text{g/L}$ . However, iron status of the mother was not an important factor determining infant iron

stores. As illustrated in Figure 2, the relationship between maternal and infant PF levels was weak ( $r = 0.081$ ,  $p = 0.283$ ). Maternal iron status explained only 6.4% of the variation in infant iron endowment. As Figure 2 shows, when the mother's iron stores were low (PF < 20  $\mu\text{g/L}$ , her infant's PF could still range from 40 to 680  $\mu\text{g/L}$ .

**Table 1.** Feedings as reported by parents. The table indicates number of infants receiving the specified food during the month preceding the visit.

Age (month)	Total subjects	Any breast	Cereal	Fruits	Vegetables	Meats	Table foods	Formula	Cow milk
1	213	213	0	0	0	0	0	0	0
2	194	194	1	0	0	0	0	0	0
3	181	181	1	0	0	0	0	2	0
4	165	165	4	2	0	0	0	1	0
5.5	153	152	76	29	40	0	5	1	0
7.5	138	138	79	92	97	12	20	4	1
9	128	124	86	96	99	30	46	9	1
12	120	92	43	70	69	44	92	23	43

**Figure 1.** Plasma ferritin concentrations of mothers and infants one month after birth.

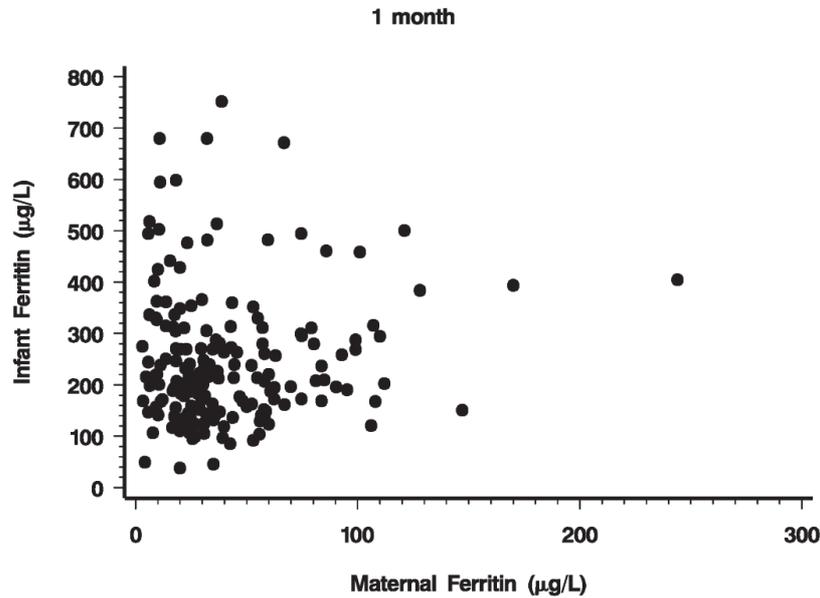


**Table 2.** Plasma concentrations of ferritin (PF) and transferrin receptor (TfR). Values are mean  $\pm$  SD unless otherwise indicated.

Age (month)	1	2	4	5.5	7.5	9	12
<b>Number determinations</b>	201	190	165	152	138	126	118
<i>Plasma ferritin (<math>\mu\text{g/L}</math>)</i>							
All	242 $\pm$ 125	184 $\pm$ 103	88 $\pm$ 57	44 $\pm$ 29	40 $\pm$ 28	26 $\pm$ 17	22 $\pm$ 18
Range	38–752	43–710	10–373	3–137	5–144	4–90	5–137
Female	256 $\pm$ 131	201 $\pm$ 106	98 $\pm$ 58	51 $\pm$ 30	44 $\pm$ 29	30 $\pm$ 18	23 $\pm$ 17
Male	227 $\pm$ 119	169 $\pm$ 99	80 $\pm$ 55	39 $\pm$ 27	36 $\pm$ 27	24 $\pm$ 16	22 $\pm$ 19
p M vs. F	0.105	0.032	0.040	0.015	0.098	0.045	0.715
Number < 10 $\mu\text{g/L}$ (M/F)	0/0	0/0	1/0	6/2	10/1	12/3	9/6
Number < 12 $\mu\text{g/L}$ (M/F)	0/0	0/0	3/0	9/3	14/2	16/7	15/9
<i>Transferrin receptor (mg/L)</i>							
All	3.21 $\pm$ 0.65	4.49 $\pm$ 1.10	6.52 $\pm$ 1.12	6.66 $\pm$ 1.17	7.05 $\pm$ 1.19	7.12 $\pm$ 1.41	6.97 $\pm$ 1.14
Female	3.08 $\pm$ 0.62	4.23 $\pm$ 0.97	6.19 $\pm$ 0.95	6.28 $\pm$ 1.01	6.74 $\pm$ 1.07	6.91 $\pm$ 1.28	6.94 $\pm$ 0.94
Male	3.34 $\pm$ 0.66	4.71 $\pm$ 1.15	6.79 $\pm$ 1.19	6.97 $\pm$ 1.21	7.30 $\pm$ 1.23	7.23 $\pm$ 1.50	6.90 $\pm$ 1.29
p M vs. F	0.0037	0.0027	0.0006	0.0003	0.005	0.200	0.85
Correl. coeff. PF vs. TfR	0.026	-0.182 <sup>a</sup>	-0.218 <sup>a</sup>	-0.433 <sup>a</sup>	-0.360 <sup>a</sup>	-0.104	-0.135
<i>Body iron (mg/kg)</i>							
All	13.7 $\pm$ 1.91	11.4 $\pm$ 2.32	7.20 $\pm$ 2.67	4.60 $\pm$ 2.90	3.03 $\pm$ 2.91	2.67 $\pm$ 2.48	2.12 $\pm$ 2.32
Female	14.1 $\pm$ 1.70	12.1 $\pm$ 2.01	7.94 $\pm$ 2.25	5.45 $\pm$ 2.57	4.63 $\pm$ 2.57	3.27 $\pm$ 2.24	2.26 $\pm$ 2.18
Male	13.3 $\pm$ 2.02	10.9 $\pm$ 2.43	6.58 $\pm$ 2.84	3.91 $\pm$ 2.59	3.33 $\pm$ 3.06	2.22 $\pm$ 2.58	2.01 $\pm$ 2.44
p M vs. F	0.0031	0.0004	0.0011	0.0010	0.0091	0.0188	0.579

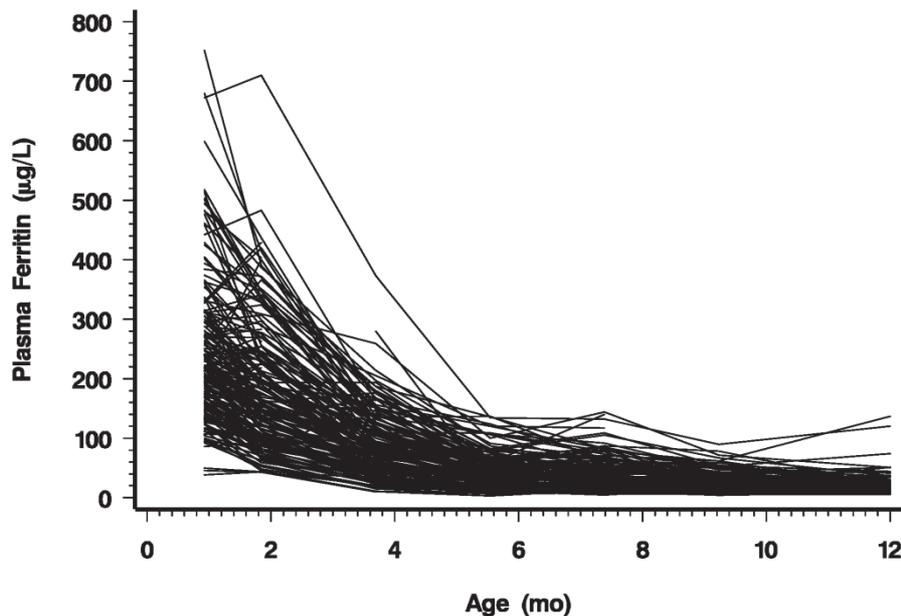
<sup>a</sup> correlation statistically significant ( $p < 0.05$ ).

**Figure 2.** Relationship between maternal and infant plasma ferritin one month after birth ( $r = 0.081, p = 0.283$ ).

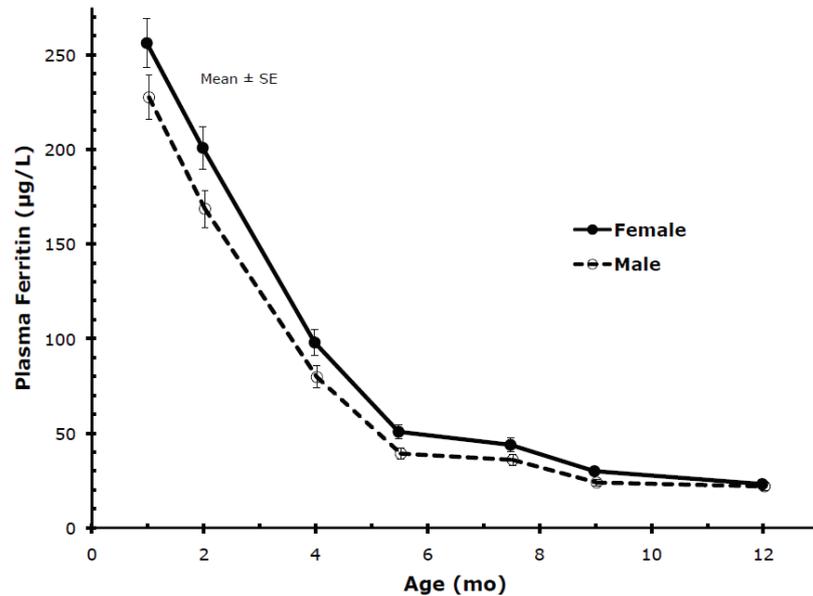


As shown in Figure 3 and Table 2, infant PF decreased rapidly with age but leveled off after 5.5 months, indicating exhaustion of the iron endowment. In some infants, PF increased between 1 and 4 months, presumably indicating continuing recycling of iron from hemoglobin breakdown. Transient increases of PF at other ages indicate acute phase reactions. From 2 to 5.5 months, average PF declined by 1.1 (SD 0.40) % each day. The decline was strongly inversely correlated with gain in weight and length ( $p \leq 0.0001$ ). This is consistent with the notion that growth is the main cause of the decline of PF. As PF decreased, the range of values narrowed progressively. This is illustrated by the percentile values shown in Figure 4.

**Figure 3.** PF of individual infants from 1 to 12 months.



**Figure 4.** PF of males and females. Differences were statistically significant except at 1 and at 12 months.



Concentrations of TfR increased with age until 7.5 months and then leveled off, mirroring PF values (Table 2). TfR was significantly inversely correlated with PF at most ages. Body iron was highest at 1 month and declined progressively thereafter, in essence paralleling the course of PF.

Tracking: In spite of the marked decrease of PF, there was a strong tendency for infants to preserve their rank. PF values correlated (tracked) strongly over time (Table 3). Although tracking is to be expected given the wide range of PF values at 1 month, tracking continued at 9 and 12 months, which suggests that other factors, including genetic, may be operating. TfR also showed tracking which was less strong than that of PF but was still quite strong (Table 3).

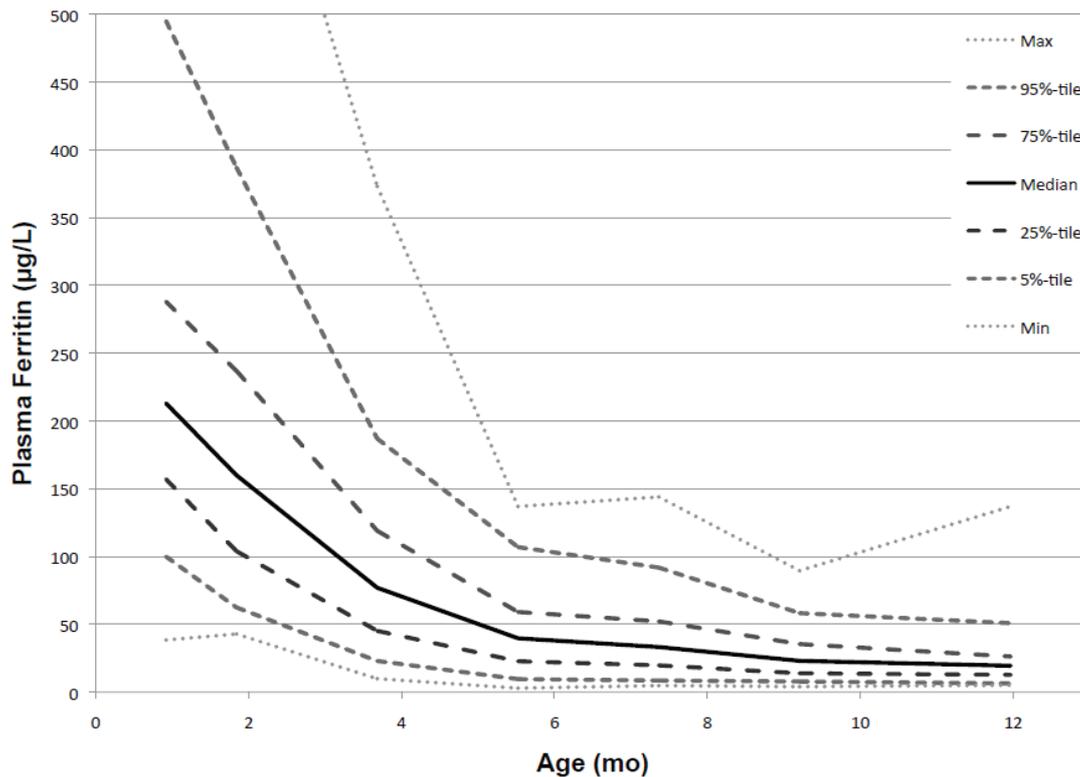
**Table 3.** Pearson correlations among PF (a) values and TfR (b) values at different ages.

<b>(a) PF</b>					
Age	4 months	5.5 months	7.5 months	9 months	12 months
<b>1 month</b>	0.670 <sup>a</sup>	0.638 <sup>a</sup>	0.496 <sup>a</sup>	0.546 <sup>a</sup>	0.465 <sup>a</sup>
<b>4 months</b>	-	0.738 <sup>a</sup>	0.680 <sup>a</sup>	0.700 <sup>a</sup>	0.438 <sup>a</sup>
<b>5.5 months</b>	-	-	0.751 <sup>a</sup>	0.747 <sup>a</sup>	0.511 <sup>a</sup>
<b>7.5 months</b>	-	-	-	0.804 <sup>a</sup>	0.515 <sup>a</sup>
<b>9 months</b>	-	-	-	-	0.579 <sup>a</sup>
<b>(b) TfR</b>					
Age	4 months	5.5 months	7.5 months	9 months	12 months
<b>1 month</b>	0.321 <sup>a</sup>	0.357 <sup>a</sup>	0.319 <sup>a</sup>	0.297 <sup>a</sup>	0.146
<b>4 months</b>	-	0.616 <sup>a</sup>	0.613 <sup>a</sup>	0.423 <sup>a</sup>	0.321 <sup>a</sup>
<b>5.5 months</b>	-	-	0.616 <sup>a</sup>	0.543 <sup>a</sup>	0.330 <sup>a</sup>
<b>7.5 months</b>	-	-	-	0.605 <sup>a</sup>	0.515 <sup>a</sup>
<b>9 months</b>	-	-	-	-	0.525 <sup>a</sup>

<sup>a</sup> correlation statistically significant ( $p < 0.05$ ).

Gender: Plasma ferritin showed marked gender-related differences as indicated in Table 2 and Figure 5, with levels of female infants being significantly higher than levels of male infants at most ages. Accordingly, female infants became iron deficient ( $PF < 10 \mu\text{g/L}$ ) less frequently than male infants (Table 2). Gender-related differences were also present in TfR but, contrary to PF, males had higher levels, with differences being statistically significant except at 9 and 12 months. Body iron was significantly higher in females than males except for 12 months.

**Figure 5.** Percentile values for PF from 1 to 12 months (males and females combined).



Infants who developed ID: PF values less than  $10 \mu\text{g/L}$  indicating exhausted iron stores were observed at almost all ages. The earliest age at which  $PF < 10 \mu\text{g/L}$  occurred was 4 months in one infant. At 5.5 months, the PF of an additional seven infants dropped below  $10 \mu\text{g/L}$ , meaning that eight (5.3%) infants had exhausted their Fe stores before 6 months. At 28 days, the mean PF of these eight infants was  $125 \pm 103 \mu\text{g/L}$ , which was significantly ( $p < 0.001$ ) lower than the PF of all other infants at 28 days (mean  $245 \pm 119 \mu\text{g/L}$ ). At 7.5 months, an additional nine infants developed a PF of  $\leq 10 \mu\text{g/L}$ , meaning that 16 (11.6%) infants had exhausted their iron stores. After 7.5 months, with the birth iron endowment exhausted, a PF  $\leq 10 \mu\text{g/L}$  was reflective of low dietary iron intake. This occurred in 15 infants at 9 months and 15 infants at 12 months. A total of 36 infants (19% of those randomized at 4 months) developed a PF  $\leq 10 \mu\text{g/L}$  at least once during the study. PF values  $< 12 \mu\text{g/L}$  were observed on 78 occasions (Table 2). Among infants with  $\leq 65 \mu\text{g/L}$  at 56 days of age ( $N = 12$ ), one developed ID at 4 months and four more by 5.5 months. Thus, among infants with a PF less than  $65 \mu\text{g/L}$  at 2 months, 42% developed ID before 6 months.

When infants developed a PF of  $\leq 10 \mu\text{g/L}$ , the parents were informed and the suggestion was made by the investigators to increase the consumption of iron-containing foods. One such infant was placed

on ferrous sulfate by his pediatrician and subsequent PF values were  $>10 \mu\text{g/L}$ . The parents of three infants chose to withdraw from the study. In all remaining infants with ID, PF was monitored closely and in seven infants the next PF was  $>10 \mu\text{g/L}$ . In cases where it remained  $<10 \mu\text{g/L}$  ( $N = 5$ ) hemoglobin was determined to check for IDA. Hemoglobin was in each case  $>105 \text{ g/100 mL}$ . With IDA ruled out, no iron treatment was recommended. The parents were always informed of findings and feeding of iron-rich foods was recommended whenever PF was  $\leq 10 \mu\text{g/L}$ .

#### 4. Discussion

We believe our cohort to be the largest group of breastfed infants in whom the birth iron endowment was assessed while the intake of iron from exogenous sources was minimal. Thus, the iron endowment was observed in its undisturbed state while not being modified by dietary iron. The size of the iron endowment was assessed by determination of plasma ferritin concentration (PF). At the earliest assessment at one and two months, the iron endowment showed enormous variation. This wide range in the size of the iron endowment has been previously shown by us [4–6] and others [7–18]. Only a small proportion of that variation was explained by iron status of the mother whereas its bulk remained unexplained. Earlier studies found for the most part no association or only a weak association between maternal iron status and infant iron status at birth [7,9,10,16–18]. Some studies did find a reduction of infant iron status when maternal iron status was very poor [8,11,12]. Except for some maternal conditions that are known to reduce the size of the iron endowment [19], the cause of the variation is unknown.

Because infants of the present cohort were not permitted to receive supplemental formula, a source of dietary iron, we expected the iron endowment to be somewhat smaller than in the control infants of our earlier study [5] who received complementary foods like the present cohort but did also receive supplementary formula. However, PF values were nearly identical in that earlier group [5] and in the present cohort. For example, at 5.5 months mean PF in the earlier group was  $42 \pm 29 \mu\text{g/L}$  and was  $44 \pm 29 \mu\text{g/L}$  in the present study. The explanation may be that infants who did receive formula received only modest amounts, and many infants probably received no formula at all.

While the cause of the variation in the size of the iron endowment remains obscure, its consequences are readily apparent. The small size of the iron endowment means that it becomes exhausted early and places the infant at risk of ID. We [5,6] and others [19] have before noted this association between size of the iron endowment and risk of ID early in life. In the present cohort a full 5.3% of infants developed ID by 6 months. The incidence is comparable to the incidence observed earlier by us [5,6]. Others have reported somewhat lower [26] and also higher [27] incidences of ID by 6 months. The present estimate of the incidence of ID in breastfed infants who receive only modest amounts of dietary iron is solid thanks to the design of the study. On the other hand, the present study provides no estimates of the incidence of IDA. The reasons are that hemoglobin was not determined with all PF determinations but was determined only when PF remained low in spite of the parents being requested to increase the amount of iron-containing foods. Without that intervention, some infants with ID might have gone on to develop IDA on subsequent assessment.

The present study shows marked gender-related differences in the size of the iron endowment, with girls having significantly greater size than boys. There was also a gender-related difference in TfR

except in the opposite direction, with boys having higher values than girls. Similar gender-related differences have been reported by Hay and colleagues [16,26], Domellöf *et al.* [28] and were seen in our earlier studies [5,6]. PF and TfR were inversely correlated at most ages. Our data clearly show that there are gender-related differences in the size of the iron endowment. This means that whatever is causing gender-related differences at later ages is already operating *in utero*.

We showed strong tracking of PF and TfR that decreases in strength somewhat with increasing age but is still strong at 12 months for PF. Tracking of PF has been reported before by Hay *et al.* [16] and by us [5,6]. Tracking, together with gender-related differences, provides a strong suggestion that the size of the iron endowment and iron status in general are controlled by genetic factors.

The strengths of the present study are that it involved a large cohort of breastfed infants in whom the intake of dietary iron was kept to a practical minimum, permitting study of the natural course of the birth iron endowment without modification by dietary iron. Another strength was that all observations were made in longitudinal fashion and that detailed dietary information was recorded.

## 5. Conclusions

It was concluded that the birth iron endowment is highly variable in size and differs significantly between males and females. Its size decreases as iron is used for growth. Some infants develop ID by 6 months of age. A small iron endowment places infants at increased risk of iron deficiency. The fact that PF and TfR track strongly during infancy suggests the operation of genetic factors.

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## Author Contributions

EEZ and SEN designed the study, analyzed the data and wrote the manuscript. JMJ had responsibility for carrying out the study. All authors read the manuscript and agree with it.

## Conflicts of Interest

The authors have no conflicts of interest to disclose.

## Financial Disclosure

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## Ethnic Variation in Breastfeeding and Complimentary Feeding in the Republic of Ireland

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**Abstract:** Early nutrition plays a pivotal role in long-term health. The World Health Organization (WHO) recommends exclusive breastfeeding during the first six months of life, with the gradual introduction of solids after this period. However, studies in the Republic of Ireland (ROI) have shown poor compliance with guidelines. The ROI continues to have one of the lowest breastfeeding rates worldwide. Our objective was to analyse differences in breastfeeding and complimentary feeding behaviours between Irish and non-Irish mothers residing in the ROI, as well as the role of acculturation on these behaviours, using the national longitudinal study, Growing Up in Ireland (GUI). Mothers ( $n = 11,134$ ) residing in the ROI were interviewed when their infants were nine months of age. The percentage of Irish mothers who initiated breastfeeding was 49.5%, as opposed to 88.1% among the non-Irish cohort ( $p < 0.001$ ). Breastfeeding initiation reduced from 89.4% of non-Irish mothers who had arrived within the last year to five years ago to 67.5% for those who had arrived 11 to >20 years ago ( $p < 0.001$ ). Our results indicate that cultural differences are an important factor in shaping patterns of infant feeding in the ROI. Reviewing existing support and education policies for parents is required to achieve the implementation of desirable infant feeding practices.

**Keywords:** infant feeding; breastfeeding; complimentary feeding; acculturation

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## 1. Introduction

Early nutrition plays a pivotal role in long-term health. Breastfeeding has been shown to have a protective role in the development of being overweight, obesity and chronic diseases later in life [1–3]. The World Health Organization (WHO) recommends exclusive breastfeeding during the first six months of life of the infant, with the gradual introduction of complimentary foods after this period [4]. The European Society of Paediatric, Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommends not to introduce complimentary foods before 17 weeks and no later than 26 weeks, while also giving the advice to commence the introduction of solids near six months of age [5]. Early complimentary feeding has been shown by studies to increase the risk of overweight and obesity during childhood and adulthood [6–9]. Moreover, the transition from milk to solid foods can have a life-long influence on dietary patterns [6,10–12]. The introduction of complimentary foods cannot be studied in isolation from the type of milk feeding early in life, as milk type influences the type of solid foods introduced and the timing of their introduction [13]. Studies on the predictors of early complimentary feeding have shown that breastfeeding reduces the likelihood of early solid food introduction [14–18].

In the Republic of Ireland (ROI), the Department of Health and Children updated their advice in 2003 to recommend adherence to the WHO advice of exclusive breastfeeding during the first six months of the infants' life [19]. The new Infant Feeding Guidelines released by the Food safety Authority of Ireland (FSAI) in November, 2012, maintain the recommendation made by ESPGHAN [20]. Despite these guidelines, the ROI continues to have one of the lowest breastfeeding rates worldwide, and compliance with complimentary feeding guidelines is poor [21]. Irish studies show rates of exclusive breastfeeding for six months of less than 1%, with 75% of infants being introduced to complimentary feeding before 17 weeks, 22.6% of these being introduced prematurely by 12 weeks [15,22].

Rates of breastfeeding in Ireland have increased since 2004, but they are still below national targets, and a large percentage of this increase has been attributed to changes in maternal characteristics, such as older age and an increase in non-national mothers [23–25]. Previous studies in the ROI have pointed out different patterns of breastfeeding rates by maternal origin of birth [23,26,27]. However, we are not aware of any studies in the ROI analysing different patterns in complimentary feeding introduction by ethnic group. Given the fact that the time of complimentary feeding introduction seems to be linked to the type of milk feeding early in life and given the low breastfeeding rates in the ROI, it could be hypothesized that Irish born mothers are more likely to introduce complimentary foods early in the life of their infant, thus increasing the risk of their infants suffering adverse health effects in the short and long term. Moreover, acculturation of non-Irish mothers could play a role in their infant feeding practices. The aim of this paper is to study variation in breastfeeding rates and the timing of the introduction of complimentary feeding between Irish and non-Irish mothers living in the ROI, as well as the role of acculturation on these behaviours using cross-sectional data from the national longitudinal study of children in Ireland (Growing Up in Ireland).

## 2. Methods

### 2.1. Study Design and Sample

Growing Up in Ireland (GUI) is a nationally representative cohort study of nine-month-old infants residing in the Republic of Ireland. The main aim of the study is to study the factors affecting the lives of infants in Ireland with the aim of creating evidence-based policy. The study sample consisted of 11,134 nine-month-old infants who participated in the first wave of the GUI study. These were selected from the approximately 41,000 births over the period of 1 December 2007 to 30 June 2008. The completed sample of 11,134 represents approximately one-third of all births in the ROI over the field period. Families were invited to participate in the study when the child was nine months of age. The sampling frame for the project was the Child Benefit Register for the Republic of Ireland. Of 16,136 mothers selected from the sampling frame, 11,134 agreed to take part in the study, a response rate of 69% [28].

### 2.2. Questionnaires and Measurements

Primary caregivers, defined as the person who spent more time with the child, and secondary caregivers were interviewed at home and asked to complete a main questionnaire and a sensitive questionnaire. Since only 0.5% of the primary care givers nominated were not the biological mothers, we refer to responses from the primary care giver as those of the mother. Interviews were carried out using a mixture of CAPI (computer-assisted personal interviewing) and CASI (computer-assisted self-interviewing).

The wave one sample was selected from the Child Benefit Register for the Republic of Ireland, which was provided by the Department of Social Protection. Of 16,136 mothers selected from the sampling frame, 11,134 agreed to take part in the study, a response rate of 69%. Fieldwork was carried out over 7 months, extending from September 2008, to the end of April 2009. Children were selected so as to be 9-months-old at the time of the interview; consequently, eligible children were all those born between 1 December 2007 and 30 June 2008 [28].

The sampling frame for the study was the Child Benefit Register for the Republic of Ireland. The sample was selected on a systematic basis, pre-stratifying by marital status, county of residence, nationality and number of children (where child is defined as <16 years of age) in the household, using a random start and constant sampling fraction. The completed sample was statistically grossed or reweighted on the basis of external population estimates to ensure that it was wholly representative of all children aged one year or less in Ireland [28].

Interviewers measured and recorded both parents' height and weight. A medically approved mechanical SECA 761 weighing scale was used for the adults' weight and a Leicester measuring stick for their height. All stages of the Growing Up in Ireland project were subject to rigorous ethical review by a Research Ethics Committee convened by the Department of Children and Youth Affairs of the Irish Government. This included a review of all instrumentation, recruitment, consent and implementation protocols adopted at all stages of the study [28].

### 2.3. Statistical Analysis and Dependent Variable

The Statistical Package for the Social Sciences statistical software package version 19.0 (SPSS, Inc., Chicago, IL, USA) and STATA 13 (StataCorp LP, College Station, TX, USA) were used for the statistical analysis. Several independent variables considered as risk factors for early complimentary feeding were selected from the database. These included demographic factors, such as maternal age, maternal education, socioeconomic status and parity, and biological factors, such as maternal BMI, mode of delivery and infant's health. In order to study the variations in complimentary feeding by ethnic group, as well as the influence of breastfeeding in its timing, the model was also adjusted for ethnicity, length of stay in the ROI, breastfeeding initiation and duration of exclusive breastfeeding. Data was analysed using cross-tabulations, and the  $\chi^2$  statistical test, as well as multivariate binary logistic regression. Independent variables were included in the multivariate analysis if they were significant in the bivariate analysis.

Mothers were asked to report their ethnic or cultural background. The following options were provided; Irish, Irish traveller, any other white background, African, any other black background, Chinese, any other Asian background, and other, including mixed background. A recoded binary variable was constructed with two categories: Irish ethnic background and non-Irish ethnic background. This recoded variable was then combined with a variable that asked non-Irish mothers how long they had been residing in the ROI.

The dependent variable "early complimentary feeding" was constructed from a question in the database that asked mothers to indicate when they started to give their infants solid foods at least twice a day for several weeks. Solid foods were defined as baby cereals, pureed fruits, *etc.*, and not milk or drinks. The dependent variable used is therefore the age at which complimentary feeding was established rather than the child's age when solid foods were first introduced. Following ESPGHAN's guidelines, a binary dependent variable was created with two categories <17 weeks for early complimentary feeding and  $\geq 17$  weeks for the acceptable introduction of complimentary feeding [5,28]. Statistical significance was taken as a *p*-value of < 0.05. The weights were on for all statistical analysis.

### 2.4. Definition of Covariates

Socio-economic status (SES) was assessed using three different indicators: household class, equivalised household income quintiles and household type. Income is equivalised to take into account household size and composition using the modified Organization for Economic Cooperation and Development equivalence scale (first adult value, 1; second or higher adults, 0.5; children aged < 14, 0.3). Primary and secondary caregivers were asked questions about their current occupation to derive the variable household class. Where the respondent was economically inactive (retired or unemployed) at the time of interview, previous employment was considered. The household class classification adopted was that used by the Central Statistics Office (CSO): professional workers, managerial and technical, non-manual, skilled-manual, semi-skilled, unskilled, all other gainfully occupied and unknown and never worked at all. This variable was recoded to contain only five categories: professional, managerial and technical workers; non-manual; skilled and semi-skilled

manual; unskilled and all other gainfully occupied and unknown, and never worked at all. Household type is a fourfold variable derived from whether the study child is living in a one or two parent family, as well as the number of children (<18 years) living in the household. This resulted in a classification as follows: one parent, one child; one parent, two or more children; two parents, one child; two parents, two or more children [28].

Maternal education was coded as follows: no formal or primary education, secondary education and third-level education. Maternal age was coded as follows:  $\leq 24$ , 25–34 and  $\geq 35$  years old. Measured parent BMI was classified according to the World Health Organization (WHO) classifications as underweight  $<18.5 \text{ kg/m}^2$ , normal weight  $\geq 18.5$  and  $<25 \text{ kg/m}^2$ , overweight  $\geq 25 \text{ kg/m}^2$  and  $<30 \text{ kg/m}^2$  and obese  $\geq 30 \text{ kg/m}^2$  [28].

Mothers were asked about their infants' overall health using the question: "In general, how would you describe the baby's current health" with response categories "very healthy, no problems", "healthy, but a few minor problems", "sometimes quite ill" and "always unwell". Children are defined as having been breastfed if they consumed breast milk at any stage regardless of the amount of time the baby was breastfed, including the colostrum in the first few days after birth. Exclusive breastfeeding was defined as the infant receiving only breast milk without any additional food or drink, regardless of the length of exclusive breastfeeding. The variable "duration of exclusive breastfeeding" was constructed from the question "how old was the baby when he/she stopped being exclusively breastfed" [28,29].

## 2.5. Missing Data

Some of the independent variables used in the analysis had a large percentage of missing cases: maternal BMI (5.1%) and equivalised household annual income (7.8%). This would have resulted in a proportion of the sample being lost from the analysis. In response, multiple imputation has been carried out in STATA 13 using the variables maternal age, maternal education, father's education, household class, maternal employment status, current maternal smoking and migrant status to predict the missing values in maternal BMI and equivalised household annual income.

## 3. Results

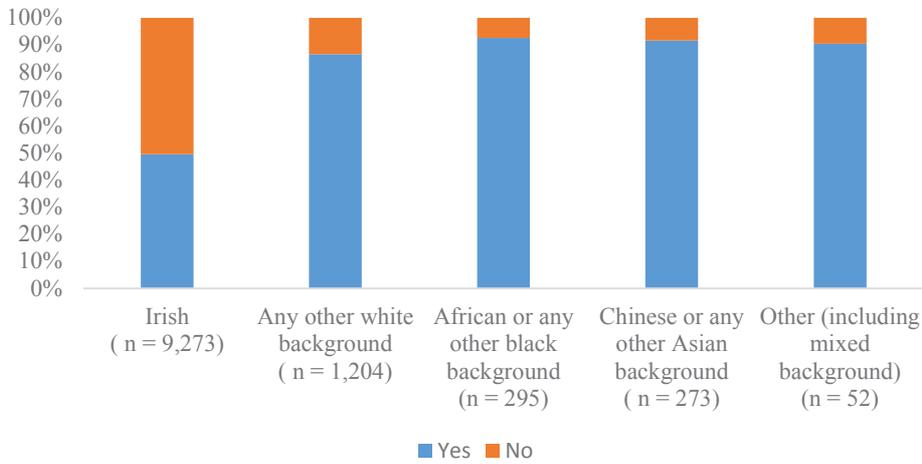
### 3.1. Characteristics of the Study Cohort

Table 1 shows the characteristics of mothers disaggregated by ethnic group. The primary caregiver was defined as the person who spent the most time with the study infant. Irish mothers were younger on average than non-Irish (31.7 years with SD 5.3 compared to 30.9 with SD 5.4). Of those mothers with an Irish ethnic background, 49.5% initiated breastfeeding compared to 88.1% of those mothers with a non-Irish ethnic background. The mean duration of any breastfeeding for those that breastfed at all was 71.1 days (SD 66.4) for Irish mothers compared to 95.8 days (SD 69.2) for non-Irish. A higher percentage of mothers with an Irish ethnic background (15.5% vs. 7.6%) introduced complimentary foods early.

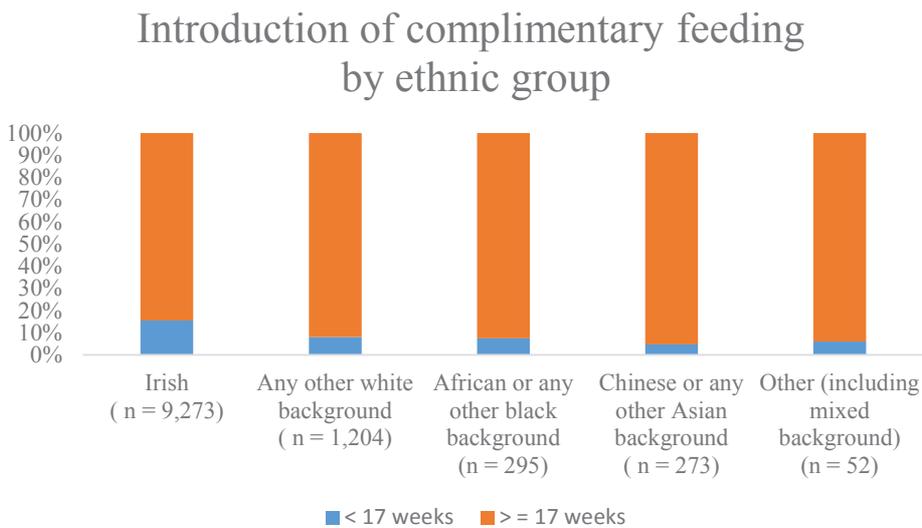
3.2. Percentage of Infants’ Breastfed and the Introduction of Complimentary Feeding in the <17 Weeks and ≥17 Weeks Categories Classified by Ethnic Group

Figure 1 shows that the ethnic group with the highest percentage of mothers who breastfed their infants was African or any other black background, with 92.5% of mothers initiating breastfeeding. This group was followed by Chinese mothers, with 91.6% of breastfeeding initiation. Figure 2 shows the percentage of mothers introducing complimentary foods in the <17 weeks and ≥17 weeks categories classified by ethnic group. Fifteen-point-five percent of mothers with an Irish ethnic background introduced complimentary foods early (<17 weeks). The group with the lowest percentage of mothers introducing complimentary foods early (4.8%) was Chinese or any other Asian background.

**Figure 1.** Breastfeeding initiation by ethnic group.



**Figure 2.** Introduction of complimentary feeding by ethnic group.



**Table 1.** Characteristics of whole sample, national and non-national primary caregivers.

Characteristic	Primary Caregiver		Irish		Non-Irish	
	Sample ( <i>n</i> *)	Mean or % †	SD	Sample ( <i>n</i> *)	Mean or % †	SD
Mean age		31.7	5.3		30.9	5.4
BMI primary carer kg/m <sup>2</sup>	Underweight (less than 18.5)	9275	2.5	1859	3.7	
	Normal weight (18.5–24.9)		50.5		51.1	
	Overweight (25–29.9)		30.1		28.5	
	Obese (≥30)		16.8		16.7	
Having ever breastfed	Yes	9273	49.5	1859	88.1	
	No		50.5		11.9	
Having exclusively breastfed ‡	Yes	4592	39.3	1634	66.5	
	No		10.2		21.4	
Mean duration of any breastfeeding (days) ‡	4019	71.1	66.4	1153	95.8	69.2
Mean duration of exclusive breastfeeding (days) ‡	3556	74.7	62	1158	95.9	63.2
Introduction of complimentary feeding	<17 weeks	9151	15.5	1755	7.6	
	≥17 weeks		84.5		92.4	

\* *n* provided is number of primary caregivers who answered each question; † Percentages provided are based on the total sample; ‡ mothers who reported not having ever breastfed were filtered out.

### 3.3. Predictors of Early Complimentary Feeding

Table 2 shows the adjusted model of significant factors that independently predicted early complimentary feeding introduction for the whole sample. After adjustment, the significant factors included the primary caregiver's age, education, BMI, ethnicity and length of stay in the ROI, household class, household type and exclusive breastfeeding duration.

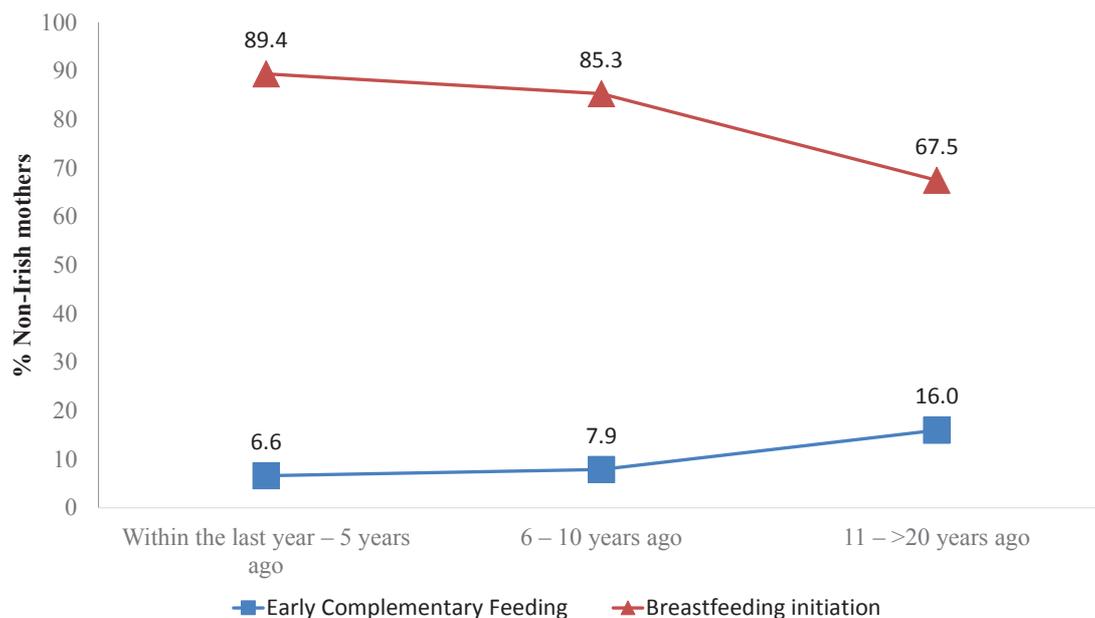
Non-Irish mothers who had been living in the ROI < 6 years were 50.7% less likely to introduce complimentary feeding early compared to Irish mothers (OR 0.493, 95% CI 0.371, 0.656). This protective effect of ethnicity decreased with the length of stay in the ROI, with non-Irish mothers who had been living in the ROI 11 to >20 years being 3.1% less likely to introduce complimentary feeding early when compared to Irish mothers (OR 0.969, 95% CI 0.613, 1.532).

Those mothers who exclusively breastfed >90 days were 93.9% less likely to introduce early complimentary feeding (OR 0.061, 95% CI 0.037, 0.101) when compared to those who did not exclusively breastfed.

### 3.4. Effects of Acculturation on Breastfeeding Initiation and Early Complimentary Feeding

Figure 3 shows the effects of acculturation on breastfeeding initiation and early complimentary feeding. Breastfeeding initiation in the non-Irish cohort reduced from 89.4 of mothers who had arrived within the last year to five years ago to 67.5% for those who had arrived 11 to >20 years ago ( $p < 0.001$ ). The percentage of non-Irish mothers introducing complimentary foods early increased from 6.6% for those who had arrived within the last year to five years ago to 16.0% for those who arrived 11 to >20 years ago ( $p < 0.001$ ).

**Figure 3.** The effect of acculturation on breastfeeding initiation and early complimentary feeding.



**Table 2.** Characteristics of Irish and non-Irish primary caregivers and households in the <17 weeks and 17 weeks complimentary feeding categories and binary logistic regression of the significant factors associated with the introduction of complimentary feeding.

Characteristics	Complimentary Feeding Introduction *														
	Total			<17 Weeks			≥17 Weeks			Unadjusted †			Adjusted ‡		
	n	% <sup>γ</sup>	n	% <sup>η</sup>	n	% <sup>η</sup>	n	% <sup>η</sup>	OR	p <sup>§</sup>	OR	95% CI	p <sup>δ</sup>		
<i>Primary caregiver age (years)</i>															
≥35	3467	31.8	426	12.3	3041	87.7			0.467		0.606	0.494	0.744	<0.001	
25–34	6119	56.1	823	13.4	5296	86.6			0.519		0.664	0.555	0.794	<0.001	
≤24	1321	12.1	304	23.0	1017	77.0			1.0 <sup>§</sup>	<0.001	1.0 <sup>§</sup>			<0.001	
<i>Maternal education</i>															
Third level education	5330	48.9	557	10.5	4773	89.5			0.534		0.820	0.602	1.117	0.208	
Secondary level education	5189	47.6	926	17.8	4263	82.2			0.994		1.031	0.773	1.374	0.835	
No formal or primary education	378	3.5	68	18	310	82			1.0 <sup>§</sup>	<0.001	1.0 <sup>§</sup>			0.004	
<i>Household class</i>															
Professional, managerial and technical workers	5228	47.9	578	11.1	4650	88.9			1.0 <sup>§</sup>		1.0 <sup>§</sup>			0.001	
Non-manual	1983	18.2	332	16.7	1651	83.3			1.620		1.179	1.001	1.387	0.048	
Skilled and semi-skilled manual	2428	22.3	377	15.5	2051	84.5			1.478		1.111	0.941	1.311	0.214	
Unskilled and all other															
gainfully occupied and unknown	283	2.6	66	23.3	217	76.7			2.430		1.850	1.346	2.544	<0.001	
Never worked at all	985	9.0	200	20.3	785	79.7			2.051	<0.001	0.972	0.752	1.258	0.830	





#### 4. Discussion

Breastfeeding is the most beneficial and nutritionally complete feeding method during infancy [30]. However, breastfeeding initiation rates in the ROI were the lowest compared to 14 European countries in 2010 [21]. Despite modest increases in breastfeeding rates, as shown in the Perinatal Statistics Report in 2012, these rates are still far from national targets and international averages [21,31–34].

In Table 1, it can be observed how a lower percentage of Irish mothers (49.5%) initiated breastfeeding compared to their non-Irish counterparts (88.1%). These findings correlate with previous studies in the ROI, which found similar percentages of breastfeeding initiation in the Irish and non-Irish mothers [23,26,27]. The percentages between the two cohorts are nearer to each other when mothers are asked about exclusive breastfeeding. However, the mean duration of any breastfeeding, as well as exclusive breastfeeding is lower for the Irish cohort. Both cohorts are far from complying with guidelines recommending six months of exclusive breastfeeding. However, a stronger predisposition towards breastfeeding, possibly due to cultural differences, is observed in the non-Irish group.

Figure 1 also shows that breastfeeding initiation was higher in all other ethnic groups when compared to the Irish cohort. The fact that any other white background has 86.5% breastfeeding initiation concurs with the study findings from 2010 in which Ireland had the lowest breastfeeding rates when compared to 14 European countries.

Differences in breastfeeding rates by ethnic background have been pointed out by other studies internationally [35–39]. Acculturation plays a role in infant feeding practices; as shown in Figure 2, the amount of non-national mothers initiating breastfeeding decreased the longer they had been living in the ROI ( $p < 0.001$ ). Moreover, the percentage of these mothers introducing complimentary foods early also increased with a longer stay in the country ( $p < 0.001$ ). This finding suggests the close relationship between the early milk feeding method chosen and the introduction of complimentary foods. Further exploration of the reasons behind these changes in infant feeding choices by non-Irish mothers is needed. Factors, such as societal pressures, language barriers and the perception of behaviours in the adopted culture as being modern, could potentially play a role in the acculturation mechanisms.

The relationship between acculturation and milk feeding choices has been reported by different studies in the United States (US) and Australia [40–44]. A study published in 2010 found that a group of Chinese mothers living in Ireland had a less positive attitude and more misconceptions about breastfeeding than a group of Chinese mothers living in Perth, Australia, suggesting a possible role of ‘acculturation’ and the mothers adapting themselves to the formula feeding culture of Ireland [45]. On the other hand, a 2013 study from Australia pointed out that Chinese mothers living in Perth had higher breastfeeding initiation rates and a longer duration of breastfeeding than Chinese mothers in Chengdu. Reported breastfeeding initiation rates in Australia are much higher than in the ROI [31,46]. These findings suggest that the culture of the adopted country may be an important influence on infant feeding practices among migrants.

Lack of breastfeeding and the use of formula feeding have been related to early complimentary feeding by many studies [14,15,17,18]. Formula feeding has been associated with impairment of appetite self-regulatory mechanisms, leading to infants demanding the introduction of solids earlier, with no subsequent reduction in milk intake during the complimentary feeding period. This

interference with self-regulating mechanisms early in life could have long-term health consequences, increasing the risk of being overweight and obesity later in life [7,8,13,47].

Several studies have linked early complimentary feeding to a higher risk of being overweight and obesity during childhood and later in life [6,7,9]. An analysis of the same cohort at three years of age found that those children who were introduced to complimentary feeding later had a lower prevalence of being overweight or obesity [48]. Previous studies on complimentary feeding in the ROI have shown poor compliance with current guidelines, with more than 70% of infants being introduced to complimentary foods <17 weeks [15]. However, these studies did not explore ethnic variations in complimentary feeding.

An important finding in this study is observed in Figure 2, which shows that a higher percentage of Irish mothers (15.5%) introduced complimentary foods early when compared to the other ethnic groups. It has to be noted that the prevalence of infants introduced early to complimentary foods in this study is probably an underestimation, because mothers were asked for the child's age at which point solid foods had been regularly given. The group with the lowest percentage of mothers introducing complimentary foods early were those of Chinese or any other Asian background (4.8%). Interestingly, this was one of the ethnic groups with one of the highest breastfeeding rates, which suggests a close relationship between early milk feeding and complimentary feeding.

The predictors of early complimentary feeding were studied for the whole sample. An important finding is that belonging to a different ethnic background than Irish had a protective role against early complimentary feeding, which was reduced with a longer length of stay in the ROI (Table 2). Figure 3 shows how the acculturation of non-Irish mothers resulted in a decrease in the breastfeeding rate, which correlates with an increase in the percentage of mothers introducing early complimentary foods. This finding highlights again the role played by acculturation and the adoption of formula milk in the timing of complimentary feeding introduction.

The inclusion of the duration of exclusive breastfeeding in the adjusted model resulted in a loss of the significance of breastfeeding initiation with little change in the rest of the significant predictors. This result suggests the importance of exclusive breastfeeding and its potential role in the timing of solids introduction and, ultimately, in the development of being overweight and obesity.

There is inconsistency in the results of studies on early complimentary feeding and the risk of developing being overweight and obesity. Moreover, a longer duration of breastfeeding is associated with the later introduction of complimentary foods. In the present study, a longer duration of exclusive breastfeeding resulted in a decrease in the probability of early complimentary feeding. Therefore, complimentary feeding could potentially be a confounder in the relationship between breastfeeding and being overweight or obesity [49–52].

Another interesting finding was the fact that maternal BMI was a predictor of early complimentary feeding. The relationship between being overweight, obesity and breastfeeding duration has been well studied, suggesting that overweight and obese women are at higher risk of early cessation of breastfeeding, due to biological and mechanical factors. [53–56].

## 5. Strengths and Limitations

GUI is a large and nationally representative sample. The results of the study can be applied at a population level, due to the application of the sampling weights. Parental BMI was measured by trained professionals using validated techniques.

However, there are several limitations to the present study. It would have been desirable to collect information on the first introduction of solids into the infant's diet to allow comparability with other studies on complimentary feeding. The results must also be interpreted with caution, as the information was collected retrospectively, when the infant was nine months of age, increasing the possibility of recall bias.

Maternal BMI was measured at the time of interview, which took place when the infant was nine months old. Therefore, we assume that those mothers who were overweight or obese at that point in time belonged to the same BMI category pre-pregnancy.

## 6. Conclusions

The results from this study suggest that, after adjusting for other maternal characteristics, inappropriate infant feeding practices are more common among Irish mothers when compared to non-Irish mothers residing in the ROI. Acculturation plays an important role in infant feeding practices among non-Irish mothers. Therefore, cultural differences are an important factor in shaping patterns of infant feeding in the ROI.

There is a strong association between breastfeeding and the early introduction of complimentary feeding. The ROI continues to have one of the lowest breastfeeding rates in the world. Existing policies to increase breastfeeding rates have been largely ineffective and with recent increases in the breastfeeding rate explained by an increase in the proportion of non-Irish mothers residing in the ROI and increasing maternal education and age, characteristics that are associated with a higher propensity to breastfeed in Ireland. The immediate revision of current support, education and policies on infant feeding practices would appear desirable to achieve the implementation of desirable infant feeding practices in line with WHO and ESPGHAN recommendations.

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## Author Contributions

Patricia Dominguez Castro contributed towards data analysis and interpretation and led the writing. Richard Layte helped in interpreting the results and provided critical feedback on the statistical analysis of the data, as well as methods used to collect the same. He also provided feedback on drafts of the paper. John Kearney helped in interpreting the results and provided feedback on drafts of the paper. All authors approved the final version of the paper.

## Conflicts of Interest

The authors declare no conflict of interest.

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## Factors Associated with Exclusive Breastfeeding in Timor-Leste: Findings from Demographic and Health Survey 2009–2010

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**Abstract:** Exclusive breastfeeding is known to have nutritional and health benefits. This study investigated factors associated with exclusive breastfeeding among infants aged five months or less in Timor-Leste. The latest data from the national Demographic and Health Survey 2009–2010 were analyzed by binary logistic regression. Of the 975 infants included in the study, overall 49% (95% confidence interval 45.4% to 52.7%) were exclusively breastfed. The exclusive breastfeeding prevalence declined with increasing infant age, from 68.0% at less than one month to 24.9% at five months. Increasing infant age, mothers with a paid occupation, who perceived their newborn as non-average size, and residence in the capital city Dili, were associated with a lower likelihood of exclusive breastfeeding. On the other hand, women who could decide health-related matters tended to breastfeed exclusively, which was not the case for others whose decisions were made by someone else. The results suggested the need of breastfeeding promotion programs to improve the exclusive breastfeeding rate. Antenatal counseling, peer support network, and home visits by health workers could be feasible options to promote exclusive breastfeeding given that the majority of births occur at home.

**Keywords:** breastfeeding; demographic and health survey; exclusive breastfeeding; Timor-Leste

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## 1. Introduction

Exclusive breastfeeding means only breastmilk is allowed with the exception of medicine, vitamin syrup and oral rehydration solution [1,2]. It is known that exclusive breastfeeding for six months can protect infants from short term illnesses such as gastroenteritis, respiratory infection and under nutrition; and in the long term, against chronic diseases such as type 2 diabetes, hypertension and obesity [3,4]. The economic benefits include cost savings from avoiding illness, workdays lost and the purchase of infant formula [5]. Moreover, it has been projected that 11.6% of child deaths in 2011 could be attributable to sub-optimal breastfeeding [6]. A variety of factors have been reported to affect the practice of exclusive breastfeeding, including maternal characteristics (education, occupation, health condition, age), infant characteristics (sex, birth order, illness), and cultural practices (initiation of breastfeeding, time of introduction of complementary feeds) [7,8]. The effects of these factors vary according to cultural context and related socioeconomic conditions.

Timor-Leste is one of the youngest countries in Asia which gained independence since 2002 [9]. The country went through a long armed conflict during 1990s, leading to destruction of most of the infrastructure [9], leaving thousands of its citizens being displaced from East to West Timor [10]. The majority of health workforce returned to Indonesia after independence. Only a small number of health professionals remained to re-establish the health system with assistance from the United Nations, expatriate workers, and other international support [10]. The population of Timor-Leste is estimated to be 1.07 million in 2010 with a growth rate of 2.4% [11]. The country has a high infant mortality (45 per 1000 live births) and under five mortality rate (64 per 1000 live births). The proportion of under five children suffering from under-nutrition is still high: stunting (53%), wasting (17%) and under-weight (52%) [11]. Exclusive breastfeeding has been found positively associated with infant stature [12] and protective against overweight and obesity in childhood [13].

Currently, information on infant nutrition in Timor-Leste is lacking. A previous survey conducted in 2003 [14] reported that breastfeeding was almost universal (97.6%), but a much lower exclusive breastfeeding rate of 30.7% for infants less than 6 months. Some health infrastructures have been restored after independence in 2002. For instance, 5 hospitals, 69 health centers and 85 health posts have been established between 2002 and 2005. A national referral hospital and community health centers were also functioning by this time with the support from the government and international community. The per capita expenditure on health was US \$45 in 2006 (7.5% of GDP), higher than many other Asian countries [9]. As part of the global measure Demographic and Health Surveys (DHS) project, an updated Timor-Leste DHS was conducted during 2009–2010 [11]. The aim of the present study was to investigate factors associated with exclusive breastfeeding among infants aged five months or less based on the updated information from the national DHS, findings from which will enable policy makers and public health researchers to develop interventions to improve exclusive breastfeeding in the country.

## 2. Methods

### 2.1. Survey Design

DHS are conducted every five years in more than 50 countries using a validated questionnaire. The Timor-Leste DHS 2009–2010, conducted in two stages, was the second survey after the initial 2003 DHS. During the first stage, 455 enumeration areas were selected based on probability proportionate to size: 116 urban and 339 rural areas. More rural samples were included because the majority of the Timor-Leste population lives in rural areas. At the second stage, 27 households were selected randomly from each enumeration area following a systematic sampling procedure.

### 2.2. Participants

The final survey included 11,463 households, comprising 9806 children under five years of age. The present study focused on the subgroup of 975 infants (1) with a singleton birth; (2) who were aged less than six months; (3) alive and living with the respondent; and (4) who were the youngest child in the family; in order to avoid the selection of children from the same household and parents. The DHS was approved by the ethics committee of Macro International Inc. and the Ministry of Health of Timor-Leste. The data were de-identified and made available for public use [15].

### 2.3. Exclusive Breastfeeding

The operational definition of exclusive breastfeeding, as defined by the World Health Organization (WHO) infant feeding guidelines [2], was adopted: “infants 0–5 months of age who are fed exclusively with breastmilk”. Apart from breastmilk or wet nurse’s milk, no other fluid was allowed, with the exception of oral rehydration solution, drops or syrups (vitamins, minerals and medicine). The binary status of exclusive breastfeeding (coded as ‘1’ for yes and ‘0’ for no) was determined for each of the 975 selected infants. Previous published studies have used such definition to report a period prevalence of exclusive breastfeeding based on 24-h recall [16,17], but it should not be treated as the rate of exclusive breastfeeding for six months.

### 2.4. Independent Variables

Selection and categorization of independent variables in this study were based on literature review [14,18]. Maternal age was recoded into three groups: 15–19, 20–34 and 35–49 years. Frequency of antenatal care (ANC) visit was categorized as: 0, 1–3 and  $\geq 4$ . Mother’s perceived size of newborn was coded as: small, average and large. Religion was originally recorded as: Roman Catholic, Muslim, Protestant, Hindu and others. Because the vast majority of population follows Roman Catholic, the other religions were grouped together. Maternal occupation was re-categorized as: no paid work (housewives and household work), agriculture (self-employed or employee), professional, clerical, sales and services, and manual work (skilled or unskilled). Education level was classified as: no education, primary, secondary and higher. Decision making on health had three categories: respondent alone, respondent with others (e.g., husband), others only (e.g., husband or someone else) [19]. Place of delivery was regrouped as either health facility (national hospital, referral hospitals,

community health centers, health post, SisCa post, private sectors, Marrie stops) or home (home of respondent or others). Birth order referred to: first time birth, second or third, and fourth or above. Residential location was defined as either rural or urban.

### 2.5. Statistical Analysis

Timor-Leste is divided into 13 administrative districts, which has been incorporated into the analysis to examine geographical differences in the 24-h period prevalence of exclusive breastfeeding among infants aged <6 months. Further age-wise disaggregated proportions of exclusive breastfeeding were reported at age <1 month, 1, 2, 3, 4, and 5 months. Factors associated with exclusive breastfeeding were screened by Chi-square tests and then assessed by backward stepwise logistic regression [20], taking into account the apparent collinearity between independent variables. Complex sample analysis was performed to estimate proportions, odds ratios and their 95% confidence intervals (CI) [21].

## 3. Results

### 3.1. Exclusive Breastfeeding

As shown in Table 1, of the 975 infants aged  $\leq 5$  months, overall 49% (95% CI 45.4% to 52.7%) were exclusively breastfed in the 24 h preceding the 2009–2010 survey. The exclusive breastfeeding prevalence appeared to decline with increasing infant age, from 68.0% at less than one month to 24.9% at five months. In particular, a sharp decrease was observed between the 4th and 5th month postpartum for the respondents.

**Table 1.** Prevalence of exclusive breastfeeding by infant age, Timor-Leste, 2009–2010 ( $n = 975$ ).

Infant Age (months)	n	Prevalence (95% Confidence Interval)
<1	80	68.0 (55.4, 78.5)
1	177	67.6 (59.2, 75.0)
2	151	56.5 (46.5, 66.1)
3	183	48.5 (40.5, 56.6)
4	209	41.8 (34.3, 49.7)
5	175	24.9 (19.5, 31.2)
$\leq 5$	975	49.0 (45.4, 52.7)

### 3.2. Sample Characteristics

The distribution of maternal age (years) was: 15–19 (6.8%), 20–34 (67.5%), 35–49 (25.7%). The majority (78.6%) of participants resided in rural areas. About one-third (34.6%) of mothers and just over a quarter (28.5%) of fathers received no education. The majority of women had no paid employment (73.2%) and followed Roman Catholic as their religion (98.2%). More of them came from poorer (45.4%) than middle class (39.6%) and rich households (15%). Most respondents did not read newspaper (70.3%), listen to the radio (59.9%), or watch television (68.6%) at all.

Slightly more than half (52.7%) of mothers had high parity ( $\geq 4$ ), with only 17.4% being first time motherhood. Only 52% of the women had paid four or more ANC visits. There were more male (52.9%) than female (47.1%) infants. The majority of infants were born at home (74.2%) by vaginal delivery (98.3%). Surprisingly, only about a quarter (26.6%) of women could make decision by themselves with regard to health-related matters. Although 56% of mothers perceived their newborn as average size, 18.5% believed they were small size. Most mothers (88.8%) initiated breastfeeding within one hour of delivery. Only a small proportion (2.9%) of mothers continued to smoke at the time of the survey.

A number of socio-demographic and health-related variables appeared to be associated with the prevalence of exclusive breastfeeding according to Chi-square tests. These included residential location ( $P = 0.014$ ), ecological region ( $P < 0.001$ ), maternal occupation ( $P < 0.001$ ), wealth status ( $P = 0.003$ ), frequency of listening to the radio ( $P = 0.007$ ), frequency of watching television ( $P = 0.004$ ), and decision making on health ( $P = 0.026$ ).

### 3.3. Factors Affecting Exclusive Breastfeeding

Stepwise logistic regression analysis further confirmed that infant age, ecological region, maternal occupation, perceived size of newborn, and decision making on health were significantly associated with exclusive breastfeeding; results of which are shown in Table 2. In particular, increase in infant age could reduce the likelihood of exclusive breastfeeding, consistent with the previous observation on the decline in exclusive breastfeeding prevalence by advancing infant age in Table 1. When compared to the capital city Dili, mothers from other regions were more likely to exclusively breastfeed their infants. On the other hand, mothers who maintained employment seemed less likely to continue exclusive breastfeeding than their non-working counterparts. Those mothers who perceived their newborn as either large or small size were also less likely to exclusively breastfeed. Finally, mothers who could decide health-related matters by themselves tended to exclusively breastfeed, which was not the case for others whose decisions were made by someone else.

## 4. Discussion

This study found that half (49.0%, 95% CI 45.4% to 52.7%) of the infants aged five months or below were exclusively breastfed at the time of the 2009–2010 DHS, which appeared to increase substantially from the previously reported 24-h recall prevalence rate of 30.77% (95% CI 27.2% to 34.5%) in 2003 [14]. According to the report by UNICEF [22], the proportion of exclusively breastfed children of 0–5 months during the period 2000–2007 was 43% in East Asia and Pacific, 44% in South Asia, and 39% overall in developing countries. However, the differences in survey period between countries should be taken into account. The apparent increase in exclusive breastfeeding prevalence may be attributable to a number of changes in Timor-Leste since 2003. The country has become more stable after the conflict, with social and health services being restored [9,11]. While it is encouraging to note the improvement in exclusive breastfeeding practice, the rate is still much lower than the recommended 90% by the WHO [23].

**Table 2.** Factors associated with exclusive breastfeeding in Timor-Leste, 2009–2010 (*n* = 975).

Factor	n (%)	EBF (%)	Adjusted Odds Ratio (95% Confidence Interval) *	P *
<b>Infant age (months)</b>	Mean 2.81	SD 1.58	0.67 (0.60, 0.71)	<0.001
<b>Ecological region</b>				<0.001
Dili (Capital)	86 (6.3)	30 (33.2)	1.00	
Aileu	83 (8.5)	61 (73.6)	8.05 (3.43, 18.89)	
Ainaro	73 (7.5)	49 (67.2)	5.10 (2.49, 10.43)	
Baucau	65 (6.7)	36 (52.3)	2.83 (1.23, 6.51)	
Bobonaro	78 (8.0)	30 (39.4)	1.23 (0.62, 2.44)	
Cova Lima	61 (6.3)	21 (34.0)	1.06 (0.51, 2.21)	
Ermera	102 (10.5)	52 (50.4)	2.21 (1.10, 4.44)	
Liquica	84 (8.6)	53 (63.3)	3.75 (1.90, 7.38)	
Lautem	72 (7.4)	35 (46.5)	1.93 (1.03, 3.64)	
Manufahi	63 (6.5)	39 (61.6)	3.80 (1.75, 8.28)	
Manatuto	81 (8.1)	34 (41.5)	1.44 (0.76, 2.74)	
Oecussi	84 (8.6)	50(59.5)	3.98 (2.21, 7.15)	
Viqueque	43 (4.4)	24 (55.3)	2.26 (2.21, 7.15)	
<b>Maternal occupation</b>				0.003
No paid work	712 (73.2)	394 (52.8)	1.00	
Agriculture	176 (18.1)	91 (46.1)	0.68 (0.46, 1.02)	
Professional, clerical, sales, services	67 (6.9)	23 (24.3)	0.33 (0.18, 0.62)	
Manual work	18 (1.8)	5 (34.4)	0.67 (0.19, 2.31)	
<b>Perceived size of newborn</b>				0.009
Average	536 (56.0)	301 (53.0)	1.00	
Small	177 (18.5)	90 (45.6)	0.61 (0.39, 0.93)	
Large	244 (25.5)	114 (43.2)	0.58 (0.38, 0.88)	
<b>Decision making on health</b>				0.023
Others only	106 (11.1)	43 (35.1)	1.00	
Respondent alone	254 (26.6)	143 (52.1)	2.02 (1.11, 3.67)	
Respondent with others	595 (62.3)	318 (50.6)	1.63 (0.89, 2.98)	

EBF: exclusive breastfeeding. \* From backward stepwise logistic regression; variables excluded were: maternal age, residential location, maternal education, paternal education, religion, sex of infant, wealth status, frequency of reading newspaper/magazine, frequency of listening radio, frequency of watching television, birth order, frequency of antenatal care visit, maternal tobacco smoking, method of delivery, place of delivery.

The prevalence of exclusive breastfeeding declined with increasing infant age, from 68.0% at less than one month to 24.9% at five months. The inverse association between infant age and exclusive breastfeeding practice was also observed in other Asian countries such as Bangladesh, China and Nepal [18,24,25]. According to the local culture, it is common that Timorese infants are introduced complementary foods at about the 4th month. The decision is usually made by the senior women of the family such as the grandmother or grandmother-in-law.

Mothers residing in Dili were less likely to breastfeed exclusively when compared with mothers from other regions. Such regional differences have been reported by previous studies in Timor-Leste and other Asian countries [14,17]. Dili is the capital and economic center of the country, where infant

formulas are readily accessible at supermarkets. Besides, the capital city citizens are more exposed to advertisement of infant formula, consequently leading to the early cessation of exclusive breastfeeding [26].

Moreover, women who maintained employment after giving birth were less likely to provide exclusive breastfeeding to their infants than their non-working counterparts. Similarly, Chinese mothers who had to return to their office job before six months were unlikely to breastfeed their infant exclusively [24]. Another qualitative study from Bangladesh reported that caretakers introduced formula, cow or buffalo milk when mothers attended work [27]. Working mothers in Timor-Leste are entitled to less than three months of maternity leave. This short duration makes it difficult to continue exclusive breastfeeding.

Newborns perceived to be non-average size by their mothers were less likely to be exclusively breastfed. Experience in other countries has similarly shown that preterm and low birth weight infants are breastfed for shorter duration [28]. Mothers may experience a number of barriers to breastfeed smaller infants, for instance, poor sucking, infants being kept separately for intensive care, illness, and lack of confidence [29], which may lead to the early introduction of complementary foods.

In this study, Timorese mothers who could decide health-related matters tended to continue exclusive breastfeeding, when compared with those that relied on the advice from someone else. This finding was consistent with the literature, which suggested that the ability of a woman to make decision on utilization of services can lead to better maternal and child health outcomes [19,30].

Several issues should be considered when interpreting the results. This study utilized the dataset from the latest national survey with a representative sample and a high response rate, while complex sample analysis was performed to account for the sampling strategy and sample weight [21]. Therefore, the findings are generalizable to the entire country. However, the 24-h recall would inevitably induce over-reporting of exclusive breastfeeding at six months [31] so that caution should be taken [2]. The DHS data nonetheless remain the only available information to estimate exclusive breastfeeding rate in many developing countries.

There is an immediate need of breastfeeding promotion programs in Timor-Leste. Given the high infant and child mortality in the country [11], improving the practice of exclusive breastfeeding will reduce such burden and partially overcome the problem of under-nutrition. Antenatal counseling on breastfeeding and peer support network are recommended [32]. Because the majority of births occur at home, home visits by health workers/volunteers would be an effective option to consider by healthcare planners to further promote exclusive breastfeeding and to increase its duration.

## **5. Conclusions**

Slightly less than half the infants in Timor-Leste were exclusively breastfed within 24-h preceding the latest national survey. This represented a significant improvement in exclusive breastfeeding practice since 2003 when the country restored peace. Mothers should be provided with continuous support to sustain their initial high rate of exclusive breastfeeding for six months. It is desirable to target mothers who are working, who perceive their newborns as non-average size and those residing in the capital Dili for breastfeeding promotion programs. In addition, mothers must be involved in the decision making process so that they can sustain breastfeeding exclusively.

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## Conflicts of Interest

The authors declare no conflict of interest.

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## Predictors of Breastfeeding Duration among Women in Kuwait: Results of a Prospective Cohort Study

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**Abstract:** The purposes of this paper are to report the prevalence of breastfeeding to six months among women in Kuwait and to determine the factors that are associated with the duration of breastfeeding. A cohort of 373 women recruited from maternity wards in four hospitals in Kuwait city were followed from birth to 26 weeks postpartum. The association of any and full breastfeeding duration and predictor variables were explored using multivariate Cox's proportional hazards models. At six months, 39% of all infants were receiving some breast milk and only 2% of infants had been fully breastfed to 26 weeks. Women born in other Arab countries were less likely to discontinue breastfeeding than women born in Kuwait. Other factors positively associated with breastfeeding duration were level of maternal education, higher parity, infant being demand fed in hospital and a preference for breastfeeding on the part of the infant's father and maternal grandmother. The introduction of a pacifier before four weeks of age and the mother intending to return to work by six months were negatively associated with duration. These findings present a number of opportunities for prolonging breastfeeding duration in Kuwait.

**Keywords:** breastfeeding; duration; determinants; Middle East

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## 1. Introduction

Breastfeeding is an unequalled way to feed an infant. In addition to its unique nutritional properties, human breast milk contains a wide-variety of immunoprotective factors that augment the immature immune system of the infant [1]. Infants who are formula fed are at greater risk of infections common to infancy including gastroenteritis, respiratory infection and otitis media [2]. The World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) recommend that infants be exclusively breastfed for the first six months of life with breastfeeding continuing for up to two years of age or beyond [3]. The wide-spread practice of delayed initiation of breastfeeding and prelacteal feeding [4–8], along with the early introduction of complementary feeding [9] in the Middle Eastern region however, mean that very few infants in this region are exclusively breastfed from birth for six months as recommended.

Breastfeeding practices are influenced by a complex mix of factors which are related to maternal and family socio-demographic characteristics, biomedical factors, health-care practices, psychosocial factors, social support, community attitudes, and public policy factors [10,11]. The direction of effect of these factors is not consistent across all cultures. For instance, in industrialised countries, better educated women are more likely to initiate breastfeeding and to breastfeed for longer than their less educated counterparts, whereas in poorer countries the opposite tends to be the case. In common with industrialised countries [10–12], amongst Middle Eastern women breastfeeding duration has been positively associated with maternal age [13–16] and parity [14,17,18]. Whereas inconsistent associations have been reported for level of maternal education with breastfeeding duration being associated both negatively [16,19,20] and positively [17] with a higher level of maternal education. Other factors reported to be negatively associated with duration of breastfeeding include maternal employment [17,19–22], mode of delivery [21,23–25] and the use of infant formula while in hospital [24–26].

Regular breastfeeding surveillance is essential to determine the extent to which national breastfeeding targets are being met, the impact of breastfeeding promotion interventions and how breastfeeding practices are changing over time. In addition, it is important to investigate the determinants of infant feeding practices so that breastfeeding interventions can be targeted at the most vulnerable population groups and address potentially modifiable risk factors which adversely affect breastfeeding practices. Relatively few studies have investigated infant feeding practices in Kuwait [27–29] and none have been longitudinal in nature. The reported mean duration of breastfeeding appears to have declined from 6.4 months in 1988 [27] to 4.9 months in 1997 [28] and there is a lack of more recent data to determine if this downward trend has continued. The aim of the Kuwait Infant Feeding Study (KIFS) was to identify the incidence and prevalence of breastfeeding up to 26 weeks postpartum among a population of women living in Kuwait and to identify the factors associated with the initiation and duration of breastfeeding. The determinants of breastfeeding initiation have been reported previously [30] and the purposes of this paper, therefore, are to report the

prevalence of breastfeeding to six months and to determine the factors that are associated with the duration of breastfeeding.

## 2. Experimental Section

### 2.1. Recruitment of Subjects

A prospective cohort study of infant feeding practices among women in Kuwait was conducted between October 2007 and October 2008. The study methods have been described previously [30], but briefly mothers were recruited from three major public hospitals and one private hospital located in Kuwait City. Within 72 h of delivery, eligible mothers were visited and invited to participate in the study by the researcher (MD) who provided a written and verbal description of the study. Women were considered to be eligible for the study if they were able to read or understand Arabic or English and had delivered a live, healthy, singleton of 36 weeks or more gestational age. Mothers whose infants were admitted to the Special Care Nursery (SCN) for minor illnesses or observation were eligible for recruitment.

The study was approved by the Medical Faculty Ethics Committee of the University of Glasgow, (Application No. FM03906: Approved May 29, 2007) and by the Ministry of Health in Kuwait. Participants provided signed informed consent and were advised that they could refuse to participate or withdraw from the study at any time, without prejudicing their post-natal care or the care of their baby.

### 2.2. Data Collection

Mothers who agreed to join the study were interviewed face-to-face to complete a baseline questionnaire prior to discharge from hospital. Women who declined to participate were asked to provide some basic socio-demographic data to determine if the sample were representative of the population of women giving birth at the participating hospitals. All participants were followed up by telephone interview at 6, 12, 18 and 26 weeks postpartum. Data were collected using questionnaires previously used in similarly designed studies of Australian women [31] and modified slightly to meet the needs of this study population. Information on socio-demographic characteristics, maternal lifestyle factors, infant characteristics, biomedical factors, hospital practices, psychosocial factors and feeding practices were collected at baseline. Information on current feeding practices, changes to feeding practices, breastfeeding experiences and introduction of a pacifier were collected during follow-up interviews.

### Infant Feeding Assessments

Breastfeeding terms used in this study were those defined by the World Health Organization [3]. An infant was considered to be *exclusively breastfed* when he or she had received only breast milk with no other liquids (except for oral rehydration solutions (ORS), drops or syrups) or complementary (solid) foods, and to be *predominantly breastfed* when he or she received breast milk as the main source of nourishment, with certain liquids (water, water-based fluids, fruit juices, ritual fluids, ORS, drops or syrups) but received no other liquids (including formula milk and non-human milks) or complementary foods. *Full breastfeeding* was defined as either exclusive or predominant

breastfeeding [32]. *Any breastfeeding* was defined as an infant who was receiving breast milk, with or without formula, other milks, fluids or complementary foods. Duration of exclusive, predominant and any breastfeeding was determined by using information about the age at which other types of milks, liquids (e.g., water, fruit juice) and/or complementary foods were introduced in the first six months of life. In this study, *prelacteal feeding* was defined as the act of giving any liquid or food item (except breast milk) to a newborn within the first three days after birth [33].

### 2.3. Statistical Analysis

We explored the associations of breastfeeding duration and a variety of characteristics and practices reported in the literature to be associated with the duration of breastfeeding using Cox's proportional hazards model. This model allows joint estimation of the effects of independent variables on the risk of cessation of breastfeeding and can be used to analyze data that contain censored observations [34]. We tested the role of: (1) socio-demographic factors (maternal age, education, country of birth, employment plans for six month postpartum); (2) maternal lifestyle factors (pre-pregnancy BMI calculated from self-reported weight and height, smoking during pregnancy); (3) infant factors (gender, having spent time in the SCN); (4) biomedical factors (parity, delivery method, breastfeeding problems at baseline, breastfeeding problems at six weeks postpartum, age at which pacifier was introduced) (5) hospital practices (time to first breastfeed, composition of infant's first feed, use of prelacteal feeds, infant roomed-in, infant demand fed in hospital); and (6) psychosocial factors (when decided on feeding method, whether pregnancy was planned, father's feeding preference, maternal grandmother's feeding preference, paternal grandmother's feeding preference). The association of individual variables and the duration of any and full breastfeeding was first evaluated in a univariate model. Any variable with a *P*-value of <0.100 was then included in a multivariate model which was reduced using the backward stepwise procedure. The fitness of each model was assessed at every step to avoid dropping non-significant variables that affected the model fitness. All variables in the final model were variables for which, when excluded, the change in deviance compared with the corresponding statistics on the relevant degrees of freedom was significant. Statistical analyses were performed using the Statistical Package of Social Sciences version 19 (SPSS Inc., Chicago, IL, USA).

## 3. Results

A total of 439 women were invited to participate in the study and 373 mothers completed the baseline questionnaire while in hospital, giving a response rate of 85%. There were no significant differences between participants and those declining to participate ( $n = 66$ ) with respect to age ( $\chi^2 4.413$ ,  $P = 0.110$ ), level of education ( $\chi^2 2.455$ ,  $P = 0.117$ ) and chosen method of feeding at discharge ( $\chi^2 447$ ,  $P = 0.800$ ), suggesting that the sample was representative of the population from which it was drawn [30]. In all, 80 women dropped out of the study prior to completing the final follow-up interview at 26 weeks however, there were no differences in the age, level of education and chosen feeding method of those who completed or withdrew from the study (data not reported). Data for the duration of any and full breastfeeding for women who withdrew were censored in accordance with the woman's status at the time of last contact, allowing all participants to be included in the survival analysis.

Almost all women (92.5%) initiated breastfeeding, and at six months, just over one third of all infants (39%) were receiving some breast milk and only 2% of infants had been fully breastfed to 26 weeks (Table 1). As not all women had ceased breastfeeding it was not possible to estimate mean duration of breastfeeding, however the median duration of any breastfeeding was 13.9 weeks and the mean duration of those women who had stopped breastfeeding before 26 weeks was six weeks. The median duration of full breastfeeding was less than one week with 50% of infants having received formula feeds within the first week post-partum.

**Table 1.** Prevalence <sup>a</sup> of full and any breastfeeding at selected ages.

Age (weeks)	Any Breastfeeding (%)	Full Breastfeeding (%)
4	66	31
8	56	26
12	53	22
26	39	2

<sup>a</sup> Survival analysis using censored cases.

The majority of women who initiated breastfeeding ( $n = 345$ ) were between 25 and 34 years of age (64.3%), born in Kuwait (54.2%) and had completed 12 or more years of education (78%). Just over one third of women had delivered by Caesarean section (36.5%). Delayed initiation of breastfeeding and prelacteal feeding were the norm amongst this cohort with just over one half of women (52.8%) initiating breastfeeding more than 24 h after giving birth and the majority of infants (88.7%) receiving formula as either their first feed and/or at some time during the first three days of their hospital stay (Table 2). Only one third of women who initiated breastfeeding left hospital fully breastfeeding their infant, with the majority of women (59.4%) partially breastfeeding. A small number of women ( $n = 29$ , 8.4%) who initiated breastfeeding left hospital exclusively formula feeding.

### 3.1. Univariate Analysis

Due to the small number of breastfed infants (11.3%) who had been exclusively breastfed whilst in hospital we investigated the association of covariates with the duration of full and any breastfeeding for the first six months of life. In the univariate analysis (Table 2), the duration of any and/or full breastfeeding was associated with socio-demographic factors (maternal age, country of birth and level of education), parity, prelacteal feeding, introduction of a pacifier, whether a mother had roomed-in for 24 h with her infant and psychosocial factors (partner's and maternal grandmother's support for breastfeeding).

### 3.2. Multivariate Analysis

Table 3 shows the results of the multivariate analysis. After adjustment, maternal level of education and country of birth were independently associated with both duration of full and any breastfeeding. Mothers with 12 or more years of education were less likely to stop any (Adjusted Hazard Ratio (AdjHR) = 0.68) and full (AdjHR = 0.74) breastfeeding during the six month follow-up period compared with mothers with less than 12 years of education. Similarly, mothers born in other Arab countries were less likely to stop any (AdjHR = 0.53) and full (AdjHR = 0.65) breastfeeding compared

with women born in Kuwait. Mothers who did not intend to return to work within six months post-partum (AdjHR = 0.76) and those who did not experience breastfeeding problems in hospital (AdjHR = 0.80) were less likely to have stopped full breastfeeding. Conversely, women who did not feed on demand while in hospital (AdjHR = 1.28) or whose partner preferred formula feeding or was ambivalent as to how his child was fed (AdjHR = 1.33) were more likely to stop full breastfeeding. Multiparous women (Adj HR = 0.63) were less likely to cease any breastfeeding while those women who introduced a pacifier to their infant before four weeks (AdjHR = 1.66) or whose own mother preferred formula feeding or was ambivalent as to how her grandchild was fed (AdjHR = 2.11) were more likely to stop breastfeeding during the six month follow-up period.

### 3.3. Reasons for Discontinuing Breastfeeding

The reasons given by mothers for stopping breastfeeding are given in Table 4. The majority of women (86.8%) indicated that they were concerned about the adequacy of their breast milk in terms of either quantity or quality. Almost half (49.1%) indicated that their baby had either weaned them self, preferred a bottle or were ready for solids. A notable proportion stopped breastfeeding because they had returned to work or study. Only a small number of women cited mother-centered reasons like inconvenience and dislike of breastfeeding. The reasons for cessation did not vary markedly according to the infant age at which women stopped breastfeeding.

## 4. Discussion

While breastfeeding initiation is virtually universal amongst women living in Kuwait, targets for breastfeeding duration are not being met, with no woman in this study exclusively breastfeeding to six month of age and only 2% of women fully breastfeeding their infants to this age. As not all women had ceased breastfeeding by 26 weeks, it was not possible to estimate mean duration of breastfeeding however, the median duration of any breastfeeding was slightly more than three months and the mean duration of those women who had stopped breastfeeding before 26 weeks was six weeks. This suggests that the mean duration of any breastfeeding in Kuwait is likely to be less than the 4.9 months reported in 1997 [28] and that breastfeeding duration is declining.

In this study, women born in other Arab countries were less likely to have discontinued any and full breastfeeding than women born in Kuwait or other countries. These women are likely to be the wives of Middle Eastern guest workers employed in the oil and construction industries and the infant feeding practices of these women likely reflect those of their home country where children are breastfed for longer than infants in Kuwait. For instance, contemporaneous studies have reported a mean duration of breastfeeding of 8.6 months in the UAE [16] and 7.6 months in Bahrain [35], and a median duration of 12.4 months in Jordan [36]. A recent study in Kuwait reported that Kuwaiti mothers use bottle feeding more than the non-Kuwaiti mothers [37] which is consistent with our findings. Al Fadli *et al.* [37] proposed that such practice could be explained by the lifestyle changes that occurred in Kuwait due to oil revenue and through using modern technology similar to what happened in Western countries in the 1960s and 1970s.

**Table 2.** Characteristics of a cohort of mother-infant dyads who had breastfed ( $n = 345$ ) and the unadjusted association with the risk of discontinuing any or full breastfeeding during the six months' follow-up (Crude Hazards Ratio and 95% Confidence Interval).

Characteristic	N <sup>a</sup>	%	Any Breastfeeding			Full Breastfeeding		
			Crude HR	95% CI	P value	Crude HR	95% CI	P value
<b>Maternal Factors</b>								
Age (years)								
<25	77	22.3	1.36	0.83–2.26	0.018	0.95	0.66–1.38	0.380
25–34	222	64.3	0.82	0.52–1.29		0.83	0.61–1.14	
≥35	46	13.3	1.00			1.00		
Mother's country of birth					<0.001			0.002
Kuwait & Gulf States	187	54.2	1.00			1.00		
Other Arab countries <sup>b</sup>	119	34.5	0.44	0.31–0.63		0.66	0.52–0.83	
Other non-Arab Countries	39	11.3	0.77	0.48–1.23		0.92	0.65–1.30	
Years of education					0.002			0.090
<12	76	22.0	1.00			1.00		
≥12	269	78.0	0.59	0.42–0.83		0.80	0.62–1.04	
Employment intention for 6 months postpartum					0.156			0.055
Intend to be working	136	39.4	1.00			1.00		
Do not intend to be working or don't know	209	60.6	0.80	0.59–1.09		0.81	0.65–1.00	
Smoked pregnancy					0.358			0.706
Yes	20	5.8	1.00			1.00		
No	325	94.2	0.73	0.37–1.43		0.92	0.58–1.44	
Pre-pregnancy Body Mass Index (kg/m <sup>2</sup> )					0.623			0.964
<24.99	149	43.2	1.00			1.00		
25.00 to 29.99	124	35.9	1.02	0.72–1.45		1.03	0.81–1.30	
≥30	72	20.9	1.21	0.81–1.82		0.99	0.75–1.31	



Table 2. Cont.

Missing	14	4.1	-	-	0.162	-	0.336
Infant's first feed							
Formula/other	277	80.3	1.00			1.00	
Breast milk/colostrum	68	19.7	0.75	0.49-1.13	0.008	0.89	0.68-1.15
Prelacteal feed given							
Yes	306	88.7	1.00			1.00	
No	39	11.3	0.42	0.22-0.80		0.69	0.50-0.97
Infant roomed-in for 24 h/day					0.183		0.401
Yes	183	53.0	1.00			1.00	
No	162	47.0	0.81	0.59-1.10		1.10	0.89-1.36
Infant fed on demand in hospital					0.042		0.064
Yes	245	71.0	1.00			1.00	
No	100	29.0	1.41	1.01-1.95		1.25	0.99-1.58
<i>Psycho-social factors</i>							
When decided how to feed infant					0.884		0.089
Before pregnant	262	75.9	1.00			1.00	
After pregnant	83	24.1	0.97	0.66-1.43		1.24	0.97-1.59
Planned pregnancy					0.155		0.085
Yes	196	56.8	1.00			1.00	
No	149	43.2	0.80	0.58-1.09		0.83	0.67-1.03
Father's infant feeding preferences					0.008		0.007
Prefers breastfeeding	280	81.2	1.00			1.00	
Prefers Bottle or ambivalent	65	18.8	1.64	1.14-2.36		1.46	1.11-1.92
Maternal grandmother's infant feeding preference					0.004		0.013
Prefers breastfeeding	312	90.4	1.00			1.00	
Prefers Bottle or ambivalent	33	9.6	2.06	1.26-3.36		1.59	1.10-2.28
Paternal grandmother's infant feeding preference					0.448		0.400
Prefers breastfeeding	261	75.7	1.00			1.00	
Prefers Bottle or ambivalent	84	24.3	1.15	0.81-1.63		1.11	0.87-1.42

<sup>a</sup> N = Number of subjects; <sup>b</sup> Other Arab countries included Algeria, Egypt, Iran, Iraq, Jordan, Lebanon, Morocco, Palestine, Qatar, Saudi Arabia, Syria, Yemen.

**Table 3.** Factors independently associated with the risk for discontinuing any or full breastfeeding in a cohort of mother-infant dyads followed to six months postpartum ( $n = 345$ ).

Characteristic	Any Breastfeeding			Full Breastfeeding		
	Adjusted <sup>a</sup> HR	95% CI	P Value	Adjusted <sup>b</sup> HR	95% CI	P Value
Mother's country of birth			0.003			0.001
Kuwait & Gulf States	1.00			1.00		
Other Arab countries <sup>c</sup>	0.53	0.36–0.76		0.65	0.51–0.83	
Other non-Arab Countries	0.75	0.46–1.22		0.93	0.65–1.33	
Years of education			0.030			0.030
<12	1.00			1.00		
≥12	0.68	0.47–0.96		0.74	0.56–0.97	
Employment intention for 6 months postpartum						0.022
Intend to be working	NS <sup>d</sup>			1.00		
Do not intend to be working or don't know			0.005	0.76	0.60–0.96	
Parity						
Primiparous	1.00			NS		
Multiparous	0.63	0.46–0.87				
Age pacifier introduced			0.013			NS
<4 weeks	1.66	1.18–2.33				
At or after 4 weeks	1.25	0.71–2.20				
Not using a pacifier at 26 weeks	1.00					
Prelacteal feed given			0.111			
Yes	1.00			NS		
No	0.59	0.31–1.13				
Breastfeeding problems in hospital						0.046
Yes	NS			1.00		
No	NS			0.80	0.64–0.99	
Demand fed in hospital						0.040
Yes	NS			1.00		
No				1.28	1.01–1.62	

**Table 3. Cont.**

Father's infant feeding preferences				0.045
Prefers breastfeeding	NS	1.00		
Prefers Bottle or ambivalent		1.33	1.01–1.77	
Maternal grandmother's infant feeding preference		0.005		
Prefers breastfeeding	1.00	NS		
Prefers Bottle or ambivalent	2.11	1.26–3.54		
		-2 Log Likelihood = 1681.143		-2 Log Likelihood = 3441.922

<sup>a</sup> Adjusted for mother's age, country of birth, level of education, parity, infant received prelacteal feed, age pacifier introduced, father's and maternal grandmother's infant feeding preference; <sup>b</sup> Adjusted for mother's country of birth, level of education, employment intention for six months post-partum, parity, breastfeeding problems at baseline, infant received prelacteal feed, infant demand fed in hospital, when infant feeding decision was made, whether pregnancy was planned, father's and maternal grandmother's infant feeding preference; <sup>c</sup> Other Arab countries included Algeria, Egypt, Iran, Iraq, Jordan, Lebanon, Morocco, Palestine, Qatar, Saudi Arabia, Syria, Yemen. <sup>d</sup> NS = non-significant.

**Table 4. Reasons <sup>a</sup> for stopping breastfeeding.**

Reason	Stopped < 4 weeks (N <sup>b</sup> = 51)		Stopped 4–12 weeks (N = 68)		Stopped 13–26 weeks (N = 40)		Total (N = 159)	
	N	%	N	%	N	%	N	%
Concerned about quantity and quality of breast milk	42	82.4	59	86.8	37	92.5	138	86.8
Baby weaned self, prefers bottle or ready for solids	24	47.1	31	45.6	23	57.5	78	49.1
Returned to work or study	24	47.1	24	35.3	19	47.5	67	42.1
Breastfeeding too difficult or requires too much motivation	4	7.8	8	11.8	2	5.0	14	8.8
Mother ill, stressed or too tired	3	5.8	4	5.9	3	7.5	10	6.3
Mother-centered reasons (breastfeeding inconvenient, dislike breastfeeding, concern for effect on figure, "done my bit")	3	5.9	3	4.4	3	7.5	9	5.7
Breast-related problems (cracked or sore nipples, engorgement, mastitis)	1	2.0	1	1.5	2	5.0	4	2.5

<sup>a</sup> Women may have given more than one reason for stopping; <sup>b</sup> N = number of subjects.

This study found no independent association with maternal age but, consistent with the findings of studies of women in Western countries [11,12], breastfeeding duration was positively associated with level of maternal education and parity, and negatively associated with maternal employment. Women with 12 or more years of education were less likely to have discontinued any or full breastfeeding compared with women with less than 12 years of education. Multiparous women were less likely to discontinue any breastfeeding than primiparous women which is consistent among women from Western [11,12] and other Middle-Eastern [14,18,22,23] countries. Previous breastfeeding success is a strong predictor of breastfeeding duration [12] and in general women breastfeed for longer with each successive pregnancy.

While there was no association with the duration for any breastfeeding, women who did not intend to return to work within 26 weeks were less likely to have discontinued full breastfeeding than those who planned to return to work. This suggests that women supplement breastfeeding with formula feeding either on return to work or in preparation for a return to work. This negative association between early return to work and breastfeeding duration has been reported in studies of women from other Middle-Eastern countries [17,19,20,22] and is consistent with studies of women from Western countries [31,38–40], and suggests that women everywhere have difficulty combining working with exclusive breastfeeding.

Other factors negatively associated with the continuation of full breastfeeding were whether prior to discharge the mother had experienced breastfeeding problems or her infant had been fed on demand, both of which may be inter-related. Milk production is directly related to suckling frequency [41] and there is evidence that fixed feeding schedules lead to insufficient milk supply and breastfeeding problems [42]. These findings highlight the importance of unrestricted breastfeeding in the early post-partum period to the successful establishment of breastfeeding. Hospital staff should encourage demand feeding and support and encourage women to persevere when they are experiencing difficulty establishing breastfeeding rather than resorting quickly to supplementing breast milk with formula.

This was the first study to investigate the association between pacifier use and breastfeeding duration among Middle Eastern women. The incidence of pacifier use amongst breastfeeding women in Kuwait (40%) was approximately half that reported for women in Australia [43,44] and the USA [45], and introduction of a pacifier before four weeks of age was found to be negatively associated with any breastfeeding duration which is consistent with the international literature [46]. While the mechanism remains unclear, it has been suggested that the non-nutritive sucking on a pacifier reduces the frequency of nutritive sucking from the breast, thereby leading to less stimulation of the breast and consequently less milk production [43,45].

Finally, this study highlights the importance of social support for breastfeeding. Support can come from a woman's partner, family and friends, and the degree to which each of these groups influences a woman's decision to breastfeed varies according to the mother's age, social class and cultural or ethnic background [47]. In traditional societies, women rely more on the advice and support of their mother, whereas in Western cultures they are more likely to identify their husband as their main source of support [48]. This and other studies of Muslim women have highlighted the importance of grandmothers both in providing practical support and as major influences on infant feeding decisions [8,49]. Advice received from their mother and mother-in-law can have both a negative and positive affect on a woman's breastfeeding practices. For instance, on one hand, breastfeeding is promoted in the Quran

(*Al Baqara*, 233) and by elders as the desired way to feed an infant, and the mean duration of breastfeeding is longer in most Muslim countries than in Western countries. On the other hand, there is a common perception amongst older women that the heavier the baby the healthier he or she is. There is anecdotal evidence that Kuwaiti grandmothers often encourage topping up with formula to ensure the baby is satiated and to stop hunger cries, which explains in this study the positive association with any breastfeeding but not full breastfeeding.

This study also showed that a husband's preference for breastfeeding over formula feeding was positively associated with breastfeeding initiation [30] and longer duration of full-breastfeeding which is consistent with Western studies [31,50,51]. To the best of our knowledge, no Middle-Eastern study has investigated previously the association of paternal attitudes and breastfeeding duration. There is, however, some evidence from Middle Eastern studies that support from a woman's husband is important for breastfeeding success and a study of women in Saudi Arabia found that mothers were more likely to initiate breastfeeding if their partners supported breastfeeding [25]. A Turkish intervention study reported the positive effects of an antenatal education program for fathers on their reproductive health knowledge, attitudes and behaviours, and women whose husbands attended these classes reported that their husbands became more supportive and communicative [52].

Women everywhere doubt the adequacy of their milk supply [53] and in this study more than eight in 10 women gave this as one of their reasons for discontinuing breastfeeding. Perceived breast milk insufficiency or *insufficient milk syndrome* (IMS) is frequently associated with the premature introduction of complementary foods [9] and with the cessation of breastfeeding [14,16] in Middle Eastern countries. It has been proposed [53] that IMS is increasing with "aspects of 'modernization': urbanization, education, and female employment—factors that are repeatedly found to be inversely associated with both the prevalence and duration of breastfeeding" (p42). It has been suggested that 'insufficient milk' is given as a socially acceptable reason for discontinuing breastfeeding when a mother decides she no longer wishes to breastfeed [54] and that claims of IMS should not be taken literally when they occur in cultural contexts that present the use of infant formula as an acceptable, if not preferred, alternative [55].

As we have previously identified [30], there are a number of limitations to this study. Firstly, the sample size is relatively small and this is reflected in the wide confidence intervals around some of the adjusted hazard ratios reported. Secondly, we may have underestimated the rate of prelacteal feeding, which in this study was defined as within the first three days after birth. The average length of post-partum stay for Kuwaiti public hospitals is a maximum of two nights for uncomplicated deliveries and five nights for a caesarean section. Therefore, it is possible that some mothers discharged within 48 h may have gone on to supplement breastfeeding with formula following discharge from hospital and within this 72 h period. Given, however, that almost nine out of 10 infants received prelacteal feeding in hospital, any underestimation of prelacteal feeding is likely to have had only a negligible effect on the results. Finally, the number of women who delivered by caesarean section is three times that of the national average. While every attempt was made to recruit mothers within 72 h and in most cases 48 h, women who had undergone a caesarean section had a greater chance of being recruited because of their longer hospital stay.

The major strength of this study is that it was the first prospective study of infant feeding practices in Kuwait, all other previously reported studies being cross-sectional. Mother-infant dyads were

followed from birth to 26 weeks with data being collected at five time points during this period, thus minimizing the potential for maternal recall bias [56] as women were recalling events close to the time at which they occurred. The findings of the study are consistent with those of other studies in the region and, in most instances, studies of Western women, and can be used to inform infant feeding policy, hospital practices and the design of breastfeeding promotion interventions.

## **5. Conclusions**

The duration of breastfeeding amongst women in Kuwait, particularly Kuwaiti born women, appears to be declining. Exclusive breastfeeding is virtually non-existent with almost nine in 10 infants receiving prelacteal feeds within the first three days of birth. Full breastfeeding is also relatively uncommon, and by four weeks, less than one third of infants were fully breastfed, while at six months, only four in 10 infants were receiving some breast milk. This study identified a number of areas for intervention. Hospitals should follow the 10 Steps for Successful Breastfeeding [57] and, in particular, promote the early initiation of breastfeeding, encourage women to feed on demand and avoid the unnecessary practice of prelacteal feeding. Women and health professionals need to be alerted to the negative consequences of early pacifier use on breastfeeding duration. The role of family members should not be underestimated in planning breastfeeding interventions. Community-based interventions are needed to support women to breastfeed and to provide a supportive environment. Close family members, especially husbands and maternal grandmothers, should be targeted in these interventions to ensure higher rates of exclusive breastfeeding and prolonged duration.

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## **Author Contributions**

MD participated in the design of the study, collected the data, performed the statistical analysis and co-wrote the first draft of the manuscript. JAS conceived of the study, developed the original questionnaires on which the study instruments were based, assisted with statistical analysis and co-wrote the first draft of the manuscript. CAE advised on the statistical analysis and commented on drafts of the manuscript, and MAS provided assistance with the on-site coordination of the study and commented on drafts of the manuscript. All authors read and approved the final manuscript.

## **Conflicts of Interest**

The authors declare no conflict of interest.

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## Exclusive Breastfeeding and Developmental and Behavioral Status in Early Childhood

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**Abstract:** Breastfeeding during infancy may have beneficial effects on various developmental outcomes in childhood. In this study, exclusively breastfed infants were randomly assigned to receive complementary foods from the age of 4 months in addition to breast milk (CF,  $n = 60$ ), or to exclusively breastfeed to 6 months (EBF,  $n = 59$ ). At 18 months and again at 30–35 months of age, the children were evaluated with the Parent’s Evaluation of Developmental Status questionnaire (PEDS) and the Brigance Screens-II. The parents completed the PEDS questionnaire at both time intervals and the children underwent the Brigance Screens-II at 30–35 months. At 30–35 months, no significant differences were seen in developmental scores from the Brigance screening test ( $p = 0.82$ ). However, at 30–35 months a smaller percentage of parents in group CF (2%) had concerns about their children’s gross motor development compared to those in group EBF (19%;  $p = 0.01$ ), which remained significant when adjusted for differences in pre-randomization characteristics ( $p = 0.03$ ). No sustained effect of a longer duration of exclusive

breastfeeding was seen on selected measures of developmental and behavioral status at 18 months, although at 30–35 months, a smaller percentage of parents of children introduced to complementary foods at four months of age expressed concerns about their gross motor development.

**Keywords:** early childhood; exclusive breastfeeding; complementary feeding; developmental status; behavior; randomized trial

**Clinical Trial Registration:** ISRCTN41946519

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## 1. Introduction

Breastfeeding may have beneficial effects on development in childhood, adolescence and even in adulthood [1,2], although this has not been a consistent finding [3]. Furthermore, some studies indicate that a longer duration of exclusive breastfeeding is important for this positive association with developmental outcomes in childhood, especially for those born small for gestational age [4–6]. While most studies have focused on cognitive development, less is known about the impact of breastfeeding and the duration of exclusive breastfeeding on non-cognitive developmental and behavioral status in childhood. Some studies indicate that breastfeeding in general, and also, a longer duration of breastfeeding may be associated with decreased risk of behavioral problems and developmental delays in childhood [7–9]; however, findings on this subject are inconsistent. A large breastfeeding promotion intervention in Belarus showed no relationship between prolonged breastfeeding or longer duration of exclusive breastfeeding and childrens' behavior at 6.5 years of age [10,11]. Other studies have shown that increased duration and exclusivity of breastfeeding may have beneficial effects on language and motor development in childhood [12–18].

There has been a longstanding debate about the optimal duration of exclusive breastfeeding; whether infants should be exclusively breastfed for 4 or 6 months after birth [19]. The current recommendations of the WHO are that infants should be exclusively breastfed for the first 6 months of life [20] but until May 2001 the WHO recommended exclusive breastfeeding for 4–6 months of age [21]. We have previously reported the results of a parallel group, masked, randomized controlled trial of the effects of exclusive breastfeeding for 4 *vs.* 6 months on growth, body composition, breast-milk intake and iron status of the infant [22,23]. We now report a secondary analysis from this cohort of exclusive breastfeeding infants for 4 *vs.* 6 months on selected measures of development and behavior in early childhood. We hypothesized that infants exclusively breastfed for 6 months would have better outcomes in selected measures of developmental and behavioral status at 18 months and 30–35 months of age than those receiving complementary foods from 4 months in addition to breast milk.

## 2. Experimental Section

### 2.1. Study Design

As described previously [22,23], between November 2007 and November 2009, a total of 119 mother-infant pairs were recruited at seven health care centers in Iceland where 50% and 35% of mothers exclusively breastfeed through 4 and 5 months of age, respectively [24]. A total of 656 infants were assessed for eligibility in this randomized controlled trial. Eligibility criteria for the study were singleton birth, gestational length  $\geq 37$  weeks, exclusively breastfed, infant characterized as healthy: absence of congenital abnormalities or chronic health issues likely to affect growth, development or iron status. Mothers of eligible infants were invited to participate in the study and infants who were still exclusively breastfed and whose parents were willing to participate were enrolled in the study at 4 months of age. Eligible mother-infant pairs were randomly assigned to receive complementary foods from the age of 4 months in addition to breast milk (CF), or to continue being exclusively breastfed to the age of 6 months (EBF). Vitamin D supplements were recommended in both groups. Exclusive breastfeeding was defined as breastfeeding with no additional liquid or solid foods other than vitamins and medications [25]. The use of up to 10 feedings of formula or water during the first 6 months was allowed to avoid having to exclude infants that in fact were otherwise exclusively breastfed.

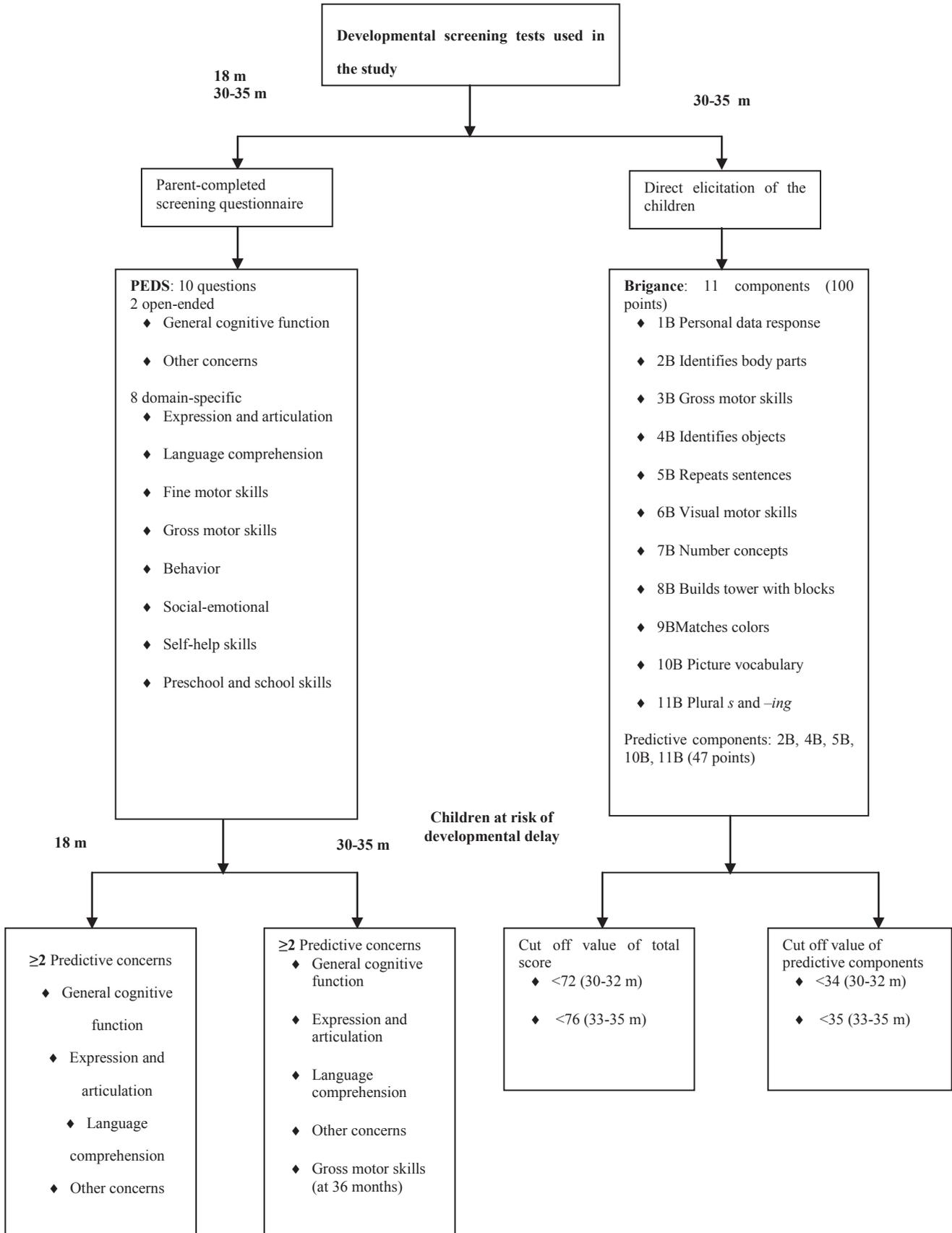
The study was reviewed and approved by the Data Protection Authority and National Bioethical Committee in Iceland and the Partners Health System IRB, Boston, MA, USA.

### 2.2. Selected Measures of Developmental and Behavioral Status

Children in the present study were assessed both at 18 months and 30–35 months of age, during their routine health care visits at the health center, where developmental and behavioral status was assessed with both the Parent's Evaluation of Developmental Status (PEDS) questionnaire and the Brigance Screens-II. The parents filled out the PEDS questionnaire at both visits, at 18 months and 30–35 months of age, and the children underwent the Brigance Screens-II at 30–35 months. Both tests were administered by trained nurses at each health care center following prescribed protocols [26–28]. PEDS questionnaire and Brigance Screens-II were both introduced in 2010 as part of routine health care visits at health centers in Iceland.

The PEDS is designed to detect parental concerns about the developmental status and behaviors of their child; it has been found to have very good reliability and has been validated for children from birth to 8 years of age [29–33]. The PEDS questionnaire consists of 10 brief questions, two open-ended about general cognitive function and other concerns and eight domain-specific items. For each of the eight domain-specific questions the parents are asked if they have any concerns about the development or behavior of their child and their response option is in a multiple-choice format (no, yes, a little). Certain parental expressions of concern in response to certain of these questions are predictive of developmental delay [26]. If parents express concern in response to  $>2$  of these predictive questions, then health center procedures require that the child be referred for further evaluation (see Figure 1). It takes parents approximately 5 min to answer the questionnaire.

**Figure 1.** Developmental screening tests used in the study, the Parent’s Evaluation of Developmental Status (PEDS) questionnaire and the Brigance Screens-II.



The Brigance Screens-II is administered by a trained nurse who observes the child and questions his/her parents and the test is completed by the child itself. It has good reliability and has been validated for measuring the developmental and behavioral status of toddlers and preschool children [34–36]. The Brigance Screens-II for 30–35 month old children is valid for children from the age of 29 months + 15 days to 35 months + 14 days old children. The Brigance Screens-II comprises 11 components and it takes children approximately 15–20 min to complete the test. The cut off points for defining children at risk of developmental delay are <72 and <76 points of 100 points for children aged 30–32 months and 33–35 months, respectively. As with the PEDS questionnaire, there are some components of the Brigance Screens-II more predictive for developmental delay than others among all the test components (see Figure 1). Cut off points for defining children at risk of developmental delay are <34 and <35 points for children aged 30–32 and 33–35 months, respectively [37]. In the current study we focused on assessment of gross motor skills (3B), fine motor skills (6B, 8B) and receptive or expressive language (5B, 10B, 11B), since studies indicate that breastfeeding may influence these factors [12–18].

### 2.3. Statistical Analysis

Data were analyzed with SPSS Windows statistical software package version 20.0 (SPSS Inc., Chicago, IL, USA) with a level of significance of  $p \leq 0.05$ . Data were presented with means and standard deviations (SD) for normally distributed variables and with median and interquartile range (IQR) for variables with skewed distribution. Group comparisons were performed using independent-samples *t*-test and Mann-Whitney U-test. Comparisons between categorical values were made using the Chi-square tests of association or two-sided Fisher's exact test. Regression analysis was performed to adjust for any pre-randomization characteristics that were different between the two intervention groups at baseline. Finally, we calculated the power to detect differences between the CF and EBF groups based on proportions. To detect a significant difference between intervention groups in developmental scores from the Brigance screening test at 30–35 months of age with a sample size of 66 and a power of 80%, the mean difference in developmental scores would have had to be approximately 11.2 points, or approximately 5.4 points if excluding the three outliers ( $n = 63$ ). Of the 100 mother-infant pairs who finished the breastfeeding intervention trial, a total of 95 children attended routine care at 18 months and 82 at 30–35 months. Fifty-four parents answered the PEDS questionnaire when their child was 18 months and 78 parents at the 30–35 months visit. These numbers are based on the calculation of the sample size.

## 3. Results

### 3.1. Sample Size and Characteristics of Participants

Since both PEDS questionnaire and Brigance Screens-II were introduced in 2010 we have 41 missing data points from PEDS questionnaire at 18 months of age for those children in the study born in 2007 and some who were born in 2008. Parents of 4 children who attended routine care at 30–35 months did not answer the PEDS questionnaire at that age. The Brigance Screens-II was undertaken by 77 children at the age of 30–35 months, but 10 of them were too old (>35 months + 14 days)

and 1 too young (<29 months + 15 days) when the Brigance Screens-II was performed and were therefore excluded from the analysis. The PEDS questionnaire is for a wider age range but we chose to use 30–35 months throughout the paper. The children that did not have developmental scores recorded from the Brigance Screens-II ( $n = 23$ ) were lost to follow-up for several reasons, such as the family had moved abroad or failure to attend the routine health care visits at the health center.

Among those children with developmental scores from the Brigance Screens-II at 30–35 months of age ( $n = 66$ ), no differences between study groups were seen in baseline characteristics, except for mode of delivery, where vaginal delivery was more common among children in the CF group (94% vs. 74% in the EBF group,  $p = 0.04$ ) (see Table 1). No difference was seen in baseline characteristics (same as seen in Table 1) among those who were followed-up ( $n = 82$ ) and those who were lost to follow-up ( $n = 18$ ), except for parity, where those parents who were lost to follow-up had more children ( $3.0 \pm 1.0$  children) than those who were followed-up ( $2.0 \pm 2.0$  children;  $p = 0.01$ ).

**Table 1.** Baseline characteristics of participants with scores from Brigance Screens-II at 30–35 months of age in the two study groups: infants who received complementary foods in addition to breast milk from 4 months (CF,  $n = 35$ ) compared with infants who were exclusively breastfed for 6 months (EBF,  $n = 31$ ).

Variables	Group CF	Group EBF
Boys	17 (49%) *	13 (42%) *
Birth weight (g)	3687 (432)	3733 (526)
Length at birth (cm)	51.3 (1.8)	51.7 (1.9)
Head circumference at birth (cm)	35.8 (1.3) †	35.9 (1.4)
Gain in head circumference from birth–18 months (cm)	12.6 (1.2) ‡	12.6 (1.7) §
Age when Brigance Screens-II was performed (months)	32.3 (1.6)	32.8 (1.6)
Gestational length (days)	280.5 (9.3)	280.8 (7.1)
Maternal age (years)	29.4 (4.4)	31.2 (4.8)
Maternal education ¶	22 (63%) *	16 (52%) *
Vaginal delivery	33 (94%) *	23 (74%) *
Parity	2.0 (2.0) ¶¶	2.0 (1.0) ¶¶
Father's education ¶¶	13 (38%) **‡	14 (45%) *

Data are presented as mean (SD) unless otherwise indicated; \* Data are presented as number (%); † One missing value,  $n = 34$ ; ‡ Three missing values,  $n = 32$ ; § Five missing values,  $n = 26$ ; ¶ Finished studies at university level; ¶¶ Data are presented as median (IQR).

### 3.2. Developmental and Behavioral Status

Table 2 shows the developmental and behavioral status measures in the two study groups at 18 months (PEDS questionnaire) and at 30–35 months (PEDS questionnaire and Brigance Screens-II). At 18 months, a significantly smaller percentage of parents had concerns about any of the domains of PEDS on their children's developmental and behavioral status in the CF group compared with those in the EBF group (17% in the CF group vs. 44% in the EBF group;  $p = 0.03$ ). A logistic regression was done to test the impact of the intervention by group, and when adjusted for mode of

delivery, the difference in parents' concerns between groups at 18 months was not statistically significant ( $p = 0.08$ ). No difference was seen between groups in the number of concerns regarding gross or fine motor skills or receptive and expressive language. At 18 months, parents most often expressed concerns about their children's expression and articulation of the eight domain-specific questions; 10% and 20% in the CF and EBF group, respectively ( $p = 0.45$ ). No significant differences were seen even when those questions with greater predictive value for developmental delay were compared among groups at 18 months (0% in the EBF group vs. 3% in the CF group;  $p = 1.0$ ).

At 30–35 months of age no significant differences were seen between study groups in number of parents with concerns about any of the domains of PEDS (42% in the EBF group vs. 33% in the CF group;  $p = 0.45$ ). A smaller proportion of parents of children in the CF group (2%) had concerns about their gross motor development compared with parents of those in the EBF group (19%;  $p = 0.01$ ). When adjusted for mode of delivery the difference was still significant ( $p = 0.03$ ). No difference was seen between groups in number of concerns regarding fine motor skills or receptive and expressive language. At 30–35 months, parents most often expressed their concerns about their children's expression and articulation of the eight domain-specific questions; 19% and 28% in the CF and EBF group, respectively ( $p = 0.36$ ). Use of the cut off of  $\geq 2$  predictive concerns for PEDS questionnaire at 30–35 months showed that 19% of parents in the EBF group were above the cut off value compared with 5% of the parents in the CF group, although the difference was not significant ( $p = 0.07$ ).

**Table 2.** Selected measures of developmental and behavioral status for children at 18 months and at 30–35 months of age in the two intervention groups: infants who received complementary foods in addition to breast milk from 4 months (CF) compared with infants who were exclusively breastfed for 6 months (EBF).

Variables	Group CF	Group EBF	P-value
<i>PEDS questionnaire</i>			
Parents with concerns according to PEDS at 18 months	5 (17%) *, $n = 29$	11 (44%) *, $n = 25$	0.03
Parents with concerns according to PEDS at 30–35 months	14 (33%) *, $n = 42$	15 (42%) *, $n = 36$	0.45
<i>Brigance Screens-II</i>			
	$n = 35$	$n = 31$	
Total score at 30–35 months	86.0 (12.5) †	86.5 (12.5) †	0.82
Total score above cut off value ‡	2 (6%) *	4 (13%) *	0.41
Score of predictive factors combined above cut off value §	7 (20%) *	3 (10%) *	0.32
<i>Components of the Brigance Screens-II</i>			
Gross motor skills	6.0 (6.0) †	6.0 (4.5) †	0.44
Fine motor skills	19.0 (3.0) †	19.0 (3.0) †	0.89
Expressive and receptive language	40.5 (8.0) †	42.0 (9.5) †	0.81

Data are presented as mean (SD) unless otherwise indicated; \* Data are presented as number (%); † Data are presented as median (IQR); ‡ Cut off values for defining risk of developmental delay were <72 and <76 points from the total score from the Brigance Screens-II for children aged 30–32 months and 33–35 months, respectively; § Cut off values for defining risk of developmental delay were <34 and <35 points from the predictive components of the Brigance Screens-II combined for children aged 30–32 months and 33–35 months, respectively; || Two missing values,  $n = 29$ .

There was no significant difference between study groups at 30–35 months by the Brigance Screens-II ( $p = 0.82$ ). Neither was there a significant difference between the groups in the number of children below the cut off value defining developmental delays for total score from the Brigance Screens-II ( $p = 0.41$ ) or number of children above the cut off value defining developmental delays from predictive components of the Brigance screening test combined ( $p = 0.32$ ). Furthermore, there was no significant difference between groups in fine or gross motor skills or receptive and expressive language according to the Brigance Screens-II at 30–35 months. Excluding three outliers found in the EBF group in the Brigance screening test did not change the mean values for the study groups or the lack of significance ( $86.1 \pm 7.8$  points in the CF group vs.  $88.0 \pm 7.4$  points in the EBF group;  $p = 0.33$ ).

#### 4. Discussion

In this study of well-nourished children at 30–35 months of age, a smaller proportion of parents in the CF group expressed their concerns about their children's gross motor development on the PEDS questionnaire, a difference that remained significant when adjusted for differences in pre-randomization characteristics. However, there were no significant intergroup differences in developmental total scores or in fine and gross motor skills or receptive and expressive language according to the Brigance Screens-II at 30–35 months. No difference was seen in the percentage of parents with concerns about their children's developmental and behavioral status at the age of 18 months.

Results from the PEDS questionnaire are based on a small number of categorical variables. Outcomes from the Brigance Screens-II, however, are based on continuous variables and therefore this test is more responsive to detecting minor developmental disabilities. The Brigance Screens-II is a comprehensive, reliable and valid screening tool of developmental status that is completed by the child itself [36,38]. Similar general developmental screening tools that are directly administered to the child and are used in primary care settings are the Battelle Developmental Inventory Screening Tool Test II, the Bayley Infant Neurodevelopmental Screener and the Denver-II Developmental Screening Test, which are all comparable to the Brigance Screens-II in sensitivity and specificity [38,39]. Per health center protocols in the Icelandic healthcare system, children identified at risk for developmental delay or behavioral problems according to the Brigance Screens-II or the PEDS questionnaire are referred for further evaluation, diagnosis and then developmental intervention, if appropriate. Early detection of developmental delay and appropriate intervention has been shown to be effective in improving developmental outcomes in childhood [40].

Although the PEDS is solely based on parental perception of their children's developmental and behavioral status, a positive correlation has been shown between the results of the PEDS questionnaire and the Brigance Screens-II [28]. The PEDS questionnaire is a valid and reliable developmental screening tool [38] and the value of parents' concerns in the detection of developmental delay has been well studied [31,41]. Comparable commonly used parent-completed screening questionnaires in primary care settings comparable are the Ages & Stages Questionnaires, the Child Development Review-Parent Questionnaire and the Infant Development Inventory [38,39]. These tests are not perfectly concordant, but are widely used and are considered appropriate for developmental evaluation

in primary care settings [42,43]. It should be noted that although some parental concerns are predictive over time, the PEDS questionnaire does not always capture longitudinal changes in developmental status since parents may have fewer concerns after their child begins a developmental intervention, even though developmental delays may still be present. In 2006, the American Academy of Pediatrics recommended systematic developmental screening in primary care using a validated screening tool for children aged 9, 18 and 30 months, but no specific guidance was provided for the specific screening tools that should be used [38]. Health care centers in Iceland chose to use the PEDS questionnaire and the Brigance screening test because of their good reliability and validity and well-established sensitivity and specificity and because they are useable for a wide range of ages in childhood [28].

To our knowledge this is the first secondary analysis of a randomized controlled trial conducted in a resource rich country to examine the effects of exclusive breastfeeding for 4 vs. 6 months on selected measures of developmental and behavioral status in early childhood. The World Health Organization (WHO) recommended exclusive breastfeeding for 4–6 months of life until the year 2001 when the recommendation was changed to breastfeed exclusively for the first 6 months of life in an effort to lower the risk of adverse health outcomes for infants during the first 6 months, particularly in resource constrained countries [44]. Developmental status is influenced by a number of genetic and environmental factors that cause cumulative risk effects of development delays that are generally not addressed in observational studies. This is one possible explanation for the inconsistent findings among such studies [13,45,46]. Studies investigating the relationship between breastfeeding and developmental status often compare formula fed infants to breastfed infants [46–49], but less is known about the impact of exclusive breastfeeding compared to partial breastfeeding.

There is strong evidence that nutrition early in life can have long-term effects on health and development later in life [50–52]. It has been suggested that the concentration of long chain polyunsaturated fatty acids in breast milk may explain the enhanced cognitive outcomes reported in some studies comparing breastfed and formula fed infants [53–56] and therefore the effect of duration of exclusive breastfeeding on developmental and behavioral status would also be a relevant factor in these outcomes. Since infants exclusively breastfed for 6 months in the present study had significantly higher breast milk intakes at 5.5–6 months of age [22], we hypothesized they would have better developmental and behavioral status in early childhood. However, no intergroup differences in measures of developmental and behavioral status outcomes were observed among those that completed the Brigance Screens-II at 30–35 months of age. The parental impressions from the PEDS questionnaire administered when the children were 30–35 months of age were thus not substantiated by the more objective and reliable Brigance Screens-II at the same age. The reason for no difference in these developmental and behavioral measures might be because both study groups consumed a significant amount of breast milk. While the infants in the EBF group consumed significantly more breast milk than those in the CF group (83 g/day, measured using the stable isotope technique) [22], the amount consumed by the CF group was consistent with the recommendations of the WHO [57]. The mothers who participated in our study were generally well-educated and well-supported, and we cannot generalize our findings to other populations. It is possible that in less well-educated or supported mothers, the introduction of a small amount of CF might result in a greater decrease in breast milk production with more impact on health outcomes, including development.

The strength of the present study lies in the fact that this is the only analysis of later developmental and behavioral data from a randomized controlled trial of 4 vs. 6 months of exclusive breastfeeding

and it therefore has a methodological advantage over previously published observational studies. Furthermore, approximately 78% of the cohort was follow-up until the age of 30–35 months. The main limitation of the present study is that data were collected in routine health care visits at the health center. We recognize that this secondary data analysis may have been underpowered to detect small effects on developmental and behavioral outcomes that may be biologically relevant, but the sample size was adequate to exclude large effects on developmental and behavioral outcomes in the two groups [58].

In addition to breast milk *per se*, other factors that influence infant development may have played a role in the outcomes we observed in this randomized trial. Infants with depleted iron stores, iron deficiency or iron deficiency anemia can have lower developmental scores in childhood [59,60], however, we have previously reported that no differences was seen in the prevalence of iron deficiency with or without anemia between both groups at 6 months of age [23]. Mothers who choose to breastfeed may differ from those who never breastfeed in many ways that can influence an infant's development, including socio-economic status and nurturing qualities. However, mothers in both study groups exclusively breastfed for the first 4 months of their infant's life all were from a similar socioeconomic background and thereafter all of them continued breastfeeding partially or exclusively until 6 months of age or beyond, minimizing the impact of these other influential developmental factors. It is possible that the mothers participating in the study might differ from other mothers in the population.

In conclusion, the present study showed no sustained effect of a longer duration of exclusive breastfeeding on selected measures of developmental and behavioral status at 18 months of age although at 30–35 months, a smaller percentage of parents of infants introduced to complementary foods at 4 months of age expressed concerns about their children's gross motor development. Further investigation is needed in a larger randomized controlled trial using the same or other measures of developmental and behavioral status to extend and confirm these findings.

## 5. Conclusions

Breastfeeding during infancy may have beneficial effects on various developmental outcomes in childhood. The association between breastfeeding and developmental status is based on observational studies that are subject to bias by confounding factors. In this randomized controlled trial, no sustained difference were seen on selected measures of development and behavior in early childhood between those receiving complementary foods in addition to breast milk from 4 months or those exclusively breastfed for 6 months. Further investigation is needed in a larger randomized controlled trial using the same or other measures of developmental and behavioral status to extend and confirm these findings.

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### Conflicts of Interest

Mary S. Fewtrell has received research funding from and undertaken advisory work for companies manufacturing infant foods and feeding products within the past 3 years, all the other authors declare that they have no conflicts of interests.

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## Breastfeeding and Active Bonding Protects against Children's Internalizing Behavior Problems

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**Abstract:** Breastfeeding is associated with numerous health benefits to offspring and mothers and may improve maternal-infant bonding. Ample evidence suggests breastfeeding can improve child neurodevelopment, but more research is needed to establish whether breastfeeding is linked to the development of child psychopathology. This paper aims to explore the effects of both breastfeeding and mother-child interactions on child behavioral outcomes at a later age. Children from the China Jintan Child Cohort Study ( $N = 1267$ ), at age six years old were assessed, along with their parents. Children who were breastfed exclusively for a period of time in the presence of active bonding were compared to those who were breastfed in the absence of active bonding as well as to children who were not exclusively breastfed, with or without active bonding. Results from ANOVA and GLM, using SPSS20, indicate that children who were breastfed and whose mothers actively engaged with them displayed the lowest risk of internalizing problems (mean = 10.01, SD = 7.21), while those who were neither exclusively breastfed nor exposed to active bonding had the least protection against later internalizing problems (mean = 12.79, SD = 8.14). The effect of breastfeeding on internalizing pathology likely represents a biosocial and holistic effect of physiological, and nutritive, and maternal-infant bonding benefits.

**Keywords:** breastfeeding; bonding; attachment; internalizing behaviors

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## 1. Introduction

Breastfeeding is associated with a wide range of positive health outcomes in children and mothers. For example, a systematic review and meta-analysis of approximately 400 studies found that breastfeeding was related to a reduced risk of acute ear infections, respiratory tract infections, asthma, obesity, type 1 and 2 diabetes mellitus, and childhood leukemia [1]. The production of prolactin and oxytocin during breastfeeding is associated with lower levels of maternal stress and enhanced bonding [2]. Furthermore, early cessation of breastfeeding or not breastfeeding at all has been linked to an increased risk of maternal postpartum depression [1].

One particular outcome of interest is that of cognitive development in breastfed children. Breast milk is rich in vital nutrients, such as essential fatty acids, vitamins, minerals, and amino acids, that are associated with improved cognitive functioning [3], language development [4], and overall neurological development [2,5]. In addition, breastfeeding has been associated with improved mother-infant bonding [6,7]. For instance, early feeding interactions between mother and infant may result in more positive feeding experiences and produce greater maternal sensitivity and responsiveness to infant needs [8].

Although previous studies have indicated a wealth of nutritional, physiological, and cognitive benefits to children from breastfeeding, little has been done on emotional development and regulation. It is known that childhood internalizing disorders, including depression and anxiety, can affect up to about 20% of children and adolescents [9]. They also increase the risk of future psychopathology in adulthood [10]. The identification of readily modifiable factors, such as breastfeeding, that may protect against childhood internalizing behaviors is therefore important. Studies have not yet found a relationship between breastfeeding and behavioral outcomes during early childhood [11,12]. However, there are limited studies conducted in older age groups. Oddy *et al.* found that breastfeeding may be associated with adverse mental health outcomes in early adolescents while Kwok *et al.* found inconsistent associations [13,14]. However, few studies have tested the long term effect of both breastfeeding as well as the mother-infant interaction during feeding on child behavioral outcomes.

The purpose of this study is to examine whether breastfeeding was related to fewer internalizing disorders later in childhood in a large, community-based sample of Chinese children and parents, and to understand whether breastfeeding and active bonding (*i.e.*, verbal interactions during feeding) were associated with the reduced risk of internalizing behaviors. Finally, this study will assess whether there was any breastfeeding duration (or dosage) effect on internalizing behaviors.

## 2. Experimental Section

### 2.1. Participants and Sample Design

The current study was part of a larger population-based community cohort study of 1656 Chinese children (55.5% boys, 44.5% girls) initially recruited from four preschools in the town of Jintan,

located in the southeastern coastal region of Mainland China. Briefly, all children and parents taking part in the original cohort study were invited to participate for assessment of children's behaviors while the children were in the final few months of their senior year in preschool (Spring 2005 to Spring 2007). At that point, some children dropped out of the study because of changing schools or because bio-markers were not obtained originally; therefore, only 1419 children in the original sample were followed up in the later waves. Detailed sampling and research procedures of this larger cohort study have been described elsewhere [15,16].

We excluded the cases where the children are over six years old (there are quite a few because it was late in the school year) for the purpose of this analysis because we are using Child Behavior Checklist (CBCL) 1.5 to 5 to measure behavioral outcome. We performed a comparison on measures such as mother's age when the children were born, neighborhood problems, gender, parent's education and occupation prestige, as well as whether parents are separated or divorced, and found there is no difference except the older children tend to be boys and have parents who are less educated. We acknowledge that Chinese tradition prefers to hold boys back in the earlier years of education because of the cultural belief that boys develop and mature later than girls, and that more educated parents would want to place their children in school as early as possible.

The analysis sample consists of 1267 complete data. The mean age of the analysis sample was 66.6 months (SD = 5, range = 50.0–71.9). This is close to the common kindergarten age in the US.

## 2.2. Measures

### 2.2.1. Internalizing Behavior Problems

We used the Internalizing Behavior scale from the Child Behavior Checklist (CBCL)/1.5-5 as the measure for the dependent variable. The factor structures of CBCL/1.5-5 have been validated in our previous study [17]. The internalizing behavior scale consists of 36 items out of 99 items in total, including Emotionally reactive, Anxious/Depressed, Withdrawn and Somatic Complaints. Items are rated on a 3-point scale (0 = not true, 1 = sometimes true, or 2 = often true) [18]. In this study, we utilized both the full Internalizing Behavior scale and the four syndromes as dependent variables. Alpha is 0.87 for the scale in our sample. We used raw scores on all behavioral assessments for the analysis, as recommended by Achenbach [19].

### 2.2.2. Breastfeeding

Mothers completed a retrospective questionnaire that asked whether they breastfed (78.3%), used formula (5.6%) or both (16%). In this study, we define exclusive breastfeeding as exclusive for a period of time with a minimum of one month. Non-exclusive breastfeeding is defined to include formula only or mixed methods. Mothers were also asked to report breastfeeding duration in months (mean = 8.79, range 0–24). Duration was categorized into three levels: less or equal to 7 months (25%), between 7 and 10 months (51%) and greater or equal to 10 months (24%).

### 2.2.3. Maternal Interaction

Mothers were asked a follow-up question for (breast) feeding: “Did you talk to the child while (breast) feeding in the first two years” These responses were: 1 = Never, 2 = Sometimes, 3 = Always. In our study, not all mothers were always talking to the child while breastfeeding. We combined the two measured into one and named it “Feeding types and bonding”, reflecting the beneficial effects of both nutritional and active communication. In our sample, we identified four groups. 34.8% of the mothers who always use breast milk and always talk to the child while feeding were classified into Breastfed and Active bonding (group 1); 43.5% who never talk to the child even when they used breast milk were assigned into Breastfed and no Active Bonding (group 2); 9% of the mothers were in the group of Non-breastfed and Active Bonding (group 3), and the final 12.7% in the Non-breastfed and no Active Bonding (group 4).

### 2.2.4. Social Adversity

Parents were asked to fill in a sociodemographic questionnaire at the same time they completed the CBCL when children were six years old. A number of researchers have demonstrated the utility of combining of several individual measurements of psychosocial risk factors in studying child behavior problems [20,21]. The adversity index was created along lines similar to those described by Rutter and Moffitt [20,21]. A total adversity score was derived based on 11 variables. This score was created by adding 1 point (for 9 of the 11 indicators) or 2 points (for 2 indicators) for 11 adversity indicators: mother’s low education (below middle school, 8.8%), father’s low education (below middle school, 5.4%), mother’s low occupational status (3-point scale: 0 = professional or skilled work, 22.2%; 1 = un-skilled worker, 38.9%; and 2 = no occupation, 31.8%), father’s low occupational status (3-point scale: 0 = professional or skilled work, 26.8%; 1 = unskilled worker, 60.3%; and 2 = no job, 3.8%), mother’s poor health status (2.1%), father’s poor health status (3.8%), obstetric complication (bleeding, hypertension, diabetes, Caesarian section, difficult birth, low birth weight, difficulty breathing, 35.6%), divorce (3.3%), absence of biological mother (4.2%), house size below 70 m<sup>2</sup> (8.4%), and poor neighborhood (overcrowded neighborhood, noise pollution, damp, 35.6%). Details on these indicators are given in Liu *et al.* [22]. The adversity score ranged from 0 to 13 (M = 3.51, SD = 2.04).

### 2.3. Statistical Methods

To test whether there is any social adversity or internalizing behavior score differences between exclusive and non-exclusive breastfeeding groups, independent *t* tests were employed. Differences in proportions for gender were tested using  $\chi^2$  tests. The same tests were conducted again to test differences between active bonding and non-active bonding group. Cohen’s *d* was calculated to show the size of the effect between two comparison groups.

To test for the combined effects of feeding and bonding types on internalizing behaviors, a series of analysis of variance (ANOVA) were performed. “Feeding and bonding types” was a new variable (four groups defined in the method section) created from the two independent binary variables. It was the grouping variable in ANOVA, and the dependent variables were emotionally reactive,

Anxious/Depressed, Somatic Complaints, Withdrawn and total internalizing problems respectively. Omega squared  $w^2$  was computed as an index of effect size with several groups.

To test for the effect of social adversity as a potential confound, general linear models were fitted with adversity entered as covariate. The effect moderator of sex was assessed by entering the measure as a factor alongside feeding and bonding type. Gender  $\times$  breastfeeding and bonding types interaction term was included in the model as well. The interaction effect of breastfeeding types and bonding types was tested in the models besides including these two variables independently in the models. To test for a dose-response relationship between breastfeeding duration and internalizing behavior, the grouping variable of duration took on three levels ( $\leq 7$  months, 7–10 months and  $\geq 10$  months) for univariate ANOVAs. All results were considered significant if  $P < 0.05$  using a two-tailed test. Statistical analyses were conducted by using SPSS version 20.0 (IBM SPSS Statistics).

### 3. Results

#### 3.1. Single Effect of Breastfeeding or Active Bonding

Of the 1267 participants with completed data, 77% exclusively breastfed their babies and 43.4% always talk to their babies while feeding. Mean (SD) scores, effect sizes (Cohen's  $d$ ) and results of specific  $t$  test comparisons for the two pairs of comparison groups are given in Table 1. The children who were exclusively breastfed had significantly lower mean scores for somatic complaints and total internalizing problems. Active bonded children had significant lower scores across emotionally reactive, anxious/depressed, withdrawn syndromes and total internalizing problems.

#### 3.2. Combined Effect of Breastfeeding and Active Bonding

ANOVA results showed significant group differences on mean behavior scores for total internalizing ( $F(3,1264) = 5.21$ ;  $P = 0.001$ ), anxious/depression ( $F(3,1263) = 2.779$ ;  $P = 0.04$ ), somatic complains ( $F(3,1264) = 3.20$ ;  $P = 0.023$ ) and withdrawn ( $F(3,1264) = 6.75$ ;  $P < 0.001$ ). Emotionally reactive showed borderline significance ( $F(3,1261) = 2.38$ ;  $P = 0.068$ ). Mean (SD) scores, effect sizes (omega squared  $w^2$ ) and  $P$  values from ANOVA for the four comparison groups are given in Table 2. Results revealed identical trend across all dependent variables, with group 1 displaying the lowest scores, followed by group 3. Group 4 had the highest scores.

#### 3.3. Potential Confounds

Demographic and social variables are known to influence breastfeeding and children's behavior. Our study showed that boys were more likely be fed by formula or a mixed method rather than pure breast milk ( $\chi^2 = 2.17$ ;  $P = 0.031$ ). Social adversity scores were significantly lower for those whom actively bonded with their baby while feeding ( $t = 5.18$ ;  $P < 0.001$ ), indicating they had a better socioeconomic background and health status (Table 1). Consequently, it is possible that social adversity and gender could account for the main effects of breastfeeding and bonding. We also tested the correlations between potential confounders and dependent variables. In our sample, Spearman correlation indicates social adversity was positively correlated to each syndrome and total internalizing problems ( $P < 0.001$  for all), although no significant correlation was detected between gender

and dependent variables. As gender and social adversity were correlated to either the breastfeeding and bonding types or the internalizing problems, we entered these two constructs in to a series of general linear models. The main effects remained significant for Anxious/Depressed, Somatic Complaints, Withdrawn and total internalizing (Table 3) after controlling for adversity ( $F(1,1252) \geq 10.98$ ;  $P \leq 0.001$  for all) and gender ( $F(1,1252) \leq 1.56$ ;  $P \geq 0.212$  for all).

### 3.4. Effect Moderator

There was no interaction between breastfeeding and bonding grouping and gender ( $F(1,1252) \leq 2.1$ ;  $P \geq 0.097$  for all), indicating that this measure did not moderate the effects of breastfeeding and bonding.

### 3.5. Dose Response Relationship

Univariate ANOVAs (with three groups: 0–7 months, 7–10 months and 10 months duration levels) were conducted on each syndrome and total internalizing scores. Results showed significant main effect for breastfeeding duration on anxious/depression ( $F(2,1170) = 3.28$ ;  $P = 0.038$ ), somatic complains ( $F(2,1171) = 3.25$ ;  $P = 0.039$ ) and total internalizing ( $F(2,1171) = 2.99$ ;  $P = 0.051$ ). No significant dose-response effect was detected for emotionally reactive ( $F(2,1170) = 1.21$ ;  $P = 0.298$ ) and withdrawn ( $F(2,1171) = 0.910$ ;  $P = 0.403$ ). The mean scores for each syndrome and total internalizing problems were plotted against breastfeeding duration levels in Figure 1a,b.

## 4. Discussion

Three key findings emerged from this study. First, compared to children whose mothers breastfed them, children who were not breastfed showed an increased number of internalizing behavioral problems, particularly anxious/depressed and somatic symptoms. Second, the group of children whose mothers both breastfed and actively interacted with their infants had the least likelihood of displaying internalizing problems. Children who were not breastfed but whose mothers still engaged in active interactions displayed the next-lowest risk, while being neither breastfed nor exposed to active bonding had the smallest effect on internalizing behaviors. Finally, a duration effect (dosage effect) appeared such that breastfeeding for 10 months or longer had the strongest impact on reducing anxious/depressed and somatic symptoms in children.

Breastfeeding confers a strong biological benefit to infants and their development [23]. From a nutritive standpoint, breast milk contains docosahexaenoic acid (DHA) omega-3 fats, the consumption of which, along with eicosapentaenoic acid (EPA) fats, may reduce the risk for affective disorders, including major depression and bipolar disorders, particularly among women [24,25]. However, the overall literature on DHA and depression remains mixed [26,27]. What is known, however, is that DHA plays a vital role in neural development, neurotransmitter transmission, and genetic expression, making it highly relevant to child neurodevelopment as well as developmental disorders, such as attention-deficit/hyperactivity disorder and motor deficits [28].

**Table 1.** Descriptives of the Breastfeeding and internalizing behavior Outcome ( $N = 1267$ ).

Variables	Breastfeeding type			Active bonding				
	Exclusive 77%	Non-exclusive 23%	Effect Size (Cohen's $d$ )	$P$	Always 43.4%	Never/Sometimes 56.6%	Effect Size (Cohen's $d$ )	$P$
Emotionally Reactive	2.63 (2.39)	2.85 (2.38)	-0.092	0.168	2.52 (2.30)	2.82 (2.43)	-0.126	0.031
Anxious/Depressed	3.22 (2.28)	3.43 (2.30)	-0.091	0.175	3.13 (2.17)	3.44 (2.36)	-0.136	0.02
Somatic Complaints	2.64 (2.47)	3.01 (2.34)	-0.153	0.025	2.60 (2.33)	2.86 (2.54)	-0.106	0.076
Withdrawn	2.18 (2.34)	2.43 (2.40)	-0.105	0.11	1.89 (2.19)	2.52 (2.44)	-0.271	<0.001
Total Internalizing	10.66 (7.64)	11.7 (7.53)	-0.137	0.04	10.14 (7.05)	11.63 (7.94)	-0.198	0.001
Boy %	53.3	60.4	\	0.031	57.3	53.1	\	0.141
Social Adversity	3.53 (2.03)	3.48 (2.11)	0.0241	0.701	3.17 (1.83)	3.77 (2.15)	-0.104	<0.001

Note: significant results were highlighted in bold; \ not available.

**Table 2.** Combined Effect of Breastfeeding and Active Bonding on Behavior Problems from ANOVA.

Variables	Group 1		Group 2		Group 3		Group 4		Effect Size (omega squared $\omega^2$ )	$P$
	Exclusive Breastfeeding with Active Bonding 34.8%	Exclusive Breastfeeding without Active Bonding 43.5%	Exclusive Breastfeeding with Active Bonding 9%	Non-exclusive Breastfeeding with Active Bonding 9%	Non-exclusive Breastfeeding without Active Bonding 12.7%	Non-exclusive Breastfeeding without Active Bonding 12.7%				
Emotionally Reactive	2.48 (2.30)	2.73 (2.45)	2.62 (2.22)	2.62 (2.22)	3.07 (2.50)	3.07 (2.50)	0.003	0.068		
Anxious/Depressed	3.11 (2.17)	3.30 (2.34)	3.15 (2.15)	3.15 (2.15)	3.71 (2.41)	3.71 (2.41)	0.004	0.04		
Somatic Complaints	2.56 (2.39)	2.70 (2.53)	2.72 (2.14)	2.72 (2.14)	3.27 (2.50)	3.27 (2.50)	0.005	0.023		
Withdrawn	1.87 (2.20)	2.40 (2.41)	2.13 (2.22)	2.13 (2.22)	2.73 (2.53)	2.73 (2.53)	0.013	<0.001		
Total Internalizing	10.01 (7.21)	11.12 (7.90)	10.61 (6.66)	10.61 (6.66)	12.79 (8.14)	12.79 (8.14)	0.01	0.001		

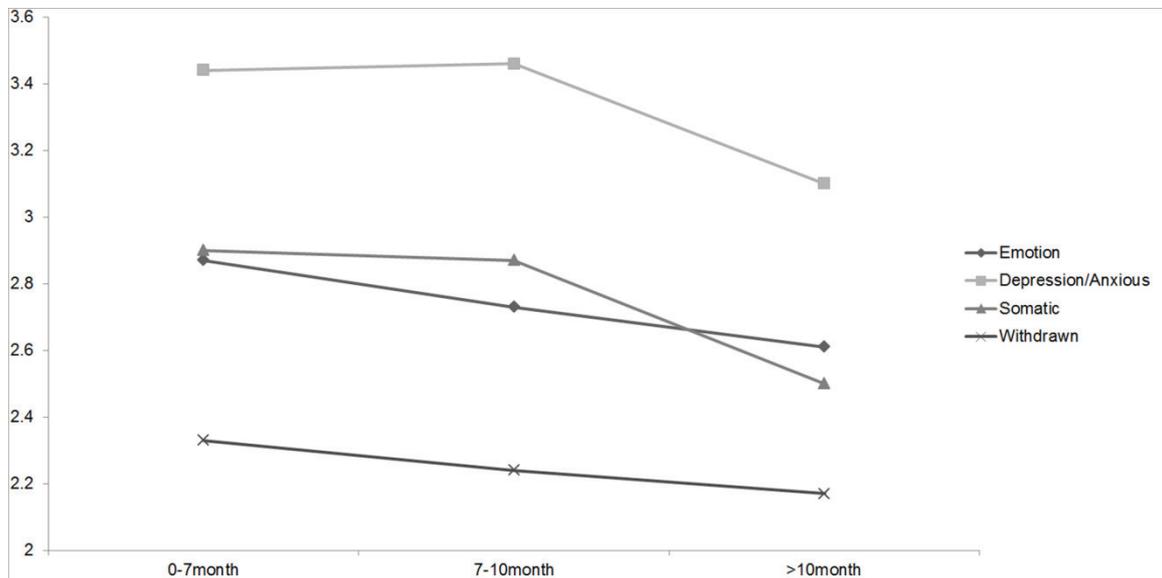
Note: significant results were highlighted in bold.

**Table 3.** General linear model statistics of breastfeeding type and bonding against internalizing problems, controlling for gender and social adversity.

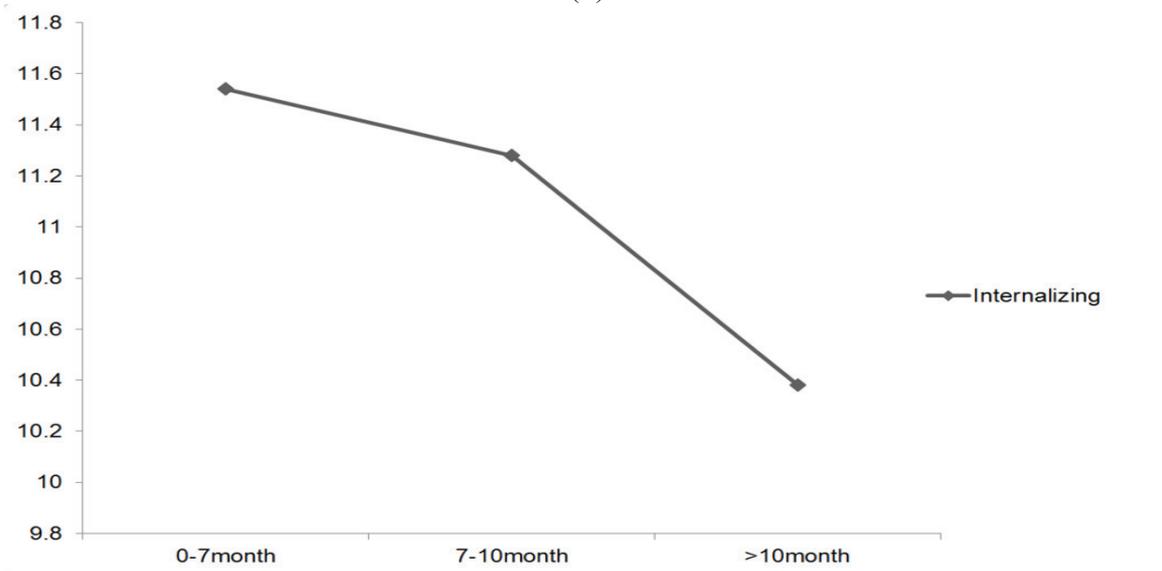
Dependent Variables	Type III Sum of Squares	df	Mean square	F	Significance
Emotionally Reactive	34.4	3, 1255	11	2.045	0.106
Anxious/Depressed	38.12	3, 1257	12.71	2.471	<b>0.06</b>
Somatic Complaints	54.6	3, 1258	18.2	3.108	<b>0.026</b>
Withdrawn	90.07	3, 1258	32.36	6.016	<b>&lt;0.001</b>
Total Internalizing	774.39	3, 1258	258.13	4.573	<b>0.003</b>

Note: significant results ( $P < 0.05$ ) were highlighted in bold.

**Figure 1.** Dose-response Relationship between duration of breastfeeding and internalizing behavior. (a) Breastfeeding duration and internalizing syndromes; (b) Breastfeeding duration and total internalizing problems.



(a)



(b)

It may be that the biological benefit offered to breastfed infants plays a role in healthy cognitive maturation which in turn lowers their risk for psychopathology [29]. Feldman and Eidelman report that breastfeeding is associated with improved motor and social skills [5], but other authors have not found an impact on emotional regulation and behavioral disruption, indicating the need for further research on breastfeeding and child psychodevelopment [30]. Interestingly, a recent study examined effects of breastfeeding on mental health outcomes among children at age 14 years and found that breastfeeding at age six months was associated with a lower rate of child psychopathology, including social and attention difficulties and aggression [31]. However, more longitudinal data is needed to better understand the potential long-term benefits of breastfeeding to child mental health. Whether the nutritional, physiological and cognitive benefits from breastfeeding directly enhance mental health via a biological route in our brain may still require further exploration. However, speculatively, the reported nutritional, physiological and cognitive benefits can confer a lot of advantages to a child to negotiate with the challenges of growing up. For example, a healthier, energetic physical body and a faster cognitive growth can help a child to cope with the arduous demands of modern-day schooling, particularly in mainland China, the tradition of which has long emphasized education as the avenue for upward socio-economic migration. A child who excels at school will also be well liked and accepted by parents, relatives, teachers and peers. School success and social popularity are both known key precursors to mental health [32]. Thus, it is likely that there may be both a direct biological route and an indirect psychosocial route from breastfeeding leading to positive mental health or fewer internalizing problems. These dual routes should both be further examined in future study.

Breastfeeding also provides a biological benefit to the mother by reducing blood pressure and pain [33]. Furthermore, the release of hormones oxytocin and prolactin not only confer analgesic and relaxation benefits, but they also appear to play a key role in mother-infant bonding [11,33], which has been shown to reduce emotional and behavior problems in children [6,7].

The attachment aspect of breastfeeding underscores the need to consider its potential mental health benefits. Psychologically, breastfeeding may enhance the mother-infant bonding process via active talking, eye contact, and skin-to-skin touch. This may help mothers form stronger attachments to offspring and improve maternal sensitivity [33], reduce postpartum depression [34], and ward off other negative mood states like maternal stress [35]. This may indirectly benefit a child's mental health, as the literature detailing the impact of maternal depression on increasing the risk of future child and adolescent psychopathology is compelling [36,37].

Infants may similarly derive a mental health benefit from being breastfed, including development of more secure attachments and reduced negative temperament [38]. Several authors have documented analgesic properties of breast milk, along with reductions in salivary cortisol, due to milk odor and skin-to-skin contact [39,40]; these are hypothesized to help alleviate child distress and strengthen bonding.

Taken together, these findings underlie a biopsychological aspect of breastfeeding wherein the physiological benefits of breastfeeding (e.g., pain reduction, stress reduction, healthy cognitive development) coupled with improved pair bonding and mother-infant attachments may provide protective effects against the formation of child internalizing behaviors. The biopsychosocial interaction may also provide indirect benefits that operate through mediating or moderating variables [41]. For example, secure parent-child attachment may improve child sleep quality [42], and reduced sleep

problems in children has been linked to better emotional and behavioral functioning [43]. In addition, breastfeeding is ultimately a holistic process and there are several aspects that facilitate the process, including how the mother responds to the infant, the physical and social environment around the mother and baby, and the level nutrients in the breast milk. These factors, such as the genetic and environmental influences of nutrient intake (e.g., breast milk) should be considered [44]. Consideration of such in larger samples will require further study before definitive conclusions can be drawn about intervening variables on breastfeeding and internalizing conditions.

In this study, breastfeeding for a longer duration (at least 10 months) had the greatest effect on reducing internalizing symptoms. This is consistent with other authors who report that longer duration of breastfeeding was associated with greater protection against child mental health problems at age five years [45]. Another recent longitudinal cohort study from Oddy and colleagues followed breastfed infants to 14 years of age and found that breastfeeding for six months or less independently predicted greater externalizing and internalizing problems in childhood and adolescence, compared to infants who were breastfed for 6 months duration or longer [14].

Some important limitations on the present study's findings exist. First, the use of retrospective data may involve recall bias. However, in the current literature, it is not rare for studies examining breastfeeding practices to use maternal recall data after much longer periods. For example, a study by Promislow looked at maternal recall of breastfeeding duration of elderly US women from 34 to 50 years ago [46]. Nevertheless, future prospective designs should be considered. As previously mentioned, we also did not include holistic measures in our assessment of breastfeeding as we do not have data available due to the retrospective nature of this study. Instead, we included confounding factors, such as demographic and social background, in our analysis of breastfeeding. Another limitation of the present study is that it does not take into consideration the exact duration of exclusive breastfeeding, which is particularly important given the fact that breastfeeding practices have decreased in recent years, especially among urban and well-educated mothers [47]. Future studies should test if there is a duration-dependent relationship between breastfeeding and internalizing behavior in children. Additionally, as active bonding was one of the key predictors, future studies should employ validated, empirical-based measures on this construct. However, despite the use of such "crude" measures, they are able to produce consistent results, indicating evidently the benefits of breastfeeding on the children's mental health. Furthermore, future studies should stratify by region, given that breastfeeding practices differ by location.

## 5. Conclusions

These results indicate that children who were breastfed and exposed to active bonding during feeding displayed the lowest risks of internalizing behavior problems at age six years. Increased duration of breastfeeding ( $\geq 10$  months) could also help lower internalizing problems in children (*i.e.*, a dosage effect). These findings were independent of several socio-demographic/family characteristics, as well as gender. It is possible that both nutrients (e.g., fatty acids) and maternal bonding interactively work to promote optimal neurodevelopment in early childhood, subsequently protecting children from internalizing disorders, such as depression, anxiety, and somatic complaints [48]. The promotion of

active bonding practices during feeding (whether breastfeeding or formula feeding) may help reduce later internalizing behaviors in children by enhancing attachment between the mother and infant.

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### Conflicts of Interest

The authors declare no conflicts of interest.

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### 3. Infants

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## Comparison of the Effect of Two Human Milk Fortifiers on Clinical Outcomes in Premature Infants

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**Abstract:** The use of human milk fortifiers (HMF) helps to meet the high nutritional requirements of the human milk-fed premature infant. Previously available powdered products have not met the protein requirements of the preterm infant population and many neonatologists add powder protein modulars to help meet protein needs. The use of powdered products is discouraged in neonatal intensive care units (NICU) due to concern for invasive infection. The use of a commercially available acidified liquid product with higher protein content was implemented to address these two concerns. During the course of this implementation, poor growth and clinically significant acidosis of infants on Acidified Liquid HMF (ALHMF) was observed. The purpose of this study was to quantify those observations by comparing infant outcomes between groups receiving the ALHMF vs. infants receiving powdered HMF (PHMF). A retrospective chart review compared outcomes of human milk-fed premature infants <2000 g receiving the ALHMF ( $n = 23$ ) and the PHMF ( $n = 46$ ). Infant growth, enteral feeding tolerance and provision, and

incidence of necrotizing enterocolitis (NEC), metabolic acidosis, and diaper dermatitis were compared between the two groups. No infants were excluded from this study based on acuity. Use of ALHMF resulted in a higher incidence of metabolic acidosis ( $p = 0.002$ ). Growth while on HMF as measured in both g/kg/day (10.59 vs. 15.37,  $p < 0.0001$ ) and in g/day (23.66 vs. 31.27,  $p = 0.0001$ ) was slower in the ALHMF group, on increased mean cal/kg/day (128.7 vs. 117.3,  $p = 0.13$ ) with nearly twice as many infants on the ALHMF requiring increased fortification of enteral feedings beyond 24 cal/ounce to promote adequate growth (48% vs. 26%,  $p = 0.10$ ). Although we were not powered to study NEC as a primary outcome, NEC was significantly increased in the ALHMF group. (13% vs. 0%,  $p = 0.03$ ). Use of a LHM in an unrestricted NICU population resulted in an increase in clinical complications within a high-acuity NICU, including metabolic acidosis and poor growth. Although further research is needed to assess outcomes among infants with a variety of clinical acuities, gestational ages, and weights to confirm these findings, based on this experience, caution is urged to avoid potential risks.

**Keywords:** prematurity; human milk; fortifier; infant feeding; growth; acidosis

## 1. Introduction

Infants born prematurely have increased nutrient needs compared to those born at term [1–3]. Nutrition-related goals for premature infants aim to mimic fetal nutrient accretion and growth *in utero* [4], yet many develop extrauterine growth restriction (EUGR) [5].

Despite the availability of customized, nutrient-dense enteral formulas, the American Academy of Pediatrics strongly supports the use of human milk for premature infants [6]. However, unfortified human milk remains inadequate to meet the high nutrient requirements of premature infants [1,4,7]. Provision of unfortified human milk has subsequently been linked to suboptimal growth (development of EUGR or growth  $< 15$  g/kg/day), reduced bone density leading to osteopenia of prematurity and a clinical diagnosis of rickets, and the secondary consequences of each [1,4].

The use of commercial human milk fortifiers (HMF) allows for a more optimal provision of essential nutrients to meet premature infant requirements [1,4,7]. Macronutrient recommendations for low birth weight premature infants vary, but consensus goal ranges suggest enteral intake of 110–120 cal/kg/day and 3.4–4.4 g protein/kg/day [1]. Protein is specifically emphasized, as early and higher provisions promote more desirable growth and clinical outcomes [8,9]. The use of HMF has been shown to be both safe and effective in improving growth and nutrition status of premature infants compared to unfortified human milk [7,10,11]. In recent years the use of HMF with additional powdered protein modular has been presented as a method of supplying the preterm infant with the recommended amount of enteral protein to provide improved linear growth and neurodevelopmental outcomes [12,13].

Human milk fortifiers have primarily been available in powder form, although the United States Food and Drug Administration discourages the use of powdered forms in the neonatal intensive care units (NICU) secondary to contamination risk [14]. They additionally advise that “alternatives to

powdered forms should be chosen when possible” [14]. To comply with this recommendation and achieve improved protein intake, The Nebraska Medical Center (TNMC) NICU changed standard human milk fortification practices with a powdered product when an acidified liquid HMF (ALHMF) with improved protein delivery became available. However, in the four months following our initial use of the ALHMF, clinical observations of infants receiving the ALHMF suggested an increased feeding intolerance, increased incidence of metabolic acidosis, poor growth, and a need for higher caloric densities of enteral feedings to promote adequate growth. Due to our concern for patient outcomes, use of the ALHMF was discontinued. The purpose of this study is to objectively quantify these clinical observations by comparing outcomes of infants receiving the ALHMF to those receiving the originally-used PHMF. Our study also looked to identify potential risk factors for the development of the observed clinical complications, as previous research evaluating the ALHMF also documented changes in pH and CO<sub>2</sub> when compared to a powder HMF (PHMF) [15].

## **2. Patients and Methods**

### *2.1. Participants and Study Design*

The institutional review board at the University of Nebraska Medical Center (Omaha, NE, USA) approved this study. Data was retrospectively collected from inpatient electronic medical records of all infants admitted to the NICU, between October 2009 and July 2011, if they met the following inclusion criteria: birth weight (BW) < 2000 g, received enteral feedings as fortified maternal breast milk during NICU stay, and remained in the NICU  $\geq$  14 days. Exclusion criteria included infants with congenital abnormalities or conditions that significantly inhibited growth, such as Trisomy 13. No infants were excluded based on clinical acuity. After extensive chart review, 69 infants were eligible for the study.

### *2.2. Comparison and Use of Human Milk Fortifiers*

Maternal breast milk (MBM) was fortified according to manufacturer directions. Ingredient and estimated nutrient compositions of fortified preterm human milk were obtained from online nutritional references [16,17]. Table 1 provides a composition comparison for key nutrients and ingredients.

**Table 1.** Comparison of ingredients and key nutrients using powder and liquid HMF.

<b>24-Calorie-Per-Ounce Fortified Premature Human Milk [16,17]</b>		
<b>Per 100 mL</b>	<b>Powder HMF</b>	<b>Liquid HMF</b>
Protein (g)	2.35	3.2
Iron (mg)	0.46	1.85
Calcium (mg)	138	141
Phosphorus (mg)	78	78
Vitamin D (IU)	119	200
pH	-	4.7
Primary Fortifier	nonfat milk, whey protein	water, whey protein isolate hydrolysate
Macronutrient Ingredients	concentrate, corn syrup solids, medium-chain triglycerides (MCT oil)	(milk), medium chain triglycerides (MCT oil), vegetable oil (soy and high oleic sunflower oils)
	-: Information not available.	

Enteral feedings are initiated in this NICU within the first one to three days of life with Human Milk (MBM as available or donor milk from the Milk Bank of Austin) at 20 mL/kg/day, trophic feedings are continued for three to five days at the discretion of the attending neonatologist, and then feedings are advanced daily by 20 mL/kg/day with human milk fortification beginning at 80–100 mL/kg/day enteral volume. A protein modular is utilized to improve protein intake to approximately 4 g/kg/day enteral protein once caloric density is 24 kcal/oz. While using the ALHMF, no additional protein modular was utilized. There were no other nutrition differences during the two time periods. Nutrition is managed closely per unit protocol and is very consistent from provider to provider.

According to unit policy, infants receiving the PHMF also received supplementation with a protein modular to provide approximately 4 g protein/kg/day when fed at goal volumes.

Sole use of the ALHMF was initiated on April 1, 2011. Infants receiving the PHMF before this date of fortification change were included in the control group (PHMF,  $n = 46$ ). Infants receiving the ALHMF following this date were included in the study group (ALHMF,  $n = 23$ ). Infants transitioned from the PHMF to the LHMFM on the date of fortification change were excluded.

### 2.3. Data Collection

Four investigators familiar with the electronic medical record and NICU terminology obtained all data in a consistent predetermined manner. Collected information was reviewed for accuracy and corrected if an electronic error occurred. All available information on each infant was included in the analysis and is displayed in the tables.

### 2.4. Demographics

Demographic information was collected for all infants including gender, gestational age at birth and discharge, and day of life (DOL) at discharge. Additional clinical outcomes were collected including the presence of bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC), diaper dermatitis, and death. Treatment

requirements were coded similarly if an infant required: oxygen at 36 weeks estimated gestational age (EGA), ROP procedure, IVH shunt, Avastin treatment, Dexamethasone use, and Bicitra use. ROP stage, IVH grade, and number of days of Dexamethasone use were included if available.

### *2.5. Anthropometrics*

Infants were weighed daily on a gram scale, and head circumference and length (centimeters) were recorded weekly by nursing staff using a measuring tape. Fenton growth curve percentile rankings [18,19] were electronically plotted for each recorded anthropometric measurement. Weight, head circumference, and length measurements with associated Fenton percentile rankings were taken for infants at birth and at 36 weeks EGA, if available.

### *2.6. Nutrition*

Enteral feeding data collected included day of life (DOL) enteral feedings were initiated, DOL full enteral feedings were reached (with a discontinuation of parenteral nutrition support), and the number of times enteral feedings were held (not secondary to preparation for a procedure). Maximum caloric density and number of days on enteral feedings >24 cal/ounce were collected for infants requiring caloric densities higher than the standard 24 cal/ounce to promote adequate growth.

Daily average provision of calories and protein (g) per kg body weight were calculated for infants in each group if they received  $\geq 50\%$  of enteral feedings as fortified MBM during NICU stay. These averages were taken when fortified enteral feedings reached a minimum of 140 mL/kg/day until either daily intake was consistently less than this amount, the infant was changed to unfortified MBM, or the infant received greater than 50% infant formula. Growth and nutrition was evaluated for the groups comparing only growth during the period where the infant received  $\geq 50\%$  of enteral feedings as fortified MBM. An electronic medical system (Intuacare<sup>®</sup>: Omaha, NE, USA) contained protein references for breast milk and specified enteral formulas and caloric density. Nursing staff documented daily intake of breast milk or specified enteral formulas, thus, daily calorie and protein provision per kg of body weight were electronically calculated using the daily-recorded weight. The electronic medical system also calculated the percentages of MBM vs. infant formula received according to nursing documentation.

### *2.7. Laboratory Measurements*

Maximum creatinine, maximum blood urea nitrogen (BUN) level, maximum base deficit value, maximum calcium level, and lowest carbon dioxide (CO<sub>2</sub>) lab values were collected, if available, after DOL 14 and DOL 30 for all infants. Values were not collected before DOL 14 to eliminate those reflective of parenteral nutrition support and unfortified enteral feedings. Phosphorus and pH were not consistently nor routinely obtained in this patient population and were therefore not collected in this retrospective study.

### *2.8. Data Analysis*

Descriptive statistics were displayed for all variables by type of milk (powder vs. liquid) given. The Wilcoxon rank sum test was used to compare continuous data between the milks groups. Associations of categorical variables were assessed with the Fisher's exact test. A  $p$ -value  $\leq 0.05$  was considered statistically significant. To assess the difference in growth patterns between infants given powder and infants given liquid, a mixed effects model was used. We included random slopes and intercepts for each subject to capture individual growth pattern as well as fixed effects for group and day and a group day interaction term. A significant interaction of day and group indicates differing growth patterns based on group. Growth Velocity (GV) was calculated using Equation 1 [20].

$$GV = [1000 \times \ln(W_n/W_1)]/(D_n - D_1) \quad (1)$$

### 3. Results

There were 46 infants in the PHMF group (21 males, 25 females) and 23 infants in the ALHMF group (13 males, 10 females) ( $p = 0.45$ ). Additional baseline characteristics were not statistically significant between the two groups, as shown in Table 2. Enteral feeding data, growth and analyzed lab values are displayed in Table 3. Clinical outcomes are displayed in Table 4. ROP stage, IVH grade, and number of days of Dexamethasone use were not statistically significant and are not included in Table 4.

**Table 2.** Baseline characteristics of the subjects.

Variable	PHMF			ALHMF			<i>p</i> -value
	<i>n</i>	Mean	SD ( $\pm$ )	<i>n</i>	Mean	SD ( $\pm$ )	
CGA at Birth	46	29.5	3.0	23	30.3	2.5	0.21
Birth Weight (g)	46	1293.7	407.5	23	1437.3	375.6	0.13
Birth Weight Percentile	46	31.4	24.7	23	36	26.5	0.82
Weight at 36 Weeks CGA (g) #	44	2245.9	450.72	18	2071.2	367.4	0.17
Weight Percentile at 36 Weeks CGA #	44	18.6	24.4	18	10.3	13.8	0.22
HC at Birth (cm)	46	27.2	3.4	22	27.9	2.1	0.19
HC Percentile at Birth	46	29.9	23.1	22	33.6	26.3	0.7
HC at 36 Weeks CGA (cm) #	42	32.5	2.6	19	31.9	1.5	0.37
HC Percentile at 36 Weeks CGA #	42	38.8	30.7	19	31.4	24.6	0.5
Length at Birth (cm)	46	38.6	3.9	21	40.4	2.8	0.07
Length Percentile at Birth	46	31.4	24.6	22	32.8	21.9	0.68
Length at 36 Weeks CGA (cm) #	42	44.2	3.3	19	43.5	4.6	0.44
Length Percentile at 36 Weeks CGA #	42	17.3	22.3	19	21.3	28.1	0.93

# Growth at these time points represents nutrition delivery throughout hospitalization not just breast milk with PHMF and ALHMF.

**Table 3.** Enteral feeding, growth and laboratory data.

Variable	PHMF		ALHMF		<i>p</i> -Value
	<i>N</i>	Median	<i>N</i>	Median	
Average Daily Provision of Protein per kg Weight	42	3.9	18	4.3	0.0014
CO2 Minimum after DOL 14	33	23	16	18.5	0.002
CO2 Minimum after DOL 30	23	25	8	20	0.002
Growth Velocity (g/kg/day) while on HMF	46	15.37	21	10.59	<0.0001
Growth (g/day, while on HMF)	46	31.27	21	23.66	0.0001
DOL Enteral Feedings Started	46	3.0	22	1.1	0.12

<b>Calcium Maximum</b>	34	10.3	16	10.45	0.17
<b>BUN Maximum after DOL 14</b>	33	18	16	20	0.28
<b>BUN Maximum after DOL 30</b>	23	18	8	16	0.91
<b>Creatinine Maximum</b>	46	0.92	22	0.9	0.52

**Table 4.** Clinical outcomes.

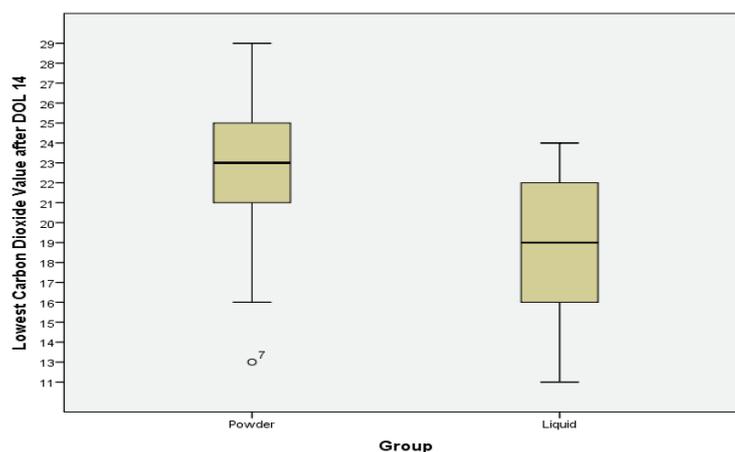
Variable	PHMF	LHMF	<i>p</i> -Value
	<i>n</i> (%)	<i>n</i> (%)	
NEC	0 (0%)	3 (13%)	0.03
ROP	16 (35%)	3 (13%)	0.09
ROP Procedure	3 (7%)	2 (9%)	1.00
IVH (any)	18 (39%)	4 (17%)	0.10
Dexamethasone Treatment	9 (20%)	1 (5%)	0.15
Bicitra Treatment	0 (0%)	1 (5%)	0.31
Death	0 (0%)	1 (4%)	0.33
Diaper Dermatitis	5 (11%)	4 (18%)	0.46
BPD	9 (20%)	3 (14%)	0.74

### 3.1. Safety and Clinical Outcomes

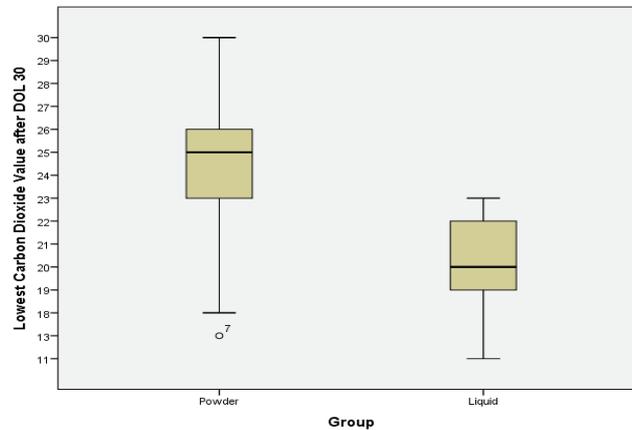
Mean lowest CO<sub>2</sub> lab values (collected while infants were enterally feeding and not acutely ill) were significantly lower in the ALHMF group compared to the PHMF group after both DOL 14 (18.5 vs. 23 mmol/L,  $p = 0.002$ ) and DOL 30 (20 vs. 25 mmol/L,  $p = 0.002$ ). Lowest CO<sub>2</sub> lab values after DOL 14 are displayed comparatively in Figure 1. Lowest values after DOL 30 are displayed similarly in Figure 2. Maximum BUN and creatinine levels were similar between the two fortifier groups and were not statistically significant. All other analyzed lab values were not statistically different. All laboratory data in this retrospective study was obtained for clinical purposes regardless of the fortifier group.

Incidence of NEC (a variable we were not powered to evaluate) was significantly higher in the ALHMF group compared to the PHMF group (13% vs. 0%,  $p = 0.03$ ).

**Figure 1.** CO<sub>2</sub> levels between groups after Day of Life 14. The lowest CO<sub>2</sub> levels after DOL 14 were collected from metabolic panels. The mean level in the powder group was 23, the mean level in the liquid group was 18.5. Laboratory clinical reference range 22–32 mmol/L. The difference is statistically significant ( $p = 0.002$ ).



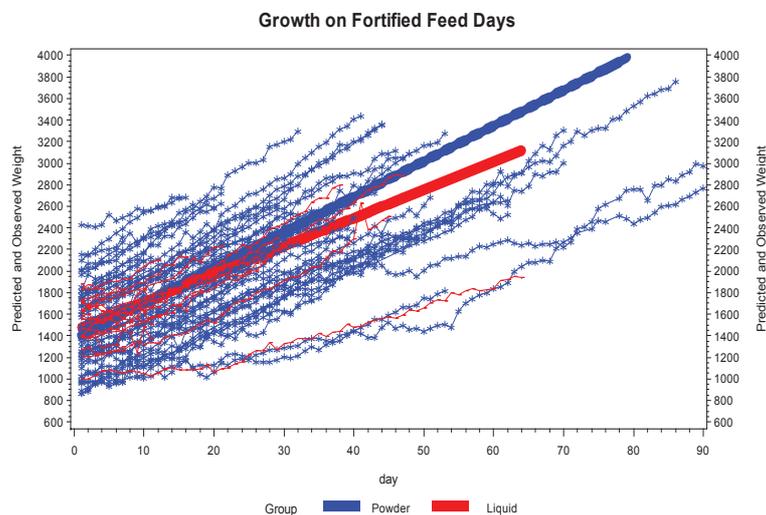
**Figure 2.** CO<sub>2</sub> levels between groups after Day of Life 30. The lowest CO<sub>2</sub> levels after DOL 30 were collected from metabolic panels. The mean level in the powder group was 25, the mean level in the liquid group was 20. Laboratory Clinical reference range 22–32 mmol/L. The difference is statistically significant ( $p = 0.002$ ).



### 3.2. Enteral Nutrition and Growth

Growth was significantly different between the two groups as measured in g/kg/day and is described in Figure 3. Infant growth as measured in g/day from birth to 36 weeks EGA was 23.7 in the PHMF group and 18.8 in the LHM group ( $p = 0.057$ ). There were no statistically significant differences in the length of time to full feedings or the number of times that feedings were held that could account for the difference in growth rates between the two groups.

**Figure 3.** The growth pattern of infants receiving powder differs from the growth pattern of infants receiving liquid on fortified feed days. The plot shows the growth pattern for each infant and the fitted line by group. Based on the plot, infants on powder grow at a faster rate than infants receiving liquid. Evaluation of growth in gm/kg/day for the days infants were fed fortified breast milk, based on the mixed effects model, shows a significant interaction between day and group ( $p = 0.0022$ ). Truncating the analysis at 45 days did not attenuate the results.



Daily average protein/kg/day provision was higher in the ALHMF group compared to the PHMF group (4.3 vs. 3.9 g,  $p = 0.0014$ ). Mean enteral calorie provisions in the ALHMF group were higher than in the PHMF group, 117.3 kcal/kg/day in the PHMF group as compared to 128.7 kcal/kg/day for infants in the ALHMF group ( $p = 0.057$ ). A higher proportion of infants in the ALHMF group required increased caloric density of feedings  $>24$  cal/ounce as compared to infants in the PHMF group, (48% vs. 26%,  $p = 0.10$ ). While this did not reach a statistical difference, clinically this was notable.

#### 4. Discussion

To our knowledge, we are the first study to date to report our clinical findings of increased complications with the use of ALHMF in a Level IIIc clinical setting. In our retrospective analysis of acidosis, growth, and clinical outcomes in NICU infants fed with human milk fortified with LHMf and PHMF we found significant acidosis and poor growth in the infants receiving LHMf. These findings were very consistent with our clinical impressions during our clinical use of the LHMf. We were also surprised to see increased NEC in the ALHMF group. Although we were not powered as a primary outcome to evaluate NEC, we strongly encourage cautious further evaluation of the product in the clinical setting with regards to this serious outcome.

A key difference in the ALHMF as compared to the PHMF is the acidification process required for sterilization. This difference is likely to explain the increased complications seen in the ALHMF group. The preterm infant's inability to buffer this acid load likely led to an increase in clinical complications including acidosis, poor growth, and, possibly, NEC.

##### 4.1. Acidosis

There was a higher incidence of clinically significant metabolic acidosis in the ALHMF group, with one infant requiring treatment with Bicitra. No infants in the PHMF group required Bicitra treatment, even with twice as many patients in this group. Premature infants are susceptible to metabolic acidosis [21] and renal tubular acidosis. However, these imbalances of acid base status should begin to normalize after the first weeks of extrauterine life [21]. Considering similar baseline characteristics, we hypothesize additional enteral acid load was a potential contributor to this increased incidence of metabolic acidosis in the LHMf group.

Premature infants are at risk for developing metabolic acidosis secondary to immature metabolic processes, a lower renal capacity to adequately excrete acid, and higher urinary losses of bicarbonate [2,4,22]. Quantity of protein may affect metabolic processes; however the median daily average protein provisions for each fortifier group were within the currently recommended ranges [1]. No clearly defined amount for maximum protein provision exists, however, it is suggested that intakes greater than 6 g/kg/day are poorly tolerated [2]. Maximum daily average protein provisions for both groups were below this level. Another reference states that protein provisions greater than 5 g/kg/day may cause azotemia [1], but each group had intakes below this value, and maximum BUN and creatinine levels were not different in the two fortifier groups. Having increased protein intake in the PHMF group as well as the ALHMF group helps to illuminate that increased protein content in the ALHMF was not likely the cause of the adverse outcomes.

We question if the acidification sterilization process of the ALHMF may contribute to this acidosis in some fragile premature infants. Our patient population included in this study was not limited by respiratory acuity, as was the population in the Moya *et al.* paper [23]. We hypothesize that our more inclusive population of both healthy infants and more fragile infants who may have less respiratory stability decreases their capability to buffer the acid load provided in the ALHMF resulting in clinical acidosis in some cases requiring medical therapy. It may be unwise in a fragile preterm infant population to minimize the clinical significance of the metabolic acidosis noted in the ALHMF groups in our study, the study conducted by Moya *et al.* [24], who reported that infants fed the ALHMF had significantly lower pH (at day six), bicarbonate (at day six and 14), and CO<sub>2</sub> (at day 14 and 28), and significantly higher chloride (at day 14 and 28). Additionally, in an abstract evaluating 100 infants, 50 fed with ALHMF and 50 fed with PHMF, published by Cibulskis *et al.*, from Saint Louis University at the 2013 AAP\_NCE, similar metabolic acidosis is described in this patient population (54% ALHMF vs. 10% PHMF,  $p = 0.0001$ ) [25]. As reported in their abstract, this group treated the acidosis as if it were clinically significant, discontinuing ALHMF on 21/50 patients due to a clinical diagnosis of acidosis [25].

#### 4.2. Enteral Nutrition and Growth

Infants in the PHMF group received a mean daily calorie intake of 117.3 kcal/kg/day as compared to infants in the ALHMF group who received a mean calorie intake of 128.7 kcal/kg/day. Infants in the ALHMF group also received a median of 0.4 g protein/kg/day more than the infants in the PHMF group. Despite higher protein and calorie provisions in the LHMFM group, growth during the HMF period was slower between the two groups as evaluated by several methods: in a mixed effects model evaluated in g/kg/day ( $p = 0.002$ ), in g/day ( $p = 0.0001$ ), and by growth velocity in g/kg/day ( $p < 0.0001$ ). Noted also, is that ALHMF infants experienced an additional decrease of 10 growth curve percentiles for weight from birth to 36 weeks EGA when compared to infants in the PHMF group (growth at 36 weeks is representative of nutrition delivery that is not limited to the period evaluated on PHMF and ALHMF). As Dexamethasone use inhibits growth in premature infants [4], we further note that fewer infants in the ALHMF group (5%) compared to the PHMF group (20%) required this drug for clinical treatment ( $p = 0.15$ ).

Maintaining appropriate growth in this patient population was a high priority, so infants with suboptimal growth were fed increased caloric density feedings above 24 cal/oz. Though not statistically significant, a higher proportion of infants in the ALHMF group (48%) required caloric densities greater than 24 cal/ounce when compared to the PHMF group (26%). Had those 48% of infants in the ALHMF group not been prescribed increased caloric densities due to clinical observations of poor growth differences in growth throughout the hospitalization would likely have been larger between the PHMF groups and ALHMF groups. The statistical significance in infant growth as noted in g/kg/day is seen in spite of the high priority our unit takes in maintaining optimal growth and the subsequent aggressive adjustment of caloric density to achieve desired results. This was ultimately the reason 26% of infants receiving ALHMF were transitioned to receive the PHMF once the ALHMF use was discontinued in the NICU.

Not only are these growth effects consistent with the findings of Moya *et al.*, they raise further questions [24]. Moya *et al.*, reported no significant differences in rate of weight gain or head

circumference growth between infants fed this same ALHMF and infants fed a PHMF, even though the human milk fortified with ALHMF contained 23% more protein (3.2 vs. 2.6 g protein/100 mL fortified preterm human milk) [24]. In our study, even though we compensated for the difference in protein content so that protein intake was similar, there was still poorer growth in infants fed ALHMF.

At least part of the inability of the additional protein to improve growth may be due to the acidosis noted above. It is well-known that infants with metabolic acidosis hyperventilate, as the expiration of CO<sub>2</sub> drives the elimination of H<sup>+</sup> ions through the bicarbonate buffering system. What is less well recognized is that protein catabolism can also be utilized to decrease acidity by the elimination of H<sup>+</sup> ions through the urinary excretion of NH<sub>4</sub><sup>+</sup>. Acidosis reduces protein synthesis in rats [26] and leads to protein catabolism in humans [27].

#### 4.3. NEC

No infants in PHMF group developed NEC compared to 13% in the ALHMF group. Reasons for these occurrences remain unclear, as similar prevention strategies were followed for each group. Previous implementation of aggressive nutrition practices in our unit demonstrated improved feeding tolerance and clinical outcomes, with no increased incidence of NEC [12]. These nutrition practices remained unchanged during the study period, and no additional clinical practices were implemented concurrent with the change in human milk fortification. Slow rate of enteral feeding advancement remained consistent between both groups, as evidenced by no statistically significant differences in length to full enteral feedings. No changes in brand or caloric density of premature infant formula were made, and infant formula was utilized equally in both groups if no MBM was available. As no additional practice changes were implemented during this study period, we can neither confirm nor exclude use of the ALHMF as a contributor to these occurrences of NEC. Although this study was not powered to detect NEC based on historical incidence in our unit with rates over the last five years ranging from 2% to 5% from our Vermont Oxford Network data, one should consider that significant differences with small sample sizes may either reflect coincidental effects due to sample size, or may be due to a real difference that is unexpectedly large.

#### 4.4. Metabolic Acidosis

Literature suggests that premature infant formulas contain a high renal acid load, though human milk contains less [2,21]. Research has additionally demonstrated that the composition of infant formulas may affect the urinary pH and nutrient excretion of premature infants [21,22]. It is further proposed that high renal acid loads contribute to maximum renal acid stimulation (urine pH < 5.4) [28] in premature infants with immature renal function. Previous research studies have demonstrated that infants with metabolic acidosis or maximum renal acid stimulation exhibit decreased growth [28,29]. This may also result in an increase in urinary sodium excretion [24,29] and a decrease in nitrogen assimilation [30]. Blood sampling for acid-base indicators may not be significantly abnormal in the presence of maximum renal acid excretion [22,28]. However, CO<sub>2</sub> values may trend low [28], which was clearly observed among infants in the ALHMF group ( $p = 0.002$ ).

#### 4.5. Summary

Our results showing increased acidosis in the ALHMF group raise further concerns with use of the ALHMF, as infants with metabolic acidosis may experience altered nutrient metabolism [28,31] and decreased bone mineralization [32], leading to poor growth and osteopenia of prematurity.

Poor growth in the ALHMF group may also be attributed to changes to the nutrient content of the milk caused by acidification as described by Erickson *et al.* [23]. This group reported significant changes in acidified breast milk, including decreased total protein content, lipase activity, and free fatty acids [23]. The nutritional changes in the composition of acidified breast milk documented by Erickson *in vitro* may have led to the *in vivo* growth deficiencies noted in our ALHMF population [23].

### 5. Strengths and Limitations

#### 5.1. Strengths

This study is the first to quantify results of use of ALHMF in a Level IIIc NICU setting. We are uniquely situated to evaluate outcomes of our use of ALHMF in our patient population for several important reasons. First, we initiated utilization of this product on all infants at one time. There was no possibility of crossover product use to decrease the validity of the data. Additionally, we used this product on all infants who would be eligible to receive fortified human milk, as would be expected in a clinical NICU practice. This makes our data very relevant and applicable to clinical NICU settings.

Second, our clinical management of nutrition in this patient population has been published and remains very successful with excellent growth and low baseline rates of NEC. Not only do we manage nutrition care of this population very closely, but we also have a defined protocol in place so that infants (except for fortification method) receive the same nutrition interventions over time regardless of which group, PHMF or ALHMF, they received.

Additionally, our nutrition management with additional protein added to the PHMF group makes the comparison of the two groups more relevant by giving them a more similar nutrient intake at baseline than a comparison of ALHMF and PHMF alone which compares a large difference in delivered protein.

Finally, we have a very detailed nutrition documentation medical record system, Intuacare. This system allows for easy retrieval of detailed nutrition information including daily percentages of breast milk, daily caloric intake, and daily protein intake in g/kg/day. This allows for minimal reporting error in a retrospective study, such as this, and provides an excellent historical representation of each infant's delivered nutrition.

#### 5.2. Limitations

This retrospective review of a clinical trial of a commercially available acidified liquid human milk fortifier has several limitations including the retrospective nature of the study, and a modest sample size, which limits the power of some data points. These limitations were partially reduced by our reliance on electronic documentation for data collection and analysis. All medical documentation remains variable between individuals and we cannot quantify unrecorded data, but the system utilized

allowed for complete assessment of all recorded data on each research subject. As with any study evaluating growth, head circumference and length measurements are also variable as length boards were not used and measuring tape placement may vary between nursing staff. Some subjects were discharged prior to 36 weeks EGA, therefore, anthropometric measurements at 36 weeks EGA were not available. Likewise, lab values were also unavailable for these infants and could not be included in data analysis.

Alterations in human milk composition are continuous, so calculated nutrient compositions of fortified human milk may only serve as general estimations for our nutrient comparisons. Standard NICU nutrition practices are followed as consistently as possible, however feeding advancement may remain variable according to infant clinical status. Furthermore, the proportion of feedings as human milk or formula remained variable among each infant. In an ideal study, all enrolled infants would receive human milk only.

Though the incidence of NEC was statistically significant, it was not powered as a primary outcome for this study. We also suspect that diaper dermatitis was under-recorded during this study period, as our clinical experience suggests that diaper dermatitis is infrequently documented in the electronic medical record even when infants experience more serious medical complications. However, perceived worsening skin breakdown in our unit while using the ALHMF prompted development of a unit list of infants with diaper dermatitis. Unfortunately, not all of the infants recorded on the unit list had diaper dermatitis electronically documented as a medical problem. As there was no way to quantify these cases, these select infants were not coded positively for diaper dermatitis in this study. Therefore, our data analysis remains limited.

## 6. Conclusions

Use of the new ALHMF resulted in an increase in clinical complications and a decrease in growth as measured in both g/day and g/kg/day. To our knowledge, this is one of the first studies assessing use of the new ALHMF within a high acuity NICU without excluding infants with significant respiratory disease or low five-min APGAR scores. Further research with the ALHMF is needed to compare infant tolerance and outcomes among infants with a variety of gestational ages, weights, and increased clinical acuity.

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## Conflicts of Interest

The authors declare no conflict of interest.

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## Vitamin D Intake and Status in 12-Month-Old Infants at 63–66° N

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**Abstract:** The objective was to assess the vitamin D status in healthy 12-month-old infants in relation to quantity and sources of dietary vitamin D, breastfeeding and seasons. Subjects were 76 12-month-old infants. Serum levels of 25-hydroxyvitamin D (25(OH)D)  $\geq 50$  nmol/L were considered indicative of vitamin D sufficiency and 25(OH)D  $< 27.5$  nmol/L as being indicative of increased risk for rickets. Additionally, 25(OH)D  $> 125$  nmol/L was considered possibly adversely high. Total vitamin D at 9–12 months (eight data collection days) included intake from diet and supplements. The mean  $\pm$  SD of vitamin D intake was  $8.8 \pm 5.2$   $\mu$ g/day and serum 25(OH)D  $98.1 \pm 32.2$  nmol/L (range 39.3–165.5). Ninety-two percent of infants were vitamin D sufficient and none at increased risk for rickets. The 26% infants using fortified products and supplements never/irregularly or in small amounts had lower 25(OH)D ( $76.8 \pm 27.1$  nmol/L) than the 22% using fortified products ( $100.0 \pm 31.4$  nmol/L), 18% using supplements ( $104.6 \pm 37.0$  nmol/L) and 33% using both ( $110.3 \pm 26.6$  nmol/L). Five of six infants with 25(OH)D  $< 50$  nmol/L had no intake of supplements or fortified products from 0 to 12 months. Supplement use increased the odds of 25(OH)D  $> 125$  nmol/L. Breastfeeding and season did not affect vitamin D status. The majority of infants were vitamin D sufficient. Our findings highlight the need for vitamin D supplements or fortified products all year round, regardless of breastfeeding.

**Keywords:** 25-hydroxyvitamin D; vitamin D; infant; dietary supplements; fortified foods

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## 1. Introduction

Vitamin D is a key nutrient for children's well-being and growth, is essential for bone health [1] and may contribute to other health benefits [2]. Infant need for vitamin D can be met by synthesis in the skin when exposed to appropriate ultraviolet B wavelengths and by sufficient vitamin D intake, either from breast milk or other dietary sources [3]. At latitudes higher than  $\sim 50^\circ$  N, little or no cutaneous vitamin D synthesis is possible during winter months [4]. Vitamin D from breast milk alone is unlikely to meet the needs of infants during complementary feeding [5]. Few common foods are naturally rich in vitamin D [6]. Vitamin D intake from supplements or fortified foods or beverages is therefore important in northern latitudes [3].

The recommended intake (RI) of vitamin D is 10  $\mu\text{g}$  (400 IU) for infants and children from six months of age according to Nordic nutrition recommendations [7]. This is in accordance with the average intake specified by the Institute of Medicine (IOM) for infants from birth to 12 months of age [8]. To ensure that the RI is met, parents are advised to give their infants a daily supplement of 10  $\mu\text{g}$  D<sub>3</sub> from the age of 1–2 weeks. Fortification schemes differ between countries [3]. In Iceland, population-based studies on infants [9,10] and pre-school children [11] have shown that less than two-thirds of young children use vitamin D supplements regularly. Several cases of rickets in the past years have given cause for concern on the vitamin D status of Icelandic infants [12]. In 2003, a follow-up formula intended for infants from 6 to 24 months, fortified with 1.2  $\mu\text{g}$  D<sub>3</sub> per 100 mL, was introduced [13], and infant porridges and breakfast cereals fortified with vitamin D are available [14]. Population-based studies on both vitamin D intake and status during the complementary feeding period in the Nordic countries are lacking [3]. The vitamin D status of Icelandic infants is unknown and has never been studied.

The objectives of this study were to assess vitamin D status measured as serum levels of 25-hydroxyvitamin D (25(OH)D) in healthy 12-month-old infants and to consider it in relation to quantity and sources of dietary vitamin D, breastfeeding and seasons.

## 2. Experimental Section

### 2.1. Subjects

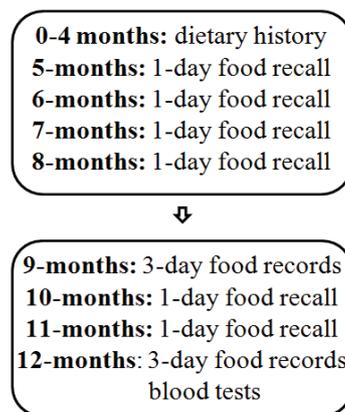
Study subjects were 76 infants with data on dietary intake in infancy and quantitative analysis of serum 25(OH)D levels at 12 months. They were a subsample of participants in a longitudinal cohort study on diet, growth and health outcomes of infants born in the year 2005. In the original study, 250 healthy Icelandic infants born at term were randomly selected from the whole country ( $63\text{--}66^\circ$  N). Blood samples were obtained with the primary aim of analyzing the iron status and blood lipids of the infants [13]. Analysis of serum 25(OH)D was only possible for those subjects with sufficient amounts of blood available, resulting in the subsample of 76. Anthropometrical variables and dietary intake in infancy, e.g., duration of breastfeeding, intake of vitamin D, formula and cod liver oil, sociodemographic

factors (parents' age and education) and parental BMI of the children included in the current analysis did not differ from those of the children in the original study. More detailed information on the original cohort is published elsewhere [13,15]. Informed written consent from the parents was obtained, and all individual information was processed with strict confidentiality. The study was approved by the Icelandic Bioethics Committee, the Icelandic Data Protection Authority and the Local Ethical Committee at Landspítali University Hospital.

## 2.2. Dietary Assessment

A flowchart of the process of the study, relevant to the present analysis, is presented in Figure 1.

**Figure 1.** Flowchart on the progress of the study.



Dietary data from 0 to 4 months of age were collected by dietary history, including questions on breastfeeding, infant formula-feeding, other food items and supplements. At 5–8 and 10–11 months, 24-h recalls using common household measures, such as cups and spoons, were made. At 9 and 12 months, weighed food records were kept for 3 consecutive days (72-h). All food and fluids were weighed on accurate scales (Philips type HR 2385, Szekesfehervar, Hungary) with 1-g precision. The amount of breast milk consumed was estimated by weighing the breastfed infants in the same clothes before and after each breastfeeding session on baby scales (Tanita model 1583, Tokyo, Japan, or Sega model 336, Hamburg, Germany) with 10-g precision. An average consumption of food and nutrients was calculated using the Icelandic food composition database [14]. The total intake of vitamin D included intake from the diet, breast milk and supplements. For the analysis presented here, the main emphasis was on dietary intake at 9–12 months, because we believe that it may influence serum 25(OH)D concentration at 12 months [3,16]. We divided infants into four groups based on regular intake of significant amounts of the main vitamin D sources at 9–12 months. The “fortified” group included infants getting on average  $\geq 2.4$   $\mu\text{g}$  of vitamin D per day from fortified products; the “supplement” group included those getting on average  $\geq 0$   $\mu\text{g}$  of vitamin D per day from supplements; the “combined” group included those fulfilling both conditions; and the “no or irregular” group included infants fulfilling neither conditions. Fortified products included infant formula, infant porridges and breakfast cereals, and the cut-off at 2.4  $\mu\text{g}$  of vitamin D was applied, because it corresponds to consumption of  $\geq 200$  mL of fortified formula, the most commonly consumed product in this category. Supplements included cod liver oil and liquid vitamin A and D supplements (vitamin

AD drops), and the cut-off at 5 µg of vitamin D was applied, because it corresponds to the recommended dose on at least half of the data collection days. We also considered whether or not infants were still partially breastfed at 12 months of age.

### 2.3. Blood Sampling and Biochemical Analyses

At 12 months of age, blood samples were collected in the morning in the fasting state. The samples were centrifuged within 6 h of data collection. Separated serum samples were then stored at  $-80^{\circ}\text{C}$  until being analyzed. The quantitative analyses of serum 25(OH)D levels were conducted by the Roche Diagnostics Vitamin D total assay (Roche Diagnostics, Mannheim, Germany), with a measuring range of 7.5–175 nmol/L and a precision of 0.1 nmol/L. In accordance with a recent Nordic systematic literature review (SLR), serum 25(OH)D  $\geq$  50 nmol/L (20 ng/mL) was considered indicative of a sufficient vitamin D status, and serum 25(OH)D  $<$  27.5 nmol/L (11 ng/mL) indicates increased risk for rickets [3]. Additionally, serum 25(OH)D  $>$  125 nmol/L (50 ng/mL) was considered as possibly adversely high, as suggested by the IOM [8]. Infants were classified according to season when blood samples were collected; winter/spring (January 2006–April 2006 and November 2006–December 2006) and summer/autumn (May 2006–October 2006).

### 2.4. Statistical Analyses

Statistical analyses were performed with SAS (Enterprise Guide 4.3; SAS Institute Inc., Cary, NC, USA). Linear regression analysis was used to examine the relation between vitamin D intake and serum 25(OH)D. Descriptive statistics were used to describe vitamin D intake and serum 25(OH)D concentrations, presented as the means  $\pm$  SD. For comparison between groups, an independent, two-sample *t*-test with equal variances and a one-way ANOVA with equal variance were used. Logistic regression was used to examine the risk of having serum 25(OH)D above 125 nmol/L among infants using supplements or not. The results were presented as odds ratios (OR), with its 95% CI. Spearman's correlation analysis was used to assess correlations between 25(OH)D and breastfeeding, presented as the correlation coefficient ( $\rho$ ) and the *p*-value for correlation. A two-sided test with a *p*-value  $<$  0.05 was considered statistically significant.

## 3. Results

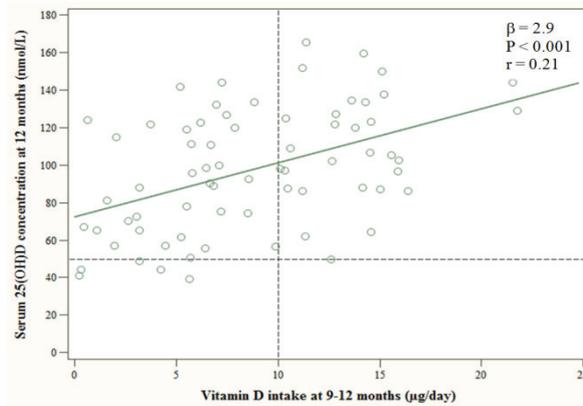
At the age of 12 months, the mean  $\pm$  SD serum 25(OH)D concentration was  $98.1 \pm 32.2$  nmol/L ( $39.3 \pm 12.9$  ng/mL) and ranged from 39.3 to 165.5 nmol/L (15.7 to 66.3 ng/mL). Seventy infants (92%) were considered vitamin D sufficient and none at increased risk for rickets. Eighteen infants (24%) were considered to have a possibly adversely high 25(OH)D concentration.

Vitamin D intake at 9–12 months predicted 25(OH)D levels at 12 months (Figure 2). The mean  $\pm$  SD intake of vitamin D was  $8.8 \pm 5.2$  µg, and 57% of the infants were below the RI of 10 µg. Those infants had significantly lower mean  $\pm$  SD 25(OH)D than infants above the RI ( $87.1 \pm 31.1$  vs.  $111.8 \pm 29.0$  nmol/L, *p* = 0.001).

Supplements provided 56% of total vitamin D at 9–12 months. Another 38% came from fortified products; thereof, 24% from formulas, 13% from infant porridges and 1% from breakfast cereals.

Among natural sources of vitamin D were meat and fish (3%) and cow's milk (1%). Breast milk provided <1% of vitamin D. As presented in Table 1, infants in the “combined” group had a higher vitamin D intake than infants in the “supplement” group ( $p < 0.001$ ), who, in turn, had a higher vitamin D intake than infants in the “fortified” group ( $p = 0.013$ ). Mean serum 25(OH)D in these three groups was, however, not significantly different ( $p > 0.05$ ). Infants not using fortified products or supplements regularly in significant amounts at 9–12 months (“no or irregular” group) had significantly lower vitamin D intake than all the other groups ( $p < 0.001$ ) and lower serum 25(OH)D ( $p < 0.001$ ).

**Figure 2.** The linear regression line for serum 25-hydroxyvitamin D (25(OH)D) at 12 months in relation to vitamin D intake from diet and supplements at 9–12 months. The dashed horizontal line at 50 nmol/L is the cut-off line applied for a sufficient vitamin D status, and the dashed vertical line at 10  $\mu\text{g}$  indicates the Nordic recommended intake (RI).



**Table 1.** Variables potentially associated with vitamin D intake at 9–12 months and serum 25(OH)D at 12 months.

Variables	<i>n</i> (%)	Vitamin D Intake ( $\mu\text{g}/\text{day}$ )	25(OH)D (nmol/L)
All	76 (100)	$8.8 \pm 5.2$	$98.1 \pm 32.2$
Boys	39 (51)	$8.6 \pm 5.7$	$96.6 \pm 34.3$
Girls	37 (49)	$8.9 \pm 4.6$	$99.7 \pm 30.3$
<i>Vitamin D sources at 9–12 months</i> <sup>a</sup>			
“No or irregular”	20 (26)	$2.5 \pm 1.9$	$76.8 \pm 27.1$
“Fortified”	17 (22)	$6.5 \pm 2.2$	$100.0 \pm 31.4$
“Supplement”	14 (18)	$8.8 \pm 2.7$	$104.6 \pm 37.0$
“Combined”	25 (33)	$14.3 \pm 3.0$	$110.3 \pm 26.6$
<i>Partially breastfed at 12 months</i>			
No	62 (82)	$8.7 \pm 5.0$	$97.7 \pm 32.7$
Yes	14 (18)	$9.1 \pm 6.0$	$101.9 \pm 31.5$
<i>Season of blood sample collection</i>			
Winter/Spring	33 (43)	$8.1 \pm 4.9$	$94.4 \pm 31.6$
Summer/Autumn	43 (57)	$9.2 \pm 5.4$	$101.0 \pm 32.8$

Abbreviation: 25(OH)D, 25-hydroxyvitamin D. Mean  $\pm$  SD. <sup>a</sup> Infants were divided into groups based on the regular intake of significant amounts of the main vitamin D sources at 9–12 months. “No or irregular”: neither fortified products nor supplements; “Fortified”: fortified products; “Supplement”: supplements; “Combined”: both fortified products and supplements.

Five out of six infants with serum 25(OH)D below 50 nmol/L belonged to the “no or irregular” group, *i.e.*, they did not use fortified products or supplements on a regular basis or in significant amounts at 9–12 months. Their intake of vitamin D was below 5 µg at 9–12 months. Further, they did not use fortified products or supplements at all from birth to nine months of age. The sixth infant with 25(OH)D below 50 nmol/L was categorized in the “supplement” group, but only got half of the recommended amount of supplements daily. Of the 18 infants with 25(OH)D levels above 125 nmol/L, one belonged to the “no or irregular” group (5% of infants in that group), three to the “fortified” group (18%), six to the “supplement” group (43%) and eight to the “combined” group (32%). Infants using supplements (*i.e.*, classified in the “supplement” or “combined” groups) were 4.6 times more likely (95% CI = 1.4, 15.8) to have 25(OH)D above 125 nmol/L than infants not using supplements (*i.e.*, classified in the “no or irregular” or “fortified” groups). Infants in the “combined” group were not more likely to have 25(OH)D above 125 mol/L than infants in the “supplement” group (OR (95% CI) = 0.6 (0.2, 2.4)).

The duration of exclusive breastfeeding ranged from 0 to 6 months, with a median (25th, 75th percentiles) of four (1, 5) months. The total duration of breastfeeding ranged from 0 to 12 months, with a median (25th, 75th percentiles) of eight (6, 10) months. There was no correlation between the duration of exclusive breastfeeding and 25(OH)D ( $\rho = -0.02$ ,  $p = 0.895$ ) or the total duration of breastfeeding and 25(OH)D ( $\rho = 0.08$ ,  $p = 0.502$ ). Among children partially breastfed at 12 months of age, breast milk intake in the age period of 9–12 months ranged from 10 mL to 750 mL per day. No difference was observed in vitamin D intake or 25(OH)D according to breastfeeding at 12 months ( $p = 0.923$  and  $0.674$ , respectively), season of blood sample collection ( $p = 0.385$  and  $p = 0.379$ , respectively) or sex ( $p = 0.859$  and  $p = 0.678$ , respectively).

#### 4. Discussion

This study provides the first information on vitamin D status in Icelandic infants. Based on thresholds proposed in a recent Nordic SLR [3], 92% of the infants were considered vitamin D sufficient and none at increased risk for rickets. Consensus has not been reached on the optimal 25(OH)D concentration in infants, and uniformity is lacking in the description of sufficient and deficient ranges for 25(OH)D levels. Using cut-off values proposed by IOM [8] or the Pediatric Endocrine Society [16] does not change our results of 92% of infants being classified as vitamin D sufficient, and according to those cut-offs, no infants are classified as vitamin D deficient. According to a European consensus statement, vitamin D deficiency occurs commonly among healthy European infants not adhering to recommendations for vitamin D supplementation [17]. However, studies on healthy infants from Denmark [18], Norway [19] and Finland [20] have previously reported a high proportion of vitamin D sufficiency amongst nine-month-olds, 12-month-olds and 14-month-olds, respectively. Those studies were not population-based, and in the Danish and Finnish studies, selection bias resulted in an unusually high frequency of infants using vitamin D supplements (97% and 100%, respectively). The Nordic countries have a well-established newborn and infant healthcare. According to protocols for the newborn and infant healthcare in Iceland [21], mothers are asked about their infants' vitamin D supplement use and encouraged to follow the recommendations on vitamin D supplements at every visit, which are scheduled at least nine times during the first year of the infant.

This may explain the relatively low proportion of vitamin D deficiency among infants in Iceland and, more broadly, the Nordic countries. To our knowledge, this is the first study on infant vitamin D status in the Nordic countries in a sample that is representative of the general infant population. Therefore, we believe it is an important contribution to the literature on the vitamin D status of healthy infants during complementary feeding in northern latitudes.

The relatively high 25(OH)D levels may, at least partly, be explained by 75% of the infants regularly using vitamin D supplements and/or fortified foods or drinks in significant amounts. The commonly used follow-up formula, fortified with vitamin D, was introduced in 2003. Before that, it was common that regular cow's milk gradually replaced breast milk in the age range of 5–12 months [9]. The main vitamin D source for Icelandic infants has, therefore, historically, been vitamin D drops or cod liver oil, and even though studies on infants and children have shown a little less than two-thirds of children complying with supplement use, the remaining one-third has been seen as a reason for concern. Studies on Icelandic infants and young children have never before assessed how frequently vitamin D fortified products are consumed or how they contribute to vitamin D status. We do not have any data on the vitamin D status of infants and young children previous to the introduction of the fortified follow-up formula.

The wide range of serum 25(OH)D concentrations observed in the study should be considered when interpreting the results. Transferring the 8% of infants in our sample with serum 25(OH)D below 50 nmol/L to the whole infant population in Iceland (around 4600 12-month-olds annually from 2005 to 2012) [22] suggests that about 275 infants every year would be vitamin D insufficient, with the possibility of some being at risk for vitamin D deficiency. Our study, showing that infants with an insufficient vitamin D status did not use fortified products or supplements at all from birth to nine months of age, in addition to a very low vitamin D intake from nine to 12 months of age, could be considered in newborn and infant healthcare in Iceland to identify, at an early age, children with undesirable diet habits that may increase the risk of vitamin D insufficiency or deficiency. Infants using supplements with or without concurrent use of fortified foods or drinks were more likely than infants not using supplements to have 25(OH)D concentrations that may be considered as possibly adversely high [8]. Correct dosing of supplements is important, as well as caution when combining the use of supplements and fortified foods or drinks. However, no infant exceeded the 25 µg vitamin D intake, which is considered the tolerable upper intake level by the European Food Safety Authority [6], and other estimations of the high end for safe concentration levels of 25(OH)D are higher than the 125 nmol/L estimated by IOM [16,23].

Since the time of this study, parents have been encouraged to give their infants vitamin D drops instead of vitamin AD drops. The vitamin D content in the two products is the same, and other infant guidelines remain unchanged. Therefore, we believe that the findings of this study are transferable to Icelandic infants born today. Iceland is among the few countries that includes cod liver oil intake or other vitamin D supplements in the population-based dietary guidelines for children and adults of all ages [24]. A recent study from Denmark showed that parents' perceived relevance of nutritional guidelines declined from the early to later phases of complementary feeding [25], and a Finnish study showed decreased use of supplements as children grew older [26]. Icelandic studies have also shown low vitamin D intakes among children [27], adolescents [28] and adults [29], and results from a follow-up of the infants participating in the current analysis reveal that only 27% used supplements

at six-years of age [30]. While the vitamin D status in our study is considered sufficient for the majority of infants, studies on Icelandic children and adults have shown lower 25(OH)D concentrations than presented here [31–33]. This study, showing the importance of supplements and/or fortified products on vitamin D status, is therefore of importance for public health policy.

We did not find differences in 25(OH)D levels between months when cutaneous synthesis is expected to be very low or totally absent at northern latitudes (November to April) and months when the quantity and quality of UV radiation might be sufficient for cutaneous synthesis (May to October). Icelandic parents are advised to keep their infants out of direct sunlight, and summer temperatures in Iceland usually require long sleeves and a hat for infants. In case infants get in contact with sun, the use of sunscreen is advised [21]. We propose that cutaneous synthesis of vitamin D does not contribute significantly to 25(OH)D in Icelandic infants and that the use of supplements and/or fortified foods and drinks is therefore essential all year round in order to maintain a sufficient vitamin D status. Seasons have, however, been shown to affect 25(OH)D in older children and adults [31,33]. No difference was seen in 25(OH)D levels between infants breastfed or not breastfed at 12 months, which may be explained by the emphasis put on supplement use regardless of feeding mode.

The main strengths of our study lie in the assessment of both vitamin D intake and status in a population-based infant sample and the longitudinal design of the study. Although we are aware of the possibility of altered dietary behavior on data collection days, the use of eight data collection days from 9 to 12 months of age in the current analysis strengthens our confidence that we have reliably estimated food and nutrient intake that may affect vitamin D status at 12 months. The dietary information from 0 to 8 months of age gives practical information. The study also has some limitations. Blood samples were obtained with the primary aim of analyzing the iron status and blood lipids of the infants [13]. Analysis of serum 25(OH)D was only possible for those subjects with sufficient amounts of blood available, resulting in a small sample size. Analyses on parameters that have been used to complement 25(OH)D levels and/or used as blood safety measurements in other studies [34,35], such as parathyroid hormone, serum calcium, alkaline phosphatase and C-reactive protein, were not performed. There is a possibility that the method used for quantitative analyses of serum 25(OH)D may overestimate the 25(OH)D concentration in infants, due to the possible presence of C-3 epimers [36–38]. As all subjects were of Icelandic origin and healthy, transferring the results to high-risk groups of vitamin D deficiency, such as infants of non-western immigrants residing in northern latitudes and infants with chronic illnesses, should not be advised [7,39,40].

## 5. Conclusions

In conclusion, the majority of infants were vitamin D sufficient. Our findings highlight the need for vitamin D supplements or fortified products all year round, regardless of breastfeeding in infant populations with little or no sun exposure.

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### Author Contributions

B.T. participated in data collection, analyzed the data and drafted the paper. I.G. and L.S. conceived of and designed the study. G.P. performed the blood sampling. I.T. supervised data collection and conceived of and designed the study. All authors contributed in writing and editing the manuscript and approved the final version of the paper as submitted.

### Conflicts of Interest

The authors declare no conflict of interest.

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## 4. Children and Adolescents

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### Dietary Intake by Dutch 1- to 3-Year-Old Children at Childcare and at Home

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**Abstract:** The goal of the current study was to assess dietary intake in a large sample ( $N = 1016$ ) of Dutch toddlers (1–3 years old), both at childcare and at home. Dietary intake during two weekdays was recorded using an observation format applied by childcare staff for intake at childcare, and partially pre-coded dietary journals filled out by parents for intake at home. Children's intake of energy, macronutrients and energy balance-related food groups (fruit, vegetables, sweet snacks, savoury snacks) were compared with Dutch dietary guidelines. In addition, differences between the dietary intake by various subgroups (based on gender, age, childcare attendance, socio-economic status of childcare centre) were explored using multilevel regression analyses, adjusting for nesting of children within centres. Energy intake was high relative to dietary guidelines, and children consumed more or less equal amounts of energy at home and at childcare. Dietary fibre, fruit and vegetable and snack intakes were low. Intake at childcare mainly consisted of carbohydrates, while intake at home contained more proteins and fat. The findings imply various opportunities for childcare centres to improve children's dietary intake, such as providing fruit and vegetables at snacking moments. In addition, the findings underline the importance of assessing dietary intake over a whole day, both at childcare and at home, to allow intake to be compared with dietary guidelines.

**Keywords:** childcare; day-care; dietary intake; dietary journal; nutrition; observation; overweight; parent; toddler

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## 1. Introduction

Worldwide, at least 42 million children under the age of five were overweight in 2010, and these numbers are expected to continue to increase [1]. Childhood overweight is a major risk factor for several chronic conditions such as cardiovascular diseases and type 2 diabetes mellitus [2]. Moreover, childhood overweight is known to track into adulthood, in that overweight children often remain overweight or obese during later life [3]. Dietary intake plays a crucial role in the development of overweight [4]. Dietary habits are often established at a young age [5] and maintained throughout life [6–8], indicating the urgency of increasing our understanding of the origin and development of dietary habits in young children.

In Europe, over half of the toddlers (below primary school age) attend some form of childcare or educational facilities [9]. It has been recommended that a child in full-time childcare (*i.e.*, 8 h or more per day) should consume one half to two-thirds of his or her daily dietary intake at childcare [10], indicating the importance of childcare for the development of children's dietary habits. Childcare use has been found to be associated with an increased overweight risk throughout childhood (e.g., [11–13]). Furthermore, various studies have shown that children attending childcare often do not meet dietary intake recommendations: they may consume excess energy [14] and excessive amounts of total fat [14,15], saturated fat [15,16] and sweets [14]. In addition, they are not consuming sufficient amounts of fruit [16], vegetables [14,16,17] and dietary fibre [18]. However, several of these studies were limited to dietary intake at childcare [16,19,20], ignoring the intake at home. As such, they have to rely on the estimated proportion of the dietary intake that takes place at childcare. Since dietary intake at home is not known, these studies assume the composition of the meals to be stable throughout the day and do not take into account possible compensation behaviour at home. The studies that have taken account of both dietary intake at home and intake at childcare [14,15,17,18,21–23] mostly had small sample sizes ( $N < 200$ ) [14,17,18,21,22], assessed dietary intake at childcare through the parents [18], or only examined specific meals instead of dietary intake during a whole day [18]. In addition, the majority of the studies examining dietary intake have been conducted in the United States [14–18,20,21], with only a few from Europe [19,22,23].

The current study aimed to assess dietary intake in terms of energy, macronutrients and the food groups of fruit, vegetables, sweet snacks and savoury snacks, both at childcare and at home, in a large sample ( $N = 1016$ ) of Dutch toddlers (aged 1–3 years). In addition, it explored the dietary intake in various subgroups (according to gender, age, childcare attendance and socio-economic status (SES) of the childcare centre's neighbourhood).

## 2. Methods

### 2.1. Respondents and Procedure

Ethical approval for this study was not required according to Dutch law, since the current research did not involve invasive procedures, and thus did not fall under the Dutch Medical Research Involving Humans Act (Wet Medisch-Wetenschappelijk Onderzoek met Mensen) [24]. All childcare centres in the Netherlands were approached to participate in the study from March 2011 onwards. Several strategies were used to recruit childcare centres. A direct mailing of letters was sent to addresses acquired by purchasing commercially available addresses. In addition, a digital mailing was sent, and childcare centers were recruited at conferences and through appointments at the head offices of the childcare organizations to which the centres belonged. If the head office was interested, the recruitment was continued at the individual centres. All childcare centres were allowed to participate. Sometimes a centre decided not to participate citing reasons such as that it would be too much effort, the centre had been closed down, the parent committee did not agree or management had changed. The 112 childcare centres that responded before August 2013 were included in the study. Data collection started as soon as a childcare centre consented to participate. All parents of the children aged 1 to 3 years from these centres were invited to participate. A total of 2788 children participated. All parents of participating children provided informed consent. Children's dietary intake was recorded on two entire weekdays, randomly chosen during one week, both at home using food diaries and at childcare using observations.

## 2.2. Assessment of Dietary Intake

In the Netherlands, children attending childcare usually consume their breakfast at home. Subsequently, they consume a morning snack, lunch and afternoon snack at childcare, and their dinner again at home.

### 2.2.1. Dietary Intake at Childcare

Staff at the childcare centre was instructed by a dietician to record the dietary intake of each of the participating children on a poster. The poster was a partially pre-coded dietary record, providing a list of the most common products that might be consumed at each different eating moment. For instance, it showed a list of sweet snacks, beverages and fruits commonly consumed at snacking moments in the Netherlands. In addition, it provided space at each eating moment to record any other products consumed which were not on the standard list. There was a separate column on the poster for each participating child, where their intake could be recorded. Childcare staff was asked to specify the type of product (e.g., whether the milk product consumed was milk, chocolate milk, butter milk or yoghurt drink), the unit (e.g., whether it was a cup or a bottle), and the amount (*i.e.*, number of units).

The first eating moment (the snacking moment of the first observation day) was recorded together with the dietician, at which point the childcare staff received detailed instructions from the dietician on how to record the dietary intake. If the childcare staff were still uncertain about any aspects, these would also be explained by the dietician. During the rest of that day, and on the second observation day, the childcare staff filled in the poster for all eating moments at the centre (*i.e.*, morning snack, lunch and afternoon snack).

An additional questionnaire was filled in by the childcare staff together with the dietician to record further information regarding the meals and foods offered at the centre, such as the standard portion

size used for certain products (e.g., how many mL were in the cups used) and the type and brand of particular products (e.g., whether regular or low-fat margarine was used and what brand).

### 2.2.2. Dietary Intake at Home

Parents were also asked to record their child's dietary intake at different eating moments at home during the two measurement days (*i.e.*, breakfast, dinner including dessert, and anything consumed after dinner, including anything consumed during the night). The questionnaire consisted of a partially pre-coded food journal, providing a list of common products that might be consumed at each different eating moment. For instance, the food journal listed the bread and bread products, butters or margarines, sandwich toppings, fruit, porridges and beverages that are often consumed at breakfast in the Netherlands. In addition there was space to record any other products consumed at each eating moment that were not on the standard list. Parents were asked to specify the type of product (e.g., whether bread was white or brown), the unit (e.g., whether it was a slice of bread or a roll), the brand, and the amount (*i.e.*, number of units).

### 2.3. Assessment of Background Characteristics

Children's age (rounded off to whole months), gender and the number of days they attended childcare were asked for in the parental questionnaire. The socio-economic status (SES) score of the population catered for by each childcare centre was derived from the centre's postal code. These SES scores are standardized scores per neighbourhood, reflecting educational level, income, and work status of the residents of that neighbourhood [25]. The SES scores were recoded into low, medium and high, based on tertile cuts of all scores in the Netherlands [25].

### 2.4. Processing of Dietary Intake Data

Only respondents for whom complete dietary intake data were available (for both measurement days, both at childcare and at home) were retained in the analyses. Of the 2788 children participating in the entire study, 1016 (43.7%) provided complete dietary intake data for both measurement days, both at home and at childcare. Of the 1773 children without complete data, the majority (75.0%) had complete data at childcare, but data at home was only available for 1 day or no days at all. Furthermore, 24.0% had only attended childcare on the day, so data for two complete days at childcare could not be provided, and 1.0% only provided data for intake at home, but not for childcare. The 1016 children with complete data were included in the final analyses.

The observed and reported dietary intake data of the children were entered by the dieticians in the FoodFigures Program [26] separately for each of the six eating moments (breakfast, morning snack, lunch, afternoon snack, dinner, evening snack). The amounts consumed as reported by the childcare staff and parents were recalculated by this program into weights and volumes using the procedures on measures and weights of the Dutch nutrient database [27] where necessary (e.g., using a standardized weight for a slice of bread). Amounts of half or a quarter of a portion were also recalculated by the program. As the focus of the current paper was on dietary intake, the average intake per day of the following nutrients was calculated by the program, based on the Dutch nutrient database [27]: energy

(in kcal), proteins (in energy percent (en%)), carbohydrates (en%), total fat (en%), saturated fat (en%), unsaturated fat (en%) and fibre (grams (g)). In addition, intake from the following energy balance-related food groups was calculated: fruit (g), vegetables (g), sweet snacks (g; including sweets (e.g., jelly candy, liquorice, marshmallows), chocolate, cookies (e.g., butter cookies) and pastry (e.g., cake, pie)) and savoury snacks (g; including salty snacks (e.g., potato chips) and fried snacks (e.g., fried meats)).

### 2.5. Data Analyses

All analyses were conducted using SPSS 20.0 [28]. *p*-Values < 0.05 were considered statistically significant. Independent *t*-tests and chi-square tests were conducted to compare the background characteristics (children's age, gender, childcare attendance, and the childcare centre's SES) of the children in the final sample with those of children who had incomplete data and were thus excluded.

Descriptive statistics were used to explore the children's background characteristics and total dietary intake. In addition, children's total dietary intake was compared with the dietary guidelines for toddlers from the Netherlands Nutrition Centre (Voedingscentrum; see Table 1) [29]. The overall dietary guidelines applied in the current study were specific Dutch guidelines [29]. The dietary guidelines for toddlers from the Netherlands Nutrition Centre refer to the guidelines for a healthy food choice of the Netherlands Nutrition Centre for a balanced dietary intake for children of one year and older. These guidelines are therefore used as a benchmark source for nutrient and food group analyses. Next, children's dietary intakes at home and at childcare were analysed separately, as well as their intakes at different eating moments (breakfast, morning snack, lunch, afternoon snack, dinner and evening snack).

**Table 1.** Total dietary intake by toddlers, compared to national guidelines (*N* = 1016).

Dietary Intake	Actual Total Dietary Intake Mean (SD)	Dietary Guideline <sup>a</sup>	Percentage of Children Meeting the Guideline
Energy (kcal)	1285.1 (238.2)	1200	37.5% <sup>b</sup>
Carbohydrates (en%)	55.7 (5.2)	≥45	98.1%
Proteins (en%)	14.3 (2.1)	≤20	99.2%
Fat			
Total (en%)	30.0 (4.8)	25–40	83.2% <sup>c</sup>
Saturated (en%)	10.7 (1.8)	≤15	98.6%
Unsaturated (en%)	16.6 (3.7)	-	-
Dietary fibre (g)	12.5 (2.7)	≥15	17.2%
Fruit (g)	124.1 (61.9)	≥150	27.5%
Vegetables (g)	64.7 (36.5)	≥50–100	69.3% <sup>d</sup> 17.0% <sup>e</sup>
Sweet snacks <sup>f</sup> (g)	13.5 (12.0)	-	-
Savoury snacks <sup>g</sup> (g)	0.7 (4.0)	-	-

Notes: en% = energy percent, g = grams, mL = millilitres, *N* = number of children, SD = standard deviation;

<sup>a</sup> Nutritional guidelines from the Netherlands Nutrition Centre ([29]). No specific guidelines are available for unsaturated fats and snacks; <sup>b</sup> 10% deviation from the guideline allowed (*i.e.*, 1080–1320 kcal). Below the guideline: 18.8%; above the guideline: 43.7%; <sup>c</sup> below the guideline: 14.9%; above the guideline: 2.0%;

<sup>d</sup> *N* (%) meeting the guideline of 50 g/day; <sup>e</sup> *N* (%) meeting the guideline of 100 g/day; <sup>f</sup> Including sweets, chocolate, cookies and pastry; <sup>g</sup> Including salty snacks and fried snacks.

In addition, the intakes by subgroups of children based on gender (boys *vs.* girls), age (2, 3 or 4 years old), childcare attendance (up to 2 days *vs.* 3 or more days a week) and the childcare centre's SES (low, medium or high) were explored. Multi-level linear regression analyses were conducted to examine the associations between these background variables and the dietary intake variables, corrected for the nesting of children within childcare centres and the background variables.

### 3. Results

Of the 1016 children, 54.8% (*N* = 554) were male. The average age was 2 years and 1 month (SD = 10 months), with 313 1-year-olds (34.1%), 330 2-year-olds (36.0%) and 274 3-year-olds (29.9%). On average, the children went to childcare for 2.4 days per week (SD = 0.6). A total of 24.5% of the children attended childcare centres that were located in low-SES neighbourhoods, 28.2% in medium-SES neighbourhoods, and 47.3% in high-SES neighbourhoods.

Children included in the final sample attended childcare for slightly more days a week than children with incomplete dietary intake data (2.4 *vs.* 2.1,  $p < 0.001$ ). There were no other significant differences in background characteristics (age, gender, childcare SES) between children who did or did not drop out.

#### 3.1. Dietary Intake and Guideline Compliance

Table 1 lists the total dietary intake of the toddlers in the current study, as well as the number and percentage of children complying with the dietary guidelines of the Netherlands Nutrition Centre [29]. About one third of the children (37.5%) complied with the guidelines regarding energy intake. A smaller group (18.8%) consumed less than the recommended amount of energy, while most children (43.7%) consumed more energy than recommended.

The vast majority of the children met the guidelines with regard to macronutrients (carbohydrates, proteins and fat). However, only 17.2% of the children consumed sufficient dietary fibre. This was also reflected in the low percentages of children consuming sufficient fruit and vegetables. Snack intake (both sweet and savoury) was generally low.

#### 3.2. Dietary Intake at Childcare and at Home

Children consumed more or less equal amounts of energy at home and at childcare. However, while their intake at childcare mainly consisted of carbohydrates, a relatively larger proportion of the intake at home consisted of proteins and fat (see Table 2). Furthermore, children consumed most of their fruit at childcare, and most of their vegetables at home. Sweet snacks were mostly eaten at childcare.

#### 3.3. Dietary Intake at Different Eating Moments

Table 3 shows the children's dietary intake at different eating moments, as well as the intake as a proportion of total daily intake (from different meals). The percentage of children consuming food

at each of the eating moments was very high (98.6%–99.8%), except for evening snacks, which were consumed by only 62.8% of the children.

The main energy sources were lunch and dinner, together accounting for over half (57.4%) of the total energy intake. The snacking moments were very high in carbohydrates, while the main meals contained relatively larger proportions of proteins and fat. The main sources of dietary fibre were the main meals. Most fruit was consumed during the morning snacking moment, while the afternoon snacking moment often involved sweet snacks (e.g., cookies, sweets, pastry). The evening snacking moment involved a relatively large proportion of saturated fat compared to the other snacking moments.

**Table 2.** Dietary intake by toddlers at childcare and at home ( $N = 1016$ ).

Dietary Intake	Dietary Intake Mean (SD)	
	At Childcare	At Home
Energy (kcal)	652.2 (169.9)	633.5 (171.9)
Carbohydrates (en%)	62.4 (6.6)	49.1 (8.4)
Proteins (en%)	12.0 (2.4)	16.8 (3.7)
Fat		
Total (en%)	25.6 (5.6)	34.1 (7.8)
Saturated (en%)	9.6 (2.3)	11.7 (2.8)
Unsaturated (en%)	13.4 (3.9)	19.5 (6.1)
Dietary fibre (g)	6.6 (1.8)	5.9 (2.1)
Fruit (g)	98.7 (46.0)	25.5 (41.4)
Vegetables (g)	10.9 (21.6)	53.8 (33.4)
Sweet snacks <sup>a</sup> (g)	10.3 (9.4)	3.3 (7.9)
Savoury snacks <sup>b</sup> (g)	0.2 (1.5)	0.5 (3.7)

Notes: en% = energy percent, g = grams, mL = millilitres; <sup>a</sup> Including sweets, chocolate, cookies and pastry;

<sup>b</sup> Including salty snacks and fried snacks.

### 3.4. Dietary Intake in Subgroups

Overall, there were few differences in total dietary intake between boys and girls. Boys consumed significantly more energy than girls (1304.8 vs. 1264.6 kcal,  $p < 0.01$ ), as well as more dietary fibre (12.7 vs. 12.2 g,  $p < 0.02$ ). There were no significant differences between boys and girls in intake specifically at childcare.

Intake of energy was significantly higher among older children, both specifically at childcare and for the day as a whole (see Table 4). Dietary fibre intake also increased with age, mainly at childcare. Sweet snacks intake increased with age, although at childcare, this increase was only significant between 2 and 3 years, while the increase in overall sweet snacks intake was only significant between 1 and 2 years. Total savoury snack intake increased between the ages of 1 and 2 years.

Children who attended childcare for 3 or more days a week had a higher total vegetables consumption (73.3 vs. 62.0 g,  $p < 0.02$ ), and consumed more savoury snacks (1.1 vs. 0.6 g,  $p < 0.04$ ; results not tabulated) than children attending childcare for 2 days or less. Childcare attendance was not significantly related to specific dietary intake at childcare.

**Table 3.** Dietary intake by toddlers at different eating moments.

Dietary Intake	Dietary Intake Mean (SD)											
	Breakfast at Home (N = 1006)		Morning Snack at Childcare (N = 1010)		Lunch at Childcare (N = 1014)		Afternoon Snack at Childcare (N = 1002)		Dinner at Home (N = 1013)		Evening Snack at Home (N = 638)	
	Mean (SD)	% of total intake	Mean (SD)	% of Total Intake	Mean (SD)	% of Total Intake	Mean (SD)	% of Total Intake	Mean (SD)	% of Total Intake	Mean (SD)	% of Total Intake
Energy (kcal)	237.7 (87.1)	18.1	126.1 (51.8)	9.6	378.5 (122.9)	28.8	147.6 (67.9)	11.2	358.8 (120.0)	27.2	66.8 (63.5)	5.1
Carbohydrates (en%)	54.9 (10.6)	14.5	85.4 (10.8)	22.6	49.6 (7.7)	13.2	81.2 (12.8)	21.5	43.0 (11.6)	11.4	63.6 (23.1)	16.8
Proteins (en%)	15.0 (4.6)	19.6	5.2 (3.5)	6.8	16.2 (3.9)	21.3	6.4 (4.6)	8.4	19.1 (5.4)	25.1	14.3 (12.4)	18.8
Fat												
Total (en%)	30.0 (10.0)	20.5	9.5 (8.5)	6.5	34.5 (7.1)	23.5	12.5 (10.2)	8.5	37.9 (11.1)	25.9	22.1 (16.1)	15.1
Saturated (en%)	11.5 (4.2)	20.8	3.1 (4.6)	5.6	12.9 (3.1)	23.3	5.0 (5.4)	9.1	12.1 (3.6)	21.9	10.7 (8.9)	19.3
Unsaturated (en%)	15.7 (7.8)	20.8	4.1 (4.5)	5.4	18.3 (5.2)	24.2	5.5 (6.2)	7.3	22.7 (9.0)	30.0	9.3 (11.1)	12.3
Dietary fibre (g)	2.6 (1.2)	20.5	1.5 (0.9)	11.8	3.9 (1.4)	30.7	1.2 (0.9)	9.5	3.0 (1.3)	23.6	0.5 (0.9)	3.9
Fruit (g)	7.5 (22.6)	5.9	70.5 (51.7)	55.9	1.2 (8.6)	1.0	30.7 (45.0)	24.3	12.8 (27.6)	10.2	3.4 (14.2)	2.7
Vegetables (g)	0.3 (3.0)	0.5	0.1 (1.0)	0.2	5.1 (15.8)	7.8	2.8 (10.2)	4.3	56.4 (32.5)	87.0	0.1 (2.0)	0.2
Sweet snacks <sup>a</sup> (g)	1.2 (4.3)	8.7	3.6 (6.4)	26.1	0.2 (1.3)	1.4	7.3 (8.2)	52.9	0.8 (4.1)	5.8	0.7 (3.0)	5.1
Savoury snacks <sup>b</sup> (g)	0.0 (0.5)	0.0	0.0 (0.5)	0.0	0.0 (0.0)	0.0	0.3 (1.6)	42.9	0.4 (3.7)	57.1	0.0 (0.4)	0.0

Notes: en% = energy percent, g = grams, mL = millilitres; <sup>a</sup> Including sweets, chocolate, cookies and pastry; <sup>b</sup> Including salty snacks and fried snacks.

Table 4. Dietary intake differences based on age.

Dietary Intake	Dietary Intake at Childcare			Dietary Intake during a Whole Day			Significance <sup>b</sup>
	1-Year-Olds Mean (SD) N = 313	2-year-olds <sup>a</sup> Mean (SD) N = 330	3-Year-Olds Mean (SD) N = 274	1-Year-Olds Mean (SD) N = 313	2-Year-Olds <sup>a</sup> Mean (SD) N = 330	3-Year-Olds Mean (SD) N = 274	
Energy (kcal)	570.7 (150.1)	662.3 (158.2)	726.8 (170.9)	1165.9 (209.1)	1305.9 (210.1)	1400.4 (231.7)	***
Carbohydrates (en%)	62.5 (7.1)	63.0(5.8)	62.3 (6.3)	55.7 (5.5)	56.0 (5.1)	55.8 (4.8)	
Proteins (en%)	12.1 (2.8)	11.8 (2.1)	12.0 (2.3)	14.5 (2.4)	14.1 (1.9)	14.2 (2.1)	
Fat							
Total (en%)	25.4 (5.8)	25.2 (5.2)	25.6 (5.3)	29.8 (5.1)	29.9 (4.7)	30.0 (4.4)	
Saturated (en%)	9.6 (2.4)	9.4 (2.1)	9.5 (2.1)	10.7 (1.9)	10.6 (1.7)	10.6 (1.6)	
Unsaturated (en%)	13.3 (4.0)	13.3 (3.8)	13.5 (3.7)	16.5 (3.9)	16.5 (3.5)	16.7 (3.5)	
Dietary fibre (g)	6.1 (1.7)	6.6 (1.9)	7.2 (1.8)	12.3 (2.6)	12.3 (2.6)	13.0 (2.7)	* <sup>c</sup>
Fruit (g)	99.8 (45.8)	98.4 (49.5)	98.9 (45.0)	122.6 (62.3)	124.3 (63.4)	127.8 (63.1)	
Vegetables (g)	13.5 (23.7)	9.8 (20.5)	9.8 (20.7)	68.5 (36.3)	61.9 (36.1)	64.6 (38.2)	
Sweet snacks <sup>d</sup> (g)	8.8 (8.3)	9.9 (8.6)	12.1 (11.4)	11.3 (10.6)	13.6 (12.2)	16.0 (13.5)	* <sup>c</sup>
Savoury snacks <sup>f</sup> (g)	0.1 (1.3)	0.2 (1.5)	0.5 (1.9)	0.3 (1.9)	1.1 (5.8)	0.9 (3.5)	* <sup>c</sup>

Notes: en% = energy percent, g = grams, mL = millilitres; <sup>a</sup> Reference category; <sup>b</sup> Adjusted significance from multivariate multi-level regression analyses, adjusted for gender, childcare attendance and socioeconomic status score of the childcare centre; <sup>c</sup> Only significant for the 1-year-olds; <sup>d</sup> Including sweets, chocolate, cookies and pastry; <sup>e</sup> Only significant for the 3-year-olds; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; <sup>f</sup> Including salty snacks and fried snacks.

There were no differences in overall intake between childcare centres with different SES. With regard to the specific intake at childcare, children at high-SES childcare centres consumed significantly less fruit (93.0 g) than children at medium- and low-SES centres (106.2 g and 101.2 g, respectively,  $p < 0.04$ ). On the other hand, they consumed significantly more vegetables at childcare (14.9 g) compared to children from medium- and low-SES centres (7.2 g and 7.3 g, respectively,  $p < 0.01$ ). Children at low-SES childcare centres consumed significantly lower amounts of energy (619.0 kcal) than those at medium- and high-SES centres (679.8 and 652.9 kcal, respectively,  $p < 0.04$ ). Finally, children at low-SES centres consumed significantly less savoury snacks (0.4 g compared to 0.1 g and 0.3 g in medium- and high-SES centres, respectively,  $p < 0.05$ ; results not tabulated).

#### 4. Discussion

The current study assessed dietary intake at childcare and at home in a large sample ( $N = 1016$ ) of Dutch toddlers (1–3 years) who attended childcare. Energy intake was high relative to dietary guidelines, while dietary fibre, fruit and vegetable intakes were low. Snack intake (both sweet and savoury) was low. In 2005 and 2006, a national food consumption survey was conducted among toddlers in the Netherlands including children who attended childcare as well as those who did not. The dietary intake among the 2- to 3-year-olds ( $N = 788$ ) from that survey was very similar to the intake we found in the current sample, specifically as regards the intake of energy, all macronutrients, dietary fibre and fruit (differences all  $<5\%$ ) [30]. This indicates that the overall dietary intake by children attending childcare does not seem to be very different from that by children not using childcare. Compared to the national survey, however, children in the current sample appeared to consume far less snacks (13.5 g of sweet snacks *vs.* 47 g in the national survey; and  $<1$  g of savoury snacks *vs.* 3 g in the national survey) and more vegetables (64.7 g compared to 40 g in the national survey) [30].

It is unclear why there were such considerable differences with regard to vegetable and snack intakes, but not with regard to any other dietary intake measures. Perhaps the fact that the current study included 1-year-olds can partly explain these differences, especially with regard to snacks, because the 1-year-olds in the current sample consumed significantly less snacks than the older children. Furthermore, it should be noted that the current study did not include days on which the children did not attend childcare. It is possible that the children from the current sample had different intake patterns during a full day at home. However, a previous study by Ziegler and colleagues [18], which compared lunch and snacking moments at childcare with the corresponding eating moments during a full day at home, found only slight differences in intake between these two locations, which were only significant for the afternoon snacking moment: at home, children seemed to consume a bit more protein and fat in the afternoon. Furthermore, Ziegler *et al.* [18] reported more frequent consumption of salty snacks in the afternoon at home than at childcare. A study by Lehtisalo [23] that compared the dietary intake of children cared for at childcare with that of children cared for at home found lower vegetable consumption and higher sweet pastry consumption by the children cared for at home. These findings are in line with the deviating vegetable and snack consumption in the current sample compared to the national survey [30]. A final explanation for the differences between the current study

and the national survey may regard the fact that the current study did not include weekend days. Several studies have shown that children's dietary intake is generally less healthy on weekend days (e.g., [22,23,31]), possibly explaining the lower snack consumption and higher vegetable intake in the current study.

In line with previous research among young children (e.g., [30,32]), the children in the current sample skipped very few meals and snacking moments: 98.6%–99.8% of the children consumed food at each of the eating moments (except for an evening snack, consumed by 62.4%). As regards the quality of children's diets, the macronutrient content of their diets seemed to be very good, with 83.2% to 99.2% of the children meeting the guidelines for carbohydrates, proteins, total fat and saturated fat. Studies from the US found excess consumption of total and saturated fat in childcare [14–16], perhaps reflecting a cultural difference between the US and the Netherlands. However, in line with previous US studies [14,16–18], many children in the current sample did not consume sufficient dietary fibre, vegetables and fruit. Furthermore, almost half of the children consumed excess amounts of energy (*i.e.*, >1320 kcal), which is in line with previous research [14]. About equal amounts of energy were consumed at home and at childcare. Energy intake at the different eating moments in the current study was comparable to that found in US studies [18,20].

There were few differences in dietary intake between subgroups, both at childcare and in total. In line with previous research [19], boys consumed more energy than girls. In addition, boys consumed more dietary fibre. Concerns about children's diet seemed to change with age: while younger children were more likely to consume insufficient dietary fibre, the older children often consumed more snacks and energy. These differences were visible specifically at childcare as well as during a whole day, with the exception of savoury snack intake, whose increase was only significant as regards intake during a whole day. With regard to childcare attendance, children who attended three or more days a week consumed more vegetables and savoury snacks, though not at childcare, indicating that this increased vegetable and snack intake took place at home. Furthermore, we found older children to consume more energy, dietary fibre, sweets and snacks. Despite the fact that The Netherlands Nutrition Centre recommends the same intake for children aged 1–4 in their guidelines [29], our results show that children within this age group have different needs. Children in the age of 1 may for example still be nursed which influences their dietary intake.

Although the overall consumption of snacks seemed to be low (13.5 g of sweet snacks and less than one gram of savoury snacks per day on average), the majority of the sweet snacks were consumed at childcare, especially during the afternoon snacking moment. Fruit was consumed especially during the morning snack at childcare, and to a lesser extent in the afternoon. This indicates an opportunity for childcare centres to improve children's fruit consumption (which was too low for almost three quarters of the children), and at the same time even further lower snack consumption, by replacing the afternoon sweet snacks with fruit. Fruit consumption seemed to be especially low in high-SES childcare centres, while intake at home was not significantly different between childcare centres with different SES. Previous studies have repeatedly shown that children from low-SES families often consume less fruit (e.g., [33,34]). However, children from high-SES childcare centres in the current study also consumed more vegetables. It seems that high-SES childcare centres place more emphasis on vegetable intake, and less on fruit intake.

Various previous studies examining dietary intake at childcare have used the guidelines of the American Dietetic Association (that a child who spends a full day at childcare [*i.e.*, 8 h or more] should consume one half to two-thirds of his or her daily dietary intake at childcare [10]) to convert daily dietary intake guidelines into estimated guidelines for intake at childcare (e.g., [16,17,19]). However, such conversion into childcare-specific guidelines ignores the fact that the composition of meals and other eating moments is not stable throughout a day (e.g., the composition of a typical lunch is different from the composition of a typical dinner), as the current study shows. It therefore makes no sense to apply the same guidelines at home and at childcare. This underlines the importance of studies assessing dietary intake during a total day, both at home and at childcare, enabling comparison with daily intake guidelines.

The current study had several limitations. There was a relatively high percentage of incomplete cases (56.3%), although the final sample included in the study can still be considered very large (over 1000 children from over 100 different childcare centres) compared to previous studies. This large sample size also provided sufficient statistical power to correct the analyses for the multi-level structure of the data. Nonetheless, there was some selective drop-out with regard to longer childcare attendance. In addition, the data collection took place over a relatively long period (30 months), which could have influenced the results. A strength of the current study was that dietary intake was assessed both at childcare and at home, making it possible to compare the intake with dietary guidelines without having to estimate the proportion of intake taking place at childcare. However, dietary intake at childcare was observed and recorded by childcare staff, while dietary intake at home was self-reported by parents, possibly introducing bias. Moreover, the dietary intake assessment methodologies used in both settings (childcare and home) were not validated, and weekend days were not assessed in the current study. With regards the software used to recalculate intake, participants were not asked whether children consumed their entire plate/glass which may have biased the reported intake. However, the ability of the program to register amounts of half or a quarter of a portion helped accurate assessment of children's intake. Moreover, the dietary intake in the current sample was very similar to the intake in a previous national survey, indicating that the assessment methods in the current study were probably sufficiently reliable.

## 5. Conclusions

In terms of energy balance, the main concern of children's dietary intake in our study was their low fibre, vegetable and fruit consumption, and their high energy intake, putting them at risk for developing overweight. Childcare has a large potential to contribute to resolving these issues, for instance by offering fruit as a snack twice instead of once a day, and by providing vegetables during lunch and snacking moments. This could potentially also further lower snack consumption and thereby lower energy intake, thus also reducing the overweight risk. Previous studies have shown that childcare staff can have an important positive influence on children's dietary intake at childcare, for example by serving sufficient healthy foods [16,35], preferably using a family serving style (in which the child can take healthy foods him/herself and can decide how much to take) [19,36,37] or indulgent feeding style (giving and offering seconds for healthy foods) [37,38]; by being a positive role model

and eating healthy foods together with the children [19]; and by talking about healthy foods with the children [19].

As regards research, the current findings underline the importance of future research assessing dietary intake during a total day, both at home and at childcare. This enables comparison with daily guidelines, instead of having to convert these guidelines to improvised childcare-specific guidelines. In addition, intake should be assessed during a full day at home as well, including weekend days, to be able to compare intake at home and at childcare and to check for variability in children's diets across locations.

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### Conflicts of Interest

The authors declare no conflict of interest.

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## Prevalence of Dietary Supplement Use in Healthy Pre-School Chinese Children in Australia and China

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**Abstract:** There is a growing use of dietary supplements in many countries including China. This study aimed to document the prevalence of dietary supplements use and characteristics of Chinese pre-school children using dietary supplements in Australia and China. A survey was carried out in Perth, Western Australia of 237 mothers with children under five years old and 2079 in Chengdu and Wuhan, China. A total of 22.6% and 32.4% of the Chinese children were taking dietary supplements in Australia and China, respectively. In China, the most commonly used dietary supplements were calcium (58.5%) and zinc (40.4%), while in Australia, the most frequently used types were multi-vitamins/minerals (46.2%) and fish oil (42.3%). In Australia, “not working”, “never breastfeed”, “higher education level of the mother” and “older age of the child” were associated with dietary supplement use in children. In China, being unwell and “having higher household income” were significantly related to dietary supplement usage. Because of the unknown effects of many supplements on growth and development and the potential for adverse drug interactions, parents should exercise caution when giving their infants or

young children dietary supplements. Wherever possible it is preferable to achieve nutrient intakes from a varied diet rather than from supplements.

**Keywords:** dietary supplements; Chinese; calcium; zinc; migrants; child; nutrition

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## 1. Introduction

Infant nutrition is important for short term and long term health. A balanced variety of nutritious foods are emphasized in the guidelines of the Australian and Chinese governments and other professional organizations as the best source of nutrition for healthy children [1–3]. However, the Chinese diet has been reported to be low in calcium, riboflavin, Vitamin A, and zinc [4,5]. A national survey in 2004 found that the average calcium intake among the city and suburban populations was 430 mg per day, well below the recommended intake [6]. The iron intake appears to be adequate in amount, but its bioavailability is very low and consequently the prevalence of iron deficiency and iron deficiency anemia was 43.7% and 7.8%, respectively, among children aged 1–3 years in 2001 [4,7].

The consumption of fortified foods and/or supplements can help some children meet their nutritional needs [8]. Examples of recommended use of supplements include the American Academy of Pediatrics' recommendation for oral Vitamin D supplementation for exclusively breastfed infants and, under certain conditions, for specific older infants and toddlers [9]. However, other countries, such as Australia, have different climatic conditions and do not recommend universal use of Vitamin D, and the excessive intakes of single nutrients may have the potential for adverse effects [10–12].

Dietary supplements enriched with vitamins, minerals, and other substances are increasingly consumed worldwide. The North America and the Asia Pacific regions are the dominant markets for vitamins and dietary supplements [13]. The prevalence of supplement use varies in different ethnic groups for a diversity of dietary and cultural reasons and economic conditions. Most published studies on the use of supplements in children have been conducted in the US and only a small number of studies have been conducted in Asian countries. It is reported that approximately 49% of the U.S. population take dietary supplements and the prevalence of supplement use was 35% among children aged 1–13 years [14,15]. In South Korea, approximately 34% of Korean children and adolescents were taking dietary supplements in a national survey in 2007–2009 [16]. A survey of urban Japanese found that 20.4% of children and adolescents between 3 and 17 years were using supplements, or had used them in the past year [17]. A cross-sectional survey carried out in Zhejiang Province, PR China in 1999 reported a prevalence of 18% of vitamin supplements and 31% of other nutritional supplements in adolescents [18]. A recent study from Taiwan reported that 34.9% of the infants had been given a dietary supplement before six months [19].

Australians have a high prevalence of taking dietary supplements. A representative population survey conducted in 2004 in South Australia reported the use of vitamin supplements by 39.2% respondents and mineral supplements by 13.6% of the population [20]. No recent data is available on the use of supplements by infants or young children in Australia.

Until recently, there have been no reported studies of dietary supplementation among Chinese young children in mainland China or overseas. The aim of this study was to document the prevalence of use of dietary supplements in these populations. A survey was carried out of Chinese mothers living in Perth, Australia and Chengdu and Wuhan, PR China.

## 2. Methods

This data was collected from October 2010 to October 2011 in Perth, Western Australia and from September to December 2011 in Chengdu and Wuhan, China. Participants in Perth were mothers who have at least one pre-school child under 5 years old. They were recruited from the Perth Chinese community, including Chinese schools and community organizations. Mothers interested in taking part in this study received an information sheet containing project details and were asked to sign the consent form. A total of 248 questionnaires were distributed in Perth and 237 mothers agreed to participate (response rate of 95.6%) and 230 mothers completed the dietary supplementation section of the questionnaire. The response rate to the dietary questionnaire was 95.6%. Participants in China were recruited from four kindergartens in four districts of Wuhan and 14 kindergartens in seven districts of Chengdu. Both private and public kindergartens were included. A total of 2400 questionnaires were distributed to mothers by kindergarten teachers and 2079 were returned, a response rate of 86.6%. The dietary supplementation questionnaire was completed by 1464 mothers in China with a response rate to the dietary questionnaire of 70.4%. The study was approved by the Curtin University Human Research Ethics Committee (approval number: HR 96/2010) and the local education authorities in China.

Demographic and dietary supplement use was collected using a validated and reliable questionnaire previously used in Chinese population studies [21]. Pre-coded questions were used to classify income into three groups using categories based on local annual household income surveys [22,23]. A Dietary Supplement Questionnaire is used to collect information on the participants' use of medicine, vitamins, minerals, herbals, and other supplements during the past two weeks. Detailed information about type, consumption frequency, and amount taken was collected for each reported dietary supplement use. Child's health status was collected using a translated version of the Australian National Health Survey Questionnaire [24].

Body mass index (BMI) was defined as weight (kg)/height (m)<sup>2</sup>. The 2012 revised international child cut-offs developed by the International Obesity Task Force (IOTF) were used to classify thinness, overweightness and obesity in children in this study [25]. They are based on BMI data from six countries, corresponding to the body mass index (BMI) cut-offs at 18 years, which are BMI 25 (overweight), 30 (obesity) and 18.5 (underweight) [25].

All statistical analyses were performed using the IBM Statistical Package for Social Sciences (SPSS) Version 20.0. Independent samples' *t*-test was used to compare means between groups. Mann-Whitney U test was applied to compare the average age of children from two countries. Chi-square ( $\chi^2$ ) test was used to compare basic characteristics of mothers and children in Australia and China. A multiple binary logistic regression model was used to evaluate the association between mother and child's characteristics and the use of dietary supplements. A backward elimination procedure was applied to obtain final models. *p* values <0.05 were considered statistically significant.

### 3. Results

A total of 230 Chinese mothers living in Perth, Australia and 1156 mothers living in Chengdu, Sichuan Province and 308 mothers living in Wuhan, Hubei Province, PR China completed the supplements questionnaire. The distribution analysis shows there were no differences between mothers who completed the supplements questionnaire and mothers who did not in age, education attainment, marital status, working status, family income status, breastfeeding initiation and duration. There was also no difference in education attainment, marital status, family income status, breastfeeding initiation and duration, between mothers in Chengdu and Wuhan. The only statistically significant difference between mothers in Wuhan and Chengdu was the average age (31.0 years in Chengdu and 30.8 years in Wuhan,  $p < 0.001$ ). Because the difference is so small in Wuhan and Chengdu mothers, their data were pooled for further analysis.

The average age of Chinese mothers in Australia was older than mothers in China ( $33.8 \pm 4.9$  years compared to  $31.0 \pm 4.1$  years,  $p < 0.001$ ). The mothers in Australia also had higher education levels. The median age of the “index child” in the China study population (median age = 3.7 years, the interquartile range = 1.1 years) was older than in Perth (median age = 1.6 years, the interquartile range = 1.9 years,  $p < 0.001$ ). More Perth Chinese children were underweight (22.7%) and fewer overweight and obese (8.0%) than children in China (11.6% underweight and 17.0% overweight and obese,  $p = 0.003$ ) (Table 1).

**Table 1.** Characteristics of Chinese mothers and their children completing dietary questionnaires in Australia and China.

Characteristic	Australia ( $n^* = 230$ )	China ( $n^* = 1464$ )	2-sided $p$ -value
	$n$ (%)	$n$ (%)	
Mothers Age (years)			<0.001
≤30	68 (30.1)	604 (53.3)	
>30	158 (69.9)	530 (46.7)	
Marital status			0.116
Married	229 (99.6)	1151 (98.1)	
Divorced/single/widow	1 (0.4)	22 (1.9)	
Educational attainment			<0.001
High school diploma/TAFE certificate/diploma or less	57 (24.8)	661 (57.1)	
University degree or higher	173 (75.2)	496 (42.9)	
Working status			<0.001
Working	105 (45.7)	968 (83.1)	
Not employed	125 (54.3)	197 (16.9)	
Household income			0.086
Low income	108 (49.5)	572 (55.9)	
High income	110 (50.5)	451 (44.1)	
Mother's birth place			
Mainland China	187 (81.3)		
Other Asian countries	43 (18.7)		

Table 1. Cont.

Duration in Australia (years)			
<5	126 (53.1)		
5–10	73 (32.3)		
>10	33 (14.6)		
Age of the child (years)			
0–1	62 (27.0)	15 (1.0)	<0.001
1–2	81 (35.2)	24 (1.7)	
2–3	38 (16.5)	268 (18.6)	
3–4	30 (13.0)	638 (44.2)	
4–5	19 (8.3)	497 (34.5)	
Gender of the child			
Boy	122 (53.0)	782 (54.2)	0.737
Girl	108 (47.0)	660 (45.8)	
Weight status of the child (aged 2–4 years old)			
Underweight	20 (22.7)	147 (11.6)	0.003
Normal	61 (69.3)	905 (71.4)	
Overweight/obesity	7 (8.0)	216 (17.0)	
Ever breastfed			
Yes	217 (94.3)	1210 (85.2)	
No	13 (5.7)	211 (14.8)	
Regular exercises			
Yes	117 (60.0)	861 (70.9)	0.002
No	78 (40.0)	353 (29.1)	
Illness during the past 4 weeks			
Yes	85 (37.3)	790 (55.4)	<0.001
No	143 (62.7)	636 (44.6)	
Dietary supplement use by child			
Yes	52 (22.6)	475 (32.4)	0.002
No	178 (77.4)	989 (67.6)	

\* The missing values vary for each variable in both countries.

A total of 22.6% of the Chinese children living in Perth were taking dietary supplements, including multi-vitamins/minerals, fish oil, protein, probiotics, colostrum, calcium, zinc and Vitamin AD (or cod liver oil) and Chinese herbs (Table 1). In Chengdu and Wuhan, China, 32.4% of young children were having dietary supplements, including multivitamins/minerals, calcium, zinc, iron, magnesium, fish oil, probiotics, Vitamin A and/or Vitamin D, Chinese herbs or other botanicals (Table 1). Compared to Chinese Australians, Chinese parents living in China were more likely to give their children dietary supplements ( $\chi^2 = 9.2$ ,  $df = 1$ ,  $p = 0.002$ ). However, in children aged over 12 months, there is no statistical difference in the prevalence of dietary supplements between Australia (28.6%) and China (32.7%,  $p = 0.284$ ). A higher percentage of children over three years old living in Australia were taking dietary supplements (40.8%) compared to children living in China (31.5%).

In China, the use of calcium supplements was very common among supplement users (58.5%). About half of the Chinese children taking calcium supplements were also taking Vitamin D ( $n = 140$ , including the use of multi-vitamins). In Australia, only four children were given specific

calcium supplements. The most common forms of supplemental calcium used in Chinese children up to five years old are gluconate (51.8%) and carbonate (37.5%). The dosage of calcium supplements ranged from 54–725 mg/day (Table 2). The average intake for calcium carbonate users (307.4 mg/day) is higher than gluconate calcium users (81 mg/day). When calculating the average intake, the intakes from multi-vitamins/minerals were also summed up if they were reported.

**Table 2.** Main dietary supplements used by Chinese children in Australia and China.

Supplement	Australia				China			
	<i>n</i>	% supplement users ( <i>n</i> = 52)	Average intake * (mg/day)	Intake range (mg/day)	<i>n</i>	% supplement users ( <i>n</i> = 475)	Average intake * (mg/day)	Intake range (mg/day)
Calcium	4	9.6	105 ( <i>n</i> = 5)	75–200	278	58.5	131.4 ( <i>n</i> = 264)	54–725
Zinc	1	1.9	3.1 ( <i>n</i> = 12)	1–7.5	192	40.4	4.4 ( <i>n</i> = 166)	1.62–8.6
Multi-vitamins/minerals	24	46.2	NA	NA	94	19.8	NA	NA
Vitamin A	4	7.7	1026 ** ( <i>n</i> = 7)	582.5–1617 **	83	17.5	1695 ** ( <i>n</i> = 71)	600–2800 **
Vitamin D	4	7.7	177 ** ( <i>n</i> = 5)	85–200 **	91	19.2	568 ** ( <i>n</i> = 75)	80–780 **
Vitamin C	10	19.2	62.1 ( <i>n</i> = 12)	20–125	33	6.9	61.4 ( <i>n</i> = 23)	30–200
Fish oil	22	42.3	859.6 ( <i>n</i> = 13)	300–1000	4	0.8	NA	NA
Probiotics	2	3.9	NA	NA	22	4.6	NA	NA
Herbs	4	7.7	NA	NA	51	10.7	NA	NA

\* When calculated the average intake, the intakes from multi-vitamins/minerals were also summed if they were reported; \*\* IU/day,

IU: international unit; NA: not available.

The prevalence of the use of zinc supplementation was also high in China. Nearly half of supplements users were using zinc supplements (40.4%). Almost all the zinc supplements were in the form of gluconate (93.2%) and the average intake of zinc was 4.4 mg/day (*n* = 166, range from 2.15–8.6 mg) (Table 2).

In Australia, the types most frequently used by supplement users were multi-vitamins/minerals (46.2%) and fish oil (42.3%). The average intake of fish oil was 859.6 mg per day (*n* = 13) with the range from 300 to 1000 mg per day (Table 2).

Chinese herbal supplements were used by children in both countries, especially in China, where 10.7% of supplements users were taking herb supplements (Table 2). Some herbal supplements were used for “better appetite” and some were believed to be beneficial to the immune system or to bring an improvement of health or well-being. In this study, traditional Chinese medicines including cinnabar, as arum, isatis root, kaladana, mangnolia officinalis, scaphium scaphigerum, coltsfoot, coptis chinensis and realgar were included as ingredients in children’s dietary supplements or medicines for (preventing) coughs or colds. Excluding dietary supplements, 7.6% of children in China reported taking medicine during the last two weeks and 82.9% (*n* = 92, 6.3% of all the samples) were taking herbal products for medical reasons, such as cough or upper respiratory tract infection. In China, a total of 16.1% of supplements users (8.6% of the total sample) were using herbal products as dietary supplements or medicine and 7.7% of supplement users (2.2% of the total sample) in Australia reported taking herbal products.

In 4–5 year old children in Australia, nearly half (47.4%) were taking at least one dietary supplement (Table 3). In Australia, older children ( $\chi^2 = 19.22$ ,  $df = 4$ ,  $p = 0.001$ ), children who were never breastfed ( $\chi^2 = 4.32$ ,  $df = 1$ ,  $p < 0.05$ ) and children who did regular physical exercises in pre-school or at home ( $\chi^2 = 10.88$ ,  $df = 2$ ,  $p = 0.001$ ) were more likely to take dietary supplements than other children. Mothers who had migrated from other Asian regions (including Hong Kong) were more likely to give their children dietary supplements than mothers from mainland China ( $\chi^2 = 4.47$ ,  $df = 1$ ,  $p < 0.05$ ) (Table 3).

**Table 3.** Dietary supplement use by children: demographic variables.

	Children used dietary supplements in Australia		Children used dietary supplements in China	
	<i>n</i> (%)	2-sided <i>p</i> -value	<i>n</i> (%)	2-sided <i>p</i> -value
Mothers Age (years)		0.201		0.551
<30	12 (17.6)		206 (34.4)	
≥30	40 (25.5)		171 (32.8)	
Education of the mother		0.283		0.942
<University	10 (17.5)		217 (33.1)	
≥University	42 (24.4)		163 (33.3)	
Working status		0.690		0.645
Working	25 (23.8)		321 (33.2)	
Not employed	27 (21.6)		62 (31.5)	
Household income		0.692		<0.001
Low	26 (24.1)		161 (28.1)	
High	24 (21.8)		186 (41.2)	
Mother's birth place		0.034		
Mainland China	37 (19.9)			
Other Asian regions	15 (34.9)			
Duration in Australia		0.160		
≤5	21 (17.6)			
5–10	21 (28.8)			
>10	9 (27.3)			
Gender of the child		0.868		0.201
Male	28 (23.1)		267 (34.2)	
Female	24 (22.2)		204 (31.1)	
Child's age (year)		0.001		0.427
<1 year	4 (6.6)		6 (40.0)	
1–2	20 (24.7)		8 (33.3)	
2–3	8 (21.1)		100 (37.5)	
3–4	11 (36.7)		203 (31.8)	
4–5	9 (47.4)		155 (31.2)	
Infant feeding		0.038		0.272
Ever breastfed	46 (21.3)		402 (33.2)	
Never breastfed	6 (46.2)		62 (29.4)	
Child's BMI		0.406		0.596
Underweight	4 (20.0)		45 (31.7)	
Normal	20 (33.9)		310 (34.3)	
Overweight or obesity	3 (42.9)		64 (31.1)	

**Table 3.** *Cont.*

Regular exercises		0.001		0.042
Yes	37 (31.6)		306 (35.5)	
No	9 (11.5)		104 (29.5)	
Illness during the past 4 weeks		0.208		0.008
Yes	27 (26.7)		354 (34.8)	
No	25 (19.7)		113 (27.6)	

In China, the prevalence of dietary supplement use was higher in children who had been sick during the past four weeks ( $\chi^2 = 6.97$ ,  $df = 1$ ,  $p < 0.01$ ) and children who had regular exercise ( $\chi^2 = 4.13$ ,  $df = 1$ ,  $p < 0.05$ ) than in their counterparts. Higher household income was significantly related to the use of child supplements ( $\chi^2 = 19.29$ ,  $df = 1$ ,  $p < 0.001$ ) (Table 3).

Mother's age, education level, working status, household income, the child's age, BMI, regular exercise, and "illness during the last month" were entered into a binary logistic regression model using backward elimination. After controlling for those potential confounding variables, the results of the binary logistic regression analysis showed that Chinese Australian mothers with higher education levels (OR = 2.51, 95% CI 1.19–5.27), older children (OR = 3.11, 95% CI 1.42–6.83), who were not employed (OR = 3.83, 95% CI 1.09–13.44), and never breastfed their children (OR = 6.75, 95% CI 1.29–35.31) were more likely to give their child dietary supplements. In China, higher household income (OR = 1.53, 95% CI 1.13–2.08) and "having illness during the past month" (OR = 1.44, 95% CI 1.05–1.97) were associated with dietary supplement use in children (Table 4).

**Table 4.** Odds ratios of factors for dietary supplement use in Chinese children in Australia and China.

	China		Australia	
	OR	95% CI	OR	95% CI
Household income			NS	
Low	1			
High	1.53	1.13–2.08		
Education of the mother	NS			
<University			1	
≥University			2.51	1.19–5.27
Working status	NS			
Working			1	
Not employed			3.83	1.09–13.4
Child age (year)			3.11	1.42–6.83
Breastfed	NS			
Yes			1	
Never			6.75	1.29–35.31
Illness during the past 4 weeks				
Yes	1			
No	1.44	1.05–1.97		

NS: not significant.

#### 4. Discussion

With the increasing prevalence of chronic disease throughout the world and increasing interest in complementary medicine, dietary supplements have become more widely used in children [26,27]. Many varieties of dietary supplements are now marketed in China and Australia, including single-ingredient products and various combinations of vitamins, minerals, botanicals, and other constituents. Their use in healthy children is addressed towards non-clinical deficiencies, the achievement of optimal status of nutrition and health [17,28].

This study investigated the prevalence of dietary supplement use in Chinese children in mainland China and in Australia. This is the first report, to our knowledge, on the use of dietary supplements in young Chinese children under the age of five years. In this study, one fifth of Chinese children in Perth and one third of children in Chengdu and Wuhan were taking at least one nutritional supplement with no gender differences. The prevalence of dietary supplement use in young children in China was similar to that of the US (35%) and South Korea (34%), but higher than Japan (20.4%) [14–17]. However, the comparison populations in these reports generally were older children. The lower prevalence of dietary supplement use in Chinese immigrant children in Australia than children in China may be due to the age difference of the subjects. In Australia, most children were under three years old. It was found that older children in Australia were more likely to take dietary supplements.

The types of supplements commonly used in Chinese children in China and in Australia were quite different. In China, calcium and zinc supplements were most commonly used, with many children taking both. Although 58.5% of supplements users were taking calcium supplementation, the average intake was still only 131 mg per day, which is about 20% of the Adequate Intake set for calcium for Chinese children in this age group [3]. It is less than half of the calcium that can be provided from one serve (250 mL) of milk; besides milk can provide other nutrients like protein to support child growth [29]. A meta-analysis on randomized, controlled trials reported little effectiveness of calcium supplementation on bone density in healthy children, either in childhood or later life [30]. The calcium dose was of 300–1200 mg per day in 19 studies included in the meta-analysis, which was much higher than the average calcium intake from supplements in this study (131 mg in China and 105 mg in Australia). Since the level of intake of calcium supplements in China is very low, it is not possible that intake from supplements would be likely to have a positive effect on bone mineral density in Chinese children.

It has been reported in many studies that Chinese children have a low daily zinc intake [31,32]. This may be due to the higher reference value used to define the adequate daily intake in those studies. The Recommended Nutrient Intakes (RNIs) for zinc for 1–7 years old Chinese children range from 9–13.5 mg/day, which are higher than in Japan (5–7 mg/day), USA (3–5 mg/day) and in Australia (3–4 mg/day) [3,29,33,34]. The recommended intake for Chinese children is even higher than the upper level of zinc intakes for those age groups in Australia and New Zealand, which is 7 mg/day for 1–3 years and 12 mg/day for 4–8 years [33]. The 2002 China National Nutrition and Health Survey found that the median intake of zinc in 2–8 year old Chinese children ranged from 5.1 to 7.1 mg/day (the interquartile range: 3.9–9.3 mg/day), which already met the RNIs for this age group in Japan, USA and Australia [35]. However, the adequacy of zinc intake depends not only on the amount, but also its bioavailability. People consuming a diet that provides marginal zinc intake may not absorb

an adequate amount of zinc if they are also consuming foods high in phytate together with high calcium [36]. The average population phytate intake of people in China (1186 mg/day) is relatively high compared to their western counterparts, but Chinese diets are low in calcium, reducing the possibility of low zinc availability [35]. The elevation of calcium intake by increasing consumption of milk is not affected by the inhibitory effect of phytate because animal sources of protein appear to promote zinc release from its phytate complex and also provides intrinsic zinc in a highly available form [36]. For young children from this study, their calcium intakes from calcium supplements were low and because of their young age, they still rely on milk products as their main calcium source. Considering the amount of zinc intake from their diet, they may not need to take zinc supplements. Together with the amount of zinc from supplements (ranging from 2.15 to 8.6 mg/day), it is a concern that some children might have reached the upper level of intakes for their age. Adverse events associated with chronic intake of supplemental zinc may include suppression of immune response, decrease in high density lipoprotein cholesterol and reduced copper status [33].

In Australia, the most popular supplements were multi-vitamins/minerals, which is consistent with previous studies in children and adolescents [16,17,37]. Fish oil supplements (42.3%) were almost as popular as multi-vitamins and minerals (46.2%). Another large sample size, cross-sectional study ( $n = 266,848$ ) undertaken in New South Wales, Australia also reported a high prevalence of fish oil supplement use in healthy elderly people [38]. Few children were on calcium supplements in Australia. This might be due to higher consumption of milk and milk products in Australia than in China. Commercial advertising may also influence the choice of dietary supplement.

The types of dietary supplements used by young children living in China were distinct from those in Australia. This may be due to the different regulations about supplements that apply to both countries. Promotion and advertising of supplements is different in both countries. There are many reports in the literature that suggest that unnecessary or reckless use of dietary supplements can lead to problems. More studies related to the clinical effectiveness and/or safety of dietary supplements in infants and children are required, especially over the longer term. In the case of Chinese children in China, the intakes of calcium and zinc deserve special considerations in relation to development of dietary supplement regulations. Further studies on fish oil supplements in young children in Australia are also required to add to our knowledge of its health effects.

Herbal products are widely used both in China and by Chinese Australians. Most herbal traditional products not only have plant-derived materials or preparations, but may also include animal products (including scorpions, cicadas and centipedes) and mineral compounds (including cinnabar and realgar) [39]. There is a public perception that these products are inherently safe, however, the therapeutic basis of many ingredients is still not clear. Some traditional ingredients can be toxic when used for inappropriate indications, or prepared inappropriately, or used in excessive dosages, or for a prolonged duration [39–42]. It is known that some Chinese medicines can have nephrotoxicity or hepatotoxicity effects and some cause increased risk of bleeding [43–46]. There is a need to increase the awareness of toxic effects of some herbal products in the public and health care professions.

There are several limitations that need to be considered when interpreting the results of the present study. First, our results may not be representative of all Chinese children in China or in Australia because of the location of the sample and the number of subjects. Secondly, the age distribution of the subjects from two countries in this study was slightly different and this may have a small influence

on the results. Nevertheless, we believe our present study to be important for understanding the present status of supplement use in Chinese pre-school children, and in monitoring future trends of supplement use.

## 5. Conclusions

It is important for pre-school children to meet their energy and nutrient needs for growth and development. Consuming a healthy diet is important to achieve adequate nutrient intakes. Dietary supplements only need to be considered when individuals are not able to obtain an adequate nutrient status from their diet alone. A large number of healthy Chinese children both in China and in Australia use dietary supplements, which for most may not be medically indicated.

Calcium and zinc are the two most popular dietary supplements in young children in China, while multi-vitamin/minerals and fish oil are the most frequently used in Australian Chinese children. However, the supplements used in China contain relatively low amounts of calcium and the same amount could easily be obtained from milk and other dairy products. For some other nutrients such as zinc, the potential over-nutrient of taking supplements should be of concern. There is also a need to increase the awareness of toxic effects of some herbal products in the public and health care professions.

There are many reports in the literature that suggest that unnecessary use of dietary supplements can lead to problems. Parents should exercise caution when giving their infants or young children dietary supplements and be aware of the potential toxicity of inappropriate use or excessive dosages. Before providing dietary supplements, parents should seek advice from appropriate health professionals. For all infants and young children, wherever possible, it is preferable to achieve nutrient intakes from a varied diet rather than from supplements.

## Conflicts of Interest

The authors declare no conflict of interest.

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## Dietary, Lifestyle and Socio-Economic Correlates of Overweight, Obesity and Central Adiposity in Lebanese Children and Adolescents

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**Abstract:** The Eastern Mediterranean region is characterized by one of the highest burdens of paediatric obesity worldwide. This study aims at examining dietary, lifestyle, and socio-economic correlates of overweight, obesity, and abdominal adiposity amongst children and adolescents in Lebanon, a country of the Eastern Mediterranean basin. A nationally representative cross-sectional survey was conducted on 6–19-year-old subjects ( $n = 868$ ). Socio-demographic, lifestyle, dietary, and anthropometric data (weight, height, waist circumference) were collected. Overweight and obesity were defined based on BMI  $z$ -scores. Elevated waist circumference (WC) and elevated waist to height ratio (WHtR) were used as indices of abdominal obesity. Of the study sample, 34.8% were overweight, 13.2% were obese, 14.0% had elevated WC, and 21.3% had elevated WHtR.

Multivariate logistic regression analyses showed that male gender, maternal employment, residence in the capital Beirut, sedentarity, and higher consumption of fast food and sugar sweetened beverages were associated with increased risk of obesity, overweight, and abdominal adiposity, while regular breakfast consumption, higher intakes of milk/dairies and added fats/oils were amongst the factors associated with decreased risk. The study's findings call for culture-specific intervention strategies for the promotion of physical activity, healthy lifestyle, and dietary practices amongst Lebanese children and adolescents.

**Keywords:** paediatric; obesity; abdominal adiposity; prevalence; correlates; diet; Lebanon; Eastern Mediterranean region

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## 1. Introduction

The Eastern Mediterranean region is characterized by one of the highest burdens of overweight and obesity worldwide [1]. Of more concern is the high level of childhood obesity in countries of the region, with approximately 10% of school-aged children being obese, an estimate that is projected to follow an escalating secular trend [2]. Paediatric obesity is associated with both immediate and longer-term risks to health [3]. Among the immediate risks are metabolic abnormalities including increased blood cholesterol, triglycerides and glucose levels, insulin resistance, metabolic syndrome, and hypertension [3–5]. Childhood obesity is also a strong risk factor for adult obesity and its consequences including type 2 diabetes, cardiovascular diseases (CVDs), and certain types of cancer, in addition to psychological disturbances, such as low self-esteem and depression [6,7].

Obesity-related comorbidities were found to be more closely associated with abdominal adiposity and visceral fat depots than with the amount of total body fat [8]. Consequently, the use of body fat distribution indices has been increasingly recommended, and particularly the use of waist circumference (WC) and waist to height ratio (WHtR). These simple and non-invasive indices were shown to correlate with visceral fat in children and to predict risk for obesity-related comorbidities beyond that predicted by Body Mass Index (BMI) alone [8–14]. Being a relatively age-independent measure, the use of WHtR for assessing central fatness in children has been recommended in paediatric primary care practice, as well as epidemiological studies [14–16]. In a cohort of almost 1500 Caucasian children aged 5 to 15 years, both WC and WtHR were able to identify children with the highest metabolic and cardiovascular risks among those who were overweight [13]. An extensive review by Huxley *et al.* (2010) concluded that measures of abdominal obesity including WC and WHtR, may be better than BMI in predicting CVD risk, although combining BMI with these measures may improve their discriminatory capability [17].

The high disease burden of childhood obesity highlights the need for rigorous investigations of its determinants, context-specific patterns and associated factors. Most of the studies investigating obesity correlates in youth have been conducted in high-income countries and, as such, findings may not be applicable to low and middle-income countries. Among the latter, the Middle-East has been largely under-represented, although the region has one of the highest rates of childhood obesity [2]. The present study aims at examining the prevalence and correlates of overweight, obesity and abdominal

adiposity in a nationally representative sample of children and adolescents, aged six years and above, in Lebanon. Gaining greater insight into factors that are associated with paediatric obesity could catalyze the development of effective interventions and policies aiming at curbing the obesity epidemic in Lebanon, orient further studies, and assist policy makers in implementing successful, culture specific childhood obesity prevention strategies in the region.

## **2. Materials and Methods**

### *2.1. Study Design and Subjects*

Data for the present study is drawn from a national cross-sectional survey that was conducted in 2009, in Lebanon, on subjects aged six years and above. The study sample was based on the sampling frame provided by the National Survey of Household Living Conditions, which was conducted by the Ministry of Social Affairs/Central Administration of Statistics in collaboration with United Nations Development Programme (UNDP) and which covered primary residences across the Lebanese territory [18]. Sample size calculation for the study was performed based on previously estimated prevalence rates for the main outcome of interest [19]. As such, a minimum of 751 participants were needed to estimate a prevalence of obesity of 4.8% in children and adolescents [19], allowing a power of 80% and a margin of error of 1.5% at 95% confidence interval (CI). Recruitment efforts targeted a sample with an age, sex and district distribution proportionate to that of the Lebanese population [18].

Lebanon is divided into six administrative regions referred to as “governorates”, which cover the totality of the country. Except for the governorate of Beirut, which is considered purely urban, the other governorates are essentially composed of rural regions inter-mixed with urban cities. In this study, the sample was drawn from randomly selected households, based on stratified cluster sampling: the strata were the Lebanese governorates, the clusters were selected further at the level of districts, urban and rural areas, and the housing units constituted the primary sampling units in the different districts of Lebanon. One adult from each household and one child/adolescent from every other household were selected from the household roster. Field-work was carried out between May 2008 and August 2009. The final sample consisted of 3636 subjects, including 939 children and adolescents aged 6 years and above [20]. Refusal rate at the household level was estimated at 10.7%, with the main reasons for refusal to participate in the survey being lack of time or disinterest in the study. The design and conduct of the survey was approved by the Institutional Review Board of the American University of Beirut, and informed consent from adults/parents and informed assent from children and adolescents were obtained prior to enrolment in the studies.

Socio-demographic and lifestyle data were collected from study participants using a multi-component questionnaire that was developed for the purpose of this study. Data collection was performed by trained nutritionists in the household setting through face to face interviews which lasted for approximately one hour. Quality control measures including training, pre-testing of the study instruments, equipment, and data collection procedure and field monitoring of data collection, were applied. Household and parental data were collected from the adult participant (mother or father) using a multicomponent questionnaire covering information on demographic, socioeconomic and lifestyle

characteristics, in addition to medical history and health seeking behavior. Data pertinent to the child/adolescent were collected using a child-specific questionnaire which enquired about sex, age, medical history, meal pattern, eating habits, dietary intake, physical activity, and sedentary time. For children aged less than 11 years old, data was obtained by proxy (typically the mother), while the interview was conducted directly with subjects aged 11 years and above.

## 2.2. Anthropometric Measurements

Anthropometric measurements were taken using standardized protocols [21] and calibrated equipment. Height and body weight were measured according to standard procedures, using a portable stadiometer (Holtain, Crymych, UK) and a Secacalibrated electronic weighing scale (Hamburg, Germany), respectively. Subjects were weighed to the nearest 0.1 kg in light indoor clothing and with bare feet or stockings. Height was measured without shoes and recorded to the nearest 0.5 cm. A calibrated plastic measuring tape was used to measure waist circumference at the level of the umbilicus to the nearest 0.1 cm, with the subject standing and after normal expiration. Anthropometric measurements were taken and recorded by trained nutritionists who were working in teams of two, the examiner and the recorder. All measurements were taken twice and the average of the 2 values was adopted.

## 2.3. Definitions of Overweight and Obesity

Body mass index (BMI) was calculated as the ratio of weight (kilograms) to the square of height (meters). Overweight and obesity were defined based on sex and age specific +1 and +2 BMI z-scores, respectively, according to the WHO new growth standards [22]. The WHO AnthroPlus software (WHO, Geneva, Switzerland) was used to calculate BMI z-score for each specific age and sex. To allow for comparisons with studies conducted in other countries, prevalence rates of overweight and obesity were also determined using the International Obesity Taskforce (IOTF) [23] and the US Centers for Disease Control and Prevention (CDC) 2000 criteria [24].

Elevated WC was defined based on the International Diabetes Federation (IDF) criteria [25], which recommend the use of:

- Adult cut-off values for subjects aged 16–19 years (WC > 94 cm for males and >80 cm for females).
- Cut-off value of WC  $\geq$  90th percentile for sex and age (or adult cut-offs if lower) for subjects aged 6 to 15 years old. As national WC percentiles are lacking in Lebanon, the WC percentiles for children and adolescents as developed by Fernandez *et al.* (2004) were used [26].

The WHtR index for abdominal obesity was calculated by dividing WC by height, both measured in centimetres [13]. The suggested cut-off point of  $\geq 0.5$  was used to identify children with elevated WHtR [13,14].

## 2.4. Dietary Intake and Physical Activity Assessment

Dietary intake was assessed using the multiple pass 24-h recall approach. Interviewers followed the 5 steps of the USDA multiple pass 24-h recall, which included (1) the quick list; (2) the forgotten foods list; (3) time and occasion at which foods were consumed; (4) the detail cycle; and (5) the final

probe review [27]. To assist subjects in assessing the portion/amount of food consumed, quantification tools, such as household measures and graduated food models, were used. 24-h recall data were converted to energy and nutrient intake using the Nutritionist IV software through a hand-coding procedure (*N*-squared Computing Nutritionist IV. Silverton, OR: *N*-squared Computing; 1995). The Nutritionist IV food database was expanded by adding analyses of traditional Lebanese foods and recipes.

Information on the weekly frequency of physical activity outside the school setting was assessed by means of a questionnaire that was developed for this study. Examples of activities proposed by the questionnaire included moderate intensity activities such as playground activities, brisk walking, dancing, bicycle riding, as well as higher intensity activities, such as ball games, jumping rope, active games involving running and chasing, and swimming. Based on the weekly frequency, individuals were classified into three levels of physical activity: Low (Never); Moderate (1–2 times/week) and High (>2 times/week).

### 2.5. Statistical Analysis

Descriptive statistics were performed and expressed as means and standard error (SE) for continuous variables (dietary variables) or as number of subjects and percentages for nominal variables (demographic, socio-economic, physical activity, meal pattern, and lifestyle variables). Crowding index was calculated as the total number of co-residents per household divided by the total number of rooms, excluding the kitchen and bathrooms. Prevalence of overweight (including obesity), obesity, elevated WC and elevated WHtR, expressed as percentage with 95% confidence interval (CI), were computed by gender and age groups (6–11-year-old children and 12–19-year-old adolescents). Independent *t*-test and chi-squared test were used to evaluate the differences between continuous and categorical variables, respectively.

Multivariate logistic regression analysis was carried out to examine the association of overweight, obesity, elevated WC and elevated WHtR as the dependent variables with baseline socio-demographic, lifestyle, and dietary characteristics as covariates. The associations between dependent and independent variables were analyzed according to two age groups: Children (6–11 years) and adolescents (12–19 years). All statistical calculations were carried out using the Statistical Analysis Package for Social Sciences, version 18.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as *p*-value <0.05.

## 3. Results

### 3.1. Study Sample

For the purpose of this study, subjects for whom dietary data were missing or incomplete were excluded (out of 939 subjects, 71 were excluded). Accordingly, the final study sample consisted of 868 subjects (439 boys and 429 girls), with a mean age of 13.06 years ( $\pm 3.91$ ) and a median age of 12.85 years. Of the study participants, 42.6% were 6–11 years old and 57.4% were 12–19 years old. The male to female ratio was of 1.02 with 50.6% boys and 49.4% females.

The proportion of parents who had attained high school level education and above was of 38.6% for fathers and 46.3% for mothers with no significant differences between age groups (Table 1).

A significantly higher proportion of working mothers was reported amongst 12–19-year-old adolescents (29.6%) compared to children (19.4%). Parental obesity (mother or father) was reported amongst 31% of the study population with no significant differences between age groups. The majority of subjects (81.7%) had a crowding index  $\geq 1$  persons/room (Table 1).

The proportions of subjects reporting a daily consumption of breakfast was significantly higher amongst children compared to adolescents (86.4% vs. 69.5% in adolescents) while a significantly higher proportion of adolescents reported eating outside home more than once per week (58.4% in adolescents compared to 44.4% in children) (Table 1). Similarly, sedentary time was significantly higher amongst 12–19-year-old adolescents compared to children ( $10.09 \pm 2.94$  vs.  $8.72 \pm 2.77$  h/day) while the proportion of subjects reporting high physical activity was significantly higher in children compared to adolescents (63.9% vs. 33.5%).

Mean weight ( $35.01 \pm 12.55$  in children vs.  $60.77 \pm 15.61$  kg in adolescents), mean height ( $135.63 \pm 12.26$  vs.  $164.17 \pm 10.07$  cm), mean BMI ( $18.53 \pm 3.99$  vs.  $22.32 \pm 4.37$  kg/m<sup>2</sup>) and mean WC ( $63.77 \pm 10.75$  vs.  $74.93 \pm 10.97$  cm) were all significantly higher in 12–19-year-old adolescents compared to children aged 6–11 years (Table 1).

**Table 1.** Socio-demographic, lifestyle and anthropometric characteristics of the study sample by age group, Lebanon ( $n = 868$ ).

Variables	Age Group (years)		Total <sup>(1)</sup>	<i>p</i> -Value <sup>(2)</sup>
	6–11 ( $n = 370$ )	12–19 ( $n = 498$ )	( $n = 868$ )	
<b>Socio-Demographic characteristics <i>n</i> (%)</b>				
<b>Gender</b>				
Male	191 (51.6)	248 (49.8)	439 (50.6)	0.595
Female	179 (48.4)	250 (50.2)	429 (49.4)	
<b>Governorates</b>				
Capital (Beirut)	26 (7.0)	36 (7.2)	62 (7.1)	0.909
Other governorates	344 (93.0)	462 (92.8)	806 (92.9)	
<b>Father's Education</b>				
Primary or less	105 (28.8)	156 (31.7)	261 (30.5)	0.571
Intermediate	112 (30.8)	153 (31.1)	265 (31.0)	
High school and above	147 (40.4)	183 (37.2)	330 (38.6)	
<b>Mother's Education</b>				
Primary or less	76 (23.0)	112 (26.3)	188 (24.9)	0.587
Intermediate	97 (29.4)	121 (28.4)	218 (28.8)	
High school and above	157 (47.6)	193 (45.3)	350 (46.3)	
<b>Mother's working status</b>				
Not working	291 (80.6)	350 (70.4)	641 (74.7)	0.001
Working	70 (19.4)	147 (29.6)	217 (25.3)	
<b>Parental Obesity <sup>(3)</sup></b>				
No	218 (71.5)	171 (66.0)	389 (69.0)	0.163
Yes	87 (28.5)	88 (34.0)	175 (31.0)	
<b>Crowding Index</b>				
<1 person/room	66 (18.0)	92 (18.5)	158 (18.3)	0.858
$\geq 1$ person/room	300 (82.0)	405 (81.5)	705 (81.7)	

Table 1. Cont.

Lifestyle characteristics <i>n</i> (%)				
<b>Breakfast consumption (per week)</b>				
Never	11 (3.0)	36 (7.2)	47 (5.4)	<0.001
Sometimes	39 (10.6)	116 (23.3)	155 (17.9)	
Daily	319 (86.4)	346 (69.5)	665 (76.7)	
<b>Frequency of eating outside home (per week)</b>				
≤1 time	205 (55.6)	207 (41.6)	412 (47.5)	<0.001
>1 time	164 (44.4)	291 (58.4)	455 (52.5)	
<b>Physical Activity <sup>(4)</sup></b>				
Low	80 (21.7)	193 (41.2)	273 (32.6)	<0.001
Moderate	53 (14.4)	119 (25.4)	172 (20.5)	
High	235 (63.9)	157 (33.5)	392 (46.8)	
<b>Sedentary time (h/day)</b> Mean ± SD	8.72 ± 2.77	10.09 ± 2.94	9.51 ± 2.95	<0.001
Anthropometric characteristics				
<b>Weight (kg)</b>				
Mean ± SD	35.01 ± 12.55	60.77 ± 15.61	49.81 ± 19.21	<0.001
10th percentile	22.15	42.67	25.88	
50th percentile	32.45	59.00	48.75	
90th percentile	50.81	80.81	74.52	
<b>Height (cm)</b>				
Mean ± SD	135.63 ± 12.26	164.17 ± 10.07	152.02 ± 17.93	<0.001
10th percentile	119.75	152.00	126.00	
50th percentile	136.00	164.00	154.00	
90th percentile	152.00	177.50	174.05	
<b>BMI (kg/m<sup>2</sup>)</b>				
Mean ± SD	18.53 ± 3.99	22.32 ± 4.37	20.71 ± 4.61	<0.001
10th percentile	14.65	17.47	15.60	
50th percentile	17.58	21.58	20.08	
90th percentile	23.56	28.17	26.80	
<b>WC (cm)</b>				
Mean ± SD	63.77 ± 10.75	74.93 ± 10.97	70.18 ± 12.19	<0.001
10th percentile	52.75	62.73	56.00	
50th percentile	61.50	73.20	69.00	
90th percentile	78.50	91.00	86.05	
<b>WHtR</b>				
Mean ± SD	0.47 ± 0.06	0.46 ± 0.06	0.46 ± 0.06	0.002
10th percentile	0.41	0.39	0.40	
50th percentile	0.46	0.44	0.45	
90th percentile	0.55	0.54	0.55	

<sup>(1)</sup> Lack of corresponding sum of frequencies with total sample size is due to missing data; <sup>(2)</sup> Differences between age groups were examined using *t*-test and chi-square test for continuous and categorical variables, respectively; <sup>(3)</sup> Total number of parents with anthropometric data was equal to 564; <sup>(4)</sup> The three categories of physical activity (Low, Moderate, High) refer to the frequency of physical activity outside the school setting (Never; 1–2 times/week; >2 times/week, respectively).

### 3.2. Prevalence of Overweight, Obesity and Abdominal Obesity

Taking both genders, 40.2% of 6–11-year-olds and 30.8% of 12–19-year-olds were found to be overweight (BMI  $z$  score  $>+1$ ), while 17.1% and 10.3% were found to be obese (BMI  $z$  score  $>+2$ ), respectively (Table 2). Gender-based differences were noted amongst 12–19-year-olds, with the prevalence of overweight (37.9% in boys vs. 23.7% in girls) and obesity (16.1% in boys vs. 4.4% in girls) being significantly higher in boys compared to girls. Similar gender-based differentials were noted in the total sample of 6–19-year-old subjects.

Based on WC as an indicator of central fatness, abdominal obesity was observed in 13.8% of 6–11-year-olds and 14.1% of 12–19-year-olds, with no significant differences between genders (Table 2). Elevated WHtR was observed in 22% and 20.9% of children and adolescents, respectively, with gender-based differences being observed amongst 12–19-year-old subjects (26.2% in boys vs. 15.6% in girls;  $p < 0.05$ ). Similar gender-based differentials were noted in the prevalence of elevated WHtR in the total sample of 6–19-year-old subjects (Table 2).

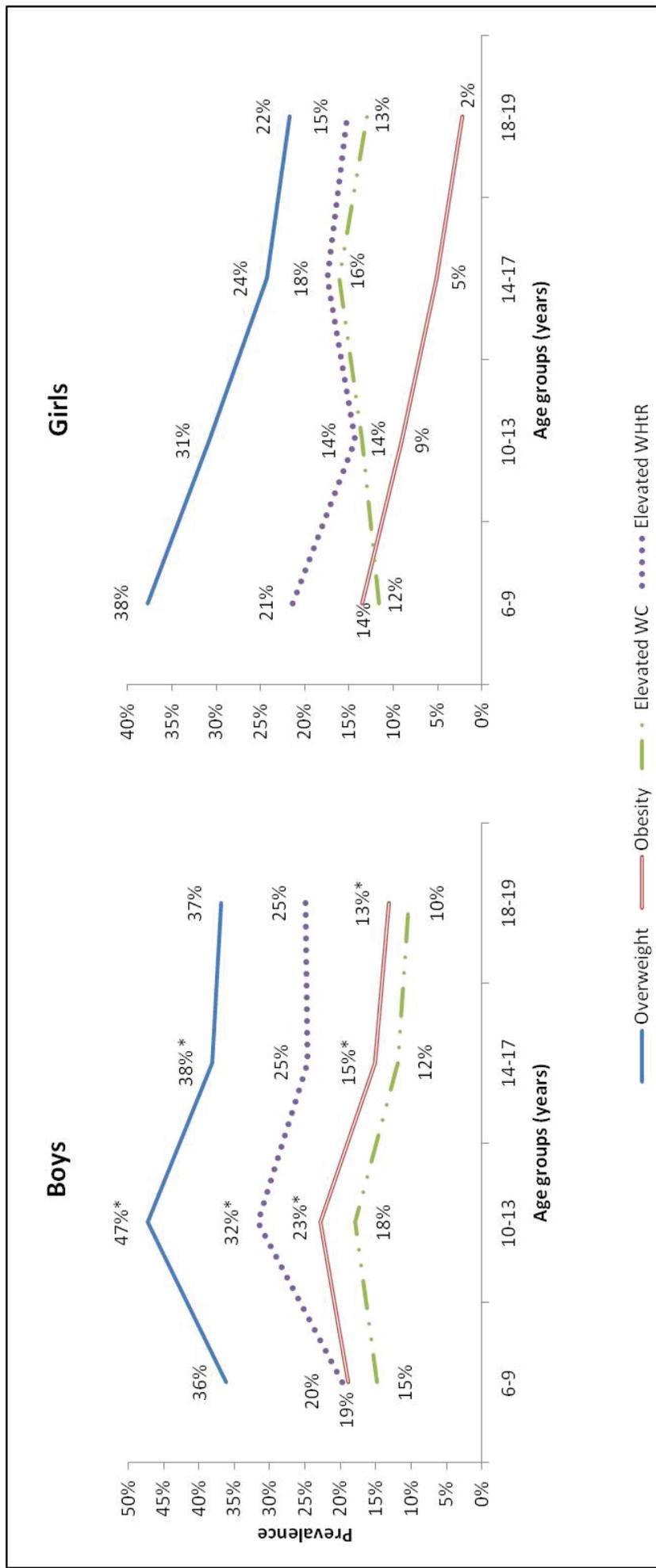
As shown in Figure 1, the prevalence of overweight, obesity, elevated WHtR and elevated WC amongst boys reached the highest rates at the age of 10–13 years (47%, 23%, 32% and 18%, respectively), while declining afterwards (Figure 1). Amongst girls, the prevalence of overweight and obesity was the highest at 6–9 years (38% and 14%, respectively) and followed a consistent declining trajectory with age. The prevalence of elevated WC reached its highest in girls aged between 14 and 17 years (16%) and declined afterwards (Figure 1).

**Table 2.** Prevalence of overweight, obesity and abdominal adiposity amongst Lebanese children and adolescents ( $n = 868$ ) by gender and by age group.

Variables	Age Groups (years)						Total		
	6–11			12–19			6–19		
	<i>n</i>	%	(95% CI)	<i>n</i>	%	(95% CI)	<i>n</i>	%	(95% CI)
<b>Male</b>									
Overweight <sup>(1)</sup>	81	42.4	(36–50)	94	37.9 <sup>a</sup>	(32–44)	175	39.9 <sup>a</sup>	(35–44)
Obesity <sup>(1)</sup>	39	20.4	(15–27)	40	16.1 <sup>b</sup>	(12–21)	79	18.0 <sup>b</sup>	(15–22)
Elevated WC <sup>(2)</sup>	30	15.7	(11–22)	32	12.9	(9–18)	62	14.1	(11–18)
Elevated WHtR <sup>(3)</sup>	46	24.1	(19–31)	65	26.2 <sup>c</sup>	(21–32)	111	25.3 <sup>c</sup>	(21–30)
<b>Female</b>									
Overweight <sup>(1)</sup>	67	37.9	(31–45)	59	23.7 <sup>a</sup>	(19–29)	126	29.6 <sup>a</sup>	(25–34)
Obesity <sup>(1)</sup>	24	13.6	(9–19)	11	4.4 <sup>b</sup>	(2–8)	35	8.2 <sup>b</sup>	(6–11)
Elevated WC <sup>(2)</sup>	21	11.8	(8–17)	38	15.2	(11–20)	59	13.8	(11–17)
Elevated WHtR <sup>(3)</sup>	35	19.7	(14–26)	39	15.6 <sup>c</sup>	(12–21)	74	17.3 <sup>c</sup>	(14–21)
<b>Both Genders</b>									
Overweight <sup>(1)</sup>	148	40.2	(35–45)	153	30.8	(27–35)	301	34.8	(32–38)
Obesity <sup>(1)</sup>	63	17.1	(14–21)	51	10.3	(8–13)	114	13.2	(11–16)
Elevated WC <sup>(2)</sup>	51	13.8	(10–17)	70	14.1	(11–17)	121	14.0	(11–16)
Elevated WHtR <sup>(3)</sup>	81	22.0	(18–27)	104	20.9	(17–24)	185	21.3	(18–24)

WC: Waist Circumference; WHtR: Waist to Height ratio; <sup>(1)</sup> Overweight and obesity defined based on sex and age specific +1 and +2 BMI  $z$ -scores, respectively [22]; <sup>(2)</sup> For subjects aged 6–15 years, abdominal obesity: WC  $>$  90th percentile [26] or adult cut-off value if lower [25]; For subjects aged 16–19 years, abdominal obesity: WC  $>$  94 cm for males and  $>$ 80 cm for females [25]; <sup>(3)</sup> Elevated WHtR defined as WHtR  $>$  0.5 [13]; <sup>a,b,c</sup> Within each age group, values with the same superscripts are significantly different by gender at  $p < 0.05$  (Using Chi-square test).

**Figure 1.** Prevalence of overweight, obesity, elevated WC and elevated WHtR amongst Lebanese children and adolescents ( $n = 868$ ) by age and gender (\* Significant difference by gender  $p < 0.05$ ).



### 3.3. Dietary Intake

As shown in Table 3, average energy intake (2255.85 vs. 1736.48 kcal/day) and percent contribution of fast food (17.27% vs. 11.35%) and legumes and nuts (3.28% vs. 2.05%) to daily energy intake were significantly higher among 12–19-year-old adolescents compared to 6–11-year-old children. On the other hand, the percent contribution of milk and dairies (8.90% vs. 6.47%) and breads and cereals (36.92% vs. 32.67%) were significantly higher in 6–11-year-old children compared to adolescents. No significant differences in macronutrient intake were observed between age groups.

**Table 3.** Energy, macronutrient and food group intake amongst Lebanese children and adolescents according to age group ( $n = 868$ ).

Dietary Variables	Age Group (years)				Total	
	6–11 ( $n = 370$ )		12–19 ( $n = 498$ )		6–19 ( $n = 868$ )	
Energy (kcal $\pm$ SE )	1736.48 $\pm$ 36.12 <sup>a</sup>		2255.85 $\pm$ 51.60 <sup>a</sup>		2033.70 $\pm$ 34.47	
	<b>Mean % Daily Energy Intake <math>\pm</math> SE</b>					
Carbohydrates	52.01 $\pm$ 0.51		51.05 $\pm$ 0.48		51.46 $\pm$ 0.35	
Protein	13.11 $\pm$ 0.18		13.53 $\pm$ 0.23		13.35 $\pm$ 0.15	
Fat	35.86 $\pm$ 0.47		36.24 $\pm$ 0.43		36.058 $\pm$ 0.32	
Breads and Cereals	36.92	$\pm$ 0.89 <sup>b</sup>	32.67	$\pm$ 0.77 <sup>b</sup>	34.49	$\pm$ 0.58
Milk and Dairies	8.90	$\pm$ 0.52 <sup>c</sup>	6.47	$\pm$ 0.37 <sup>c</sup>	7.51	$\pm$ 0.31
Meat and Equivalent	10.22	$\pm$ 0.57	10.15	$\pm$ 0.57	10.18	$\pm$ 0.40
Legumes and Nuts	2.05	$\pm$ 0.34 <sup>d</sup>	3.28	$\pm$ 0.38 <sup>d</sup>	2.76	$\pm$ 0.27
Fruits and Vegetables	5.35	$\pm$ 0.33	5.42	$\pm$ 0.33	5.39	$\pm$ 0.24
Added Fats and Oils	7.58	$\pm$ 0.47	8.42	$\pm$ 0.42	8.06	$\pm$ 0.31
Fast Food	11.35	$\pm$ 0.69 <sup>e</sup>	17.27	$\pm$ 0.92 <sup>e</sup>	14.74	$\pm$ 0.61
Sugar and Sweets	10.81	$\pm$ 0.63	9.65	$\pm$ 0.60	10.15	$\pm$ 0.44
Sugar Sweetened Beverages	6.52	$\pm$ 0.45	6.45	$\pm$ 0.32	6.48	$\pm$ 0.26

<sup>a,b,c,d,e</sup> Values with the same superscripts are significantly different by age group at  $p < 0.05$  (Using  $t$ -test).

### 3.4. Factors Associated with Overweigh, Obesity and Abdominal Obesity

Amongst 6–11-year-old children, results of the multivariate regression analysis showed that, as compared to subjects living in the capital Beirut, those residing in other governorates had significantly lower odds of being overweight (OR = 0.32; 95% CI: 0.1–0.98), obese (OR = 0.21; 95% CI: 0.06–0.71), and of having elevated WC (OR = 0.16; 95% CI: 0.04–0.56) (Table 4). Higher maternal education was associated with significantly higher odds of overweight (OR = 2.45; 95% CI: 1.13–5.31), while higher paternal education was associated with lower odds of obesity in this age group (OR = 0.32; 95% CI: 0.11–0.91). Maternal employment was shown to be associated with significantly higher odds of obesity (OR = 2.6; 95% CI: 1.18–5.70) and elevated WHtR (OR = 2.27; 95% CI: 1.19–4.33). In contrast, daily breakfast consumption was associated with significantly lower odds of overweight (OR = 0.2; 95% CI: 0.05–0.84) and obesity (OR = 0.07; 95% CI: 0.01–0.30); higher intakes of milk & dairies were associated with lower odds of elevated WC (OR = 0.35; 95% CI: 0.13–0.91), and higher intakes of added fats/oils were associated with lower odds of obesity

(OR = 0.30; 95% CI: 0.12–0.73), elevated WHtR (OR = 0.42; 95% CI: 0.20–0.88), and elevated WC (OR = 0.32; 95% CI: 0.12–0.88). High consumption of fast food was associated with a threefold increase in the risk of overweight in this age group (OR = 3.24; 95% CI: 1.21–8.69) (Table 4).

**Table 4.** Associations of socio-demographic, lifestyle and dietary factors with overweight, obesity, elevated waist to height ratio (WHtR), and elevated waist circumference (WC) in Lebanese 6–11-year-old children ( $n = 868$ ).

Variables	6–11 Years ( $n = 370$ )			
	Overweight <sup>1</sup>	Obesity <sup>1</sup>	Elevated WHtR	Elevated WC
	Odds Ratio [95% CI]			
<b>Socio-Demographic Factors</b>				
<b>Age (years)</b>	1.17 [1.00–1.36]	1.17 [0.91–1.37]	1.14 [0.97–1.34]	1.22 [0.96–1.54]
<b>Sex</b>				
Female	1.00	1.00	1.00	1.00
Male	1.17 [0.69–1.97]	1.92 [0.94–3.90]	1.47 [0.84–2.56]	2.01 [0.90–4.48]
<b>Place of Residence</b>				
Beirut (Capital)	1.00	1.00	1.00	1.00
Other Governorates	0.32 [0.10–0.98]	0.21 [0.06–0.71]	0.39 [0.14–1.05]	0.16 [0.04–0.56]
<b>Father's Education<sup>2</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	1.14 [0.57–2.29]	0.83 [0.35–1.96]	0.98 [0.46–2.07]	1.12 [0.43–2.92]
High	0.52 [0.24–1.11]	0.32 [0.11–0.91]	0.73 [0.33–1.62]	0.46 [0.14–1.48]
<b>Mother's Education<sup>2</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	1.98 [0.90–4.33]	1.59 [0.60–4.18]	0.66 [0.30–1.47]	0.68 [0.23–1.95]
High	2.45 [1.13–5.31]	1.36 [0.52–3.59]	0.68 [0.31–1.45]	0.80 [0.29–2.20]
<b>Mother's Working Status</b>				
Not Working	1.00	1.00	1.00	1.00
Working	1.06 [0.55–2.03]	2.60 [1.18–5.70]	2.27 [1.19–4.33]	1.47 [0.60–3.63]
<b>Crowding Index</b>				
<1 person/room	1.00	1.00	1.00	1.00
≥1 person/room	1.19 [0.59–2.40]	1.04 [0.40–2.70]	0.64 [0.31–1.31]	0.53 [0.20–1.40]
<b>Parental Obesity</b>				
No	1.00	1.00	1.00	1.00
Yes	1.72 [0.97–3.04]	2.67 [1.34–5.31]	2.10 [1.18–3.72]	2.46 [1.15–5.23]
<b>Lifestyle and Dietary Factors</b>				
<b>Physical Activity<sup>3</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	1.60 [0.91–2.81]	1.78 [0.90–3.52]	0.99 [0.52–1.86]	1.43 [0.71–2.89]
High	0.86 [0.56–1.33]	0.75 [0.42–1.33]	0.70 [0.43–1.14]	0.68 [0.38–1.22]
<b>Sedentary Time (h/day)</b>	1.02 [0.93–1.12]	1.10 [0.97–1.25]	1.05 [0.93–1.19]	1.08 [0.94–1.25]
<b>Daily Breakfast Consumption</b>				
No	1.00	1.00	1.00	1.00
Yes	0.20 [0.05–0.84]	0.07 [0.01–0.30]	0.32 [0.07–1.32]	0.25 [0.05–1.25]

Table 4. *Cont.*

<b>Frequency of Eating Out</b>				
≤1 time/week	1.00	1.00	1.00	1.00
>1 time/week	1.01 [0.62–1.65]	0.96 [0.48–1.93]	1.18 [0.65–2.14]	1.75 [0.82–3.70]
<b>Total Daily Energy Intake (kcal) <sup>2</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	1.21 [0.70–2.10]	1.19 [0.54–2.65]	1.43 [0.73–2.78]	1.13 [0.48–2.66]
High	1.44 [0.71–2.90]	1.10 [0.52–3.74]	1.13 [0.47–2.71]	1.54 [0.54–4.43]
<b>Bread and Cereals <sup>4</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	1.19 [0.67–2.12]	0.76 [0.32–1.79]	0.72 [0.35–1.46]	0.51 [0.20–1.32]
High	1.01 [0.53–1.86]	1.36 [0.59–3.14]	0.97 [0.46–2.01]	1.13 [0.46–2.73]
<b>Milk and Dairies <sup>4</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	0.92 [0.50–1.69]	0.99 [0.43–2.30]	1.07 [0.52–2.22]	0.53 [0.21–1.32]
High	1.16 [0.62–2.16]	0.64 [0.26–1.56]	0.66 [0.30–1.43]	0.35 [0.13–0.91]
<b>Meat and Equivalent <sup>4</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	0.86 [0.48–1.53]	1.19 [0.54–2.61]	1.24 [0.63–2.44]	1.38 [0.56–3.18]
High	0.71 [0.34–1.47]	0.64 [0.21–1.94]	1.32 [0.53–3.29]	1.26 [0.38–4.17]
<b>Legumes and Nuts <sup>4</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	1.08 [0.66–1.76]	0.65 [0.34–1.23]	0.74 [0.41–1.33]	0.86 [0.42–1.74]
High	0.90 [0.51–1.58]	0.68 [0.33–1.42]	0.93 [0.49–1.78]	1.17 [0.55–2.51]
<b>Fruits and Vegetables <sup>4</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	1.55 [0.86–2.79]	2.24 [0.99–5.08]	1.08 [0.49–2.37]	2.01 [0.83–4.86]
High	0.91 [0.50–1.67]	1.11 [0.45–2.69]	0.99 [0.48–2.03]	1.11 [0.42–2.90]
<b>Added Fats and Oils <sup>4</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	0.73 [0.40–1.32]	0.36 [0.17–0.86]	0.42 [0.20–0.88]	0.79 [0.32–1.93]
High	0.64 [0.34–1.18]	0.30 [0.12–0.73]	0.53 [0.26–1.08]	0.32 [0.12–0.88]
<b>Fast Foods <sup>4</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	2.14 [0.91–5.02]	2.41 [0.70–8.23]	1.62 [0.51–5.10]	2.86 [0.64–12.64]
High	3.24 [1.21–8.69]	1.50 [0.43–5.23]	2.46 [0.79–7.67]	1.50 [0.37–6.01]
<b>Sugar and Sweets <sup>4</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	1.04 [0.57–1.90]	0.86 [0.37–1.92]	1.03 [0.50–2.14]	1.01 [0.39–2.59]
High	1.12 [0.61–2.06]	0.76 [0.33–1.75]	1.17 [0.57–2.43]	1.59 [0.65–3.88]
<b>Sugar Sweetened Beverages <sup>4</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	1.13 [0.67–1.90]	0.66 [0.34–1.28]	0.53 [0.22–1.28]	0.57 [0.22–1.17]
High	1.32 [0.79–2.22]	0.59 [0.29–1.17]	0.81 [0.45–1.45]	0.54 [0.20–1.13]

<sup>1</sup> Overweight and obesity defined based on sex and age specific +1 and +2 BMI z-scores, respectively [22]; <sup>2</sup> Low, medium and high education levels refer to primary or less, intermediate or high school and above, respectively; <sup>3</sup> The three categories of physical activity (Low, Moderate, High) refer to the frequency of physical activity outside the school setting (Never, 1–2 times/week; >2 times/week); <sup>4</sup> Food groups' intake based on percent contribution to daily energy intake. Low, medium, and high refer to first, second, and third tertiles, respectively.

Amongst 12–19-year-old adolescents, male gender was associated with significantly higher odds of obesity (OR = 5.18; 95% CI: 1.76–15.28) and elevated WHtR (OR = 1.82; 95% CI: 1.12–2.97) (Table 5). Similar to findings amongst 6–11-year-old children, significantly lower odds of overweight were observed amongst adolescents residing in other governorates as compared to those living in the capital Beirut (OR = 0.40; 95% CI: 0.19–0.83). Parental obesity was associated with approximately a 3-fold increase in the odds of overweight (OR = 3.01; 95% CI: 1.61–5.63), obesity (OR = 2.93; 95% CI: 1.09–7.86), and elevated WHtR (OR = 2.87; 95% CI: 1.55–5.30). A borderline significant association between high physical activity and lower odds of overweight (OR = 0.62; 95% CI: 0.33–1.05) and central fatness as assessed by WHtR (OR = 0.53; 95% CI: 0.26–1.09) was also observed. Sedentary time was significantly positively associated with all adiposity indicators amongst 12–19-year-old adolescents with higher odds of overweight (OR = 1.12; 95% CI: 1.03–1.21), obesity (OR = 1.2; 95% CI: 1.06–1.35), elevated WHtR (OR = 1.27; 95% CI: 1.13–1.43), and elevated WC (OR = 1.10; 95% CI: 1.01–1.22) being observed. Higher intakes of milk and dairies were associated with significantly lower odds of overweight (OR = 0.56; 95% CI: 0.32–0.98) in this age group. In contrast, higher intakes of sugar sweetened beverages were associated with significantly higher odds of overweight (OR = 2.49; 95% CI: 1.5–4.12) and elevated WHtR (OR = 1.77; 95% CI: 1.02–3.07). A borderline significant association was found between the consumption of fruits and vegetables and lower odds of elevated WC (OR = 0.46; 95% CI: 0.21–1.00) (Table 5).

**Table 5.** Associations of socio-demographic, lifestyle and dietary factors with overweight, obesity, elevated WHtR and elevated WC in Lebanese 12–19-year-old adolescents.

Variables	12–19 Years (n = 498)			
	Overweight <sup>1</sup>	Obesity <sup>1</sup>	Elevated WHtR	Elevated WC
	Odds ratio [95%CI]			
<b>Socio-Demographic Factors</b>				
<b>Age (years)</b>	0.99 [0.83–1.18]	0.91 [0.69–1.20]	0.99 [0.89–1.10]	0.95 [0.77–1.17]
<b>Sex</b>				
Female	1.00	1.00	1.00	1.00
Male	1.68 [0.92–3.07]	5.18 [1.76–15.28]	1.82 [1.12–2.97]	0.75 [0.35–1.58]
<b>Place of Residence</b>				
Beirut (Capital)	1.00	1.00	1.00	1.00
Other Governorates	0.40 [0.19–0.83]	0.54 [0.14–2.06]	1.18 [0.47–2.96]	1.00 [0.29–3.44]
<b>Father's Education<sup>2</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	1.53 [0.67–3.50]	1.26 [0.30–5.22]	1.70 [0.87–3.31]	1.14 [0.38–3.42]
High	1.47 [0.63–3.42]	2.20 [0.56–8.64]	1.83 [0.90–3.72]	2.13 [0.75–6.08]
<b>Mother's Education<sup>2</sup></b>				
Low	1.00	1.00	1.00	1.00
Medium	0.84 [0.33–2.09]	1.33 [0.26–6.86]	0.67 [0.33–1.36]	0.48 [0.15–1.55]
High	1.30 [0.54–3.12]	2.12 [0.50–8.85]	0.73 [0.36–1.45]	0.73 [0.25–2.13]

Table 5. Cont.

<b>Mother's Working Status</b>				
Not Working	1.00	1.00	1.00	1.00
Working	1.50 [0.69–3.27]	0.47 [0.10–2.07]	1.10 [0.60–2.02]	1.11 [0.43–2.86]
<b>Crowding Index</b>				
<1 person/room	1.00	1.00	1.00	1.00
≥1 person/room	1.74 [0.69–4.40]	0.96 [0.25–3.65]	0.74 [0.39–1.41]	0.73 [0.28–1.95]
<b>Parental Obesity</b>				
No	1.00	1.00	1.00	1.00
Yes	3.01 [1.61–5.63]	2.93 [1.09–7.86]	2.87 [1.55–5.30]	1.74 [0.81–3.72]
	<b>Lifestyle and Dietary Factors</b>			
<b>Physical Activity</b> <sup>3</sup>				
Low	1.00	1.00	1.00	1.00
Medium	0.79 [0.44–1.39]	0.89 [0.37–2.12]	0.75 [0.39–1.43]	0.77 [0.35–1.69]
High	0.62 [0.33–1.05]	0.43 [0.13–1.33]	0.53 [0.26–1.09]	0.53 [0.21–1.31]
<b>Sedentary time (h/day)</b>	1.12 [1.03–1.21]	1.20 [1.06–1.35]	1.27 [1.13–1.43]	1.10 [1.01–1.22]
<b>Daily Breakfast Consumption</b>				
No	1.00	1.00	1.00	1.00
Yes	0.62 [0.28–1.40]	1.11 [0.28–4.41]	0.58 [0.24–1.37]	0.72 [0.27–1.88]
<b>Frequency of Eating Out</b>				
≤1 time/week	1.00	1.00	1.00	1.00
>1 time/week	0.74 [0.47–1.17]	0.87 [0.42–1.79]	0.87 [0.52–1.45]	1.09 [0.60–1.97]
<b>Total Daily Energy Intake (Kcal)</b> <sup>2</sup>				
Low	1.00	1.00	1.00	1.00
Medium	1.05 [0.56–1.96]	0.62 [0.23–1.69]	0.67 [0.33–1.36]	0.62 [0.28–1.39]
High	0.80 [0.42–1.54]	0.85 [0.31–2.31]	0.75 [0.37–1.52]	0.91 [0.41–2.04]
<b>Bread and Cereals</b> <sup>4</sup>				
Low	1.00	1.00	1.00	1.00
Medium	1.18 [0.69–2.02]	1.71 [0.75–3.92]	1.21 [0.66–2.23]	1.21 [0.61–2.39]
High	0.57 [0.32–1.02]	1.07 [0.43–2.65]	0.79 [0.41–1.50]	0.76 [0.36–1.62]
<b>Milk and Dairies</b> <sup>4</sup>				
Low	1.00	1.00	1.00	1.00
Medium	1.04 [0.61–1.78]	0.79 [0.36–1.75]	1.26 [0.69–2.32]	1.39 [0.70–2.75]
High	0.56 [0.32–0.98]	0.50 [0.21–1.20]	1.17 [0.64–2.15]	1.04 [0.51–2.12]
<b>Meat and equivalent</b> <sup>4</sup>				
Low	1.00	1.00	1.00	1.00
Medium	0.78 [0.44–1.39]	0.89 [0.35–2.27]	0.94 [0.49–1.79]	1.38 [0.64–2.95]
High	1.12 [0.63–1.99]	1.01 [0.39–2.57]	1.01 [0.52–1.93]	1.45 [0.66–3.14]

Table 5. Cont.

<b>Legumes and Nuts</b> <sup>4</sup>				
Low	1.00	1.00	1.00	1.00
Medium	0.66 [0.39–1.12]	1.59 [0.76–3.31]	1.34 [0.76–2.46]	1.28 [0.64–2.54]
High	0.97 [0.61–1.53]	0.81 [0.38–1.75]	1.33 [0.77–2.30]	1.25 [0.66–2.36]
<b>Fruits and Vegetables</b> <sup>4</sup>				
Low	1.00	1.00	1.00	1.00
Medium	0.87 [0.50–1.54]	0.73 [0.30–1.73]	0.62 [0.29–1.33]	0.46 [0.21–1.00]
High	0.69 [0.39–1.21]	0.87 [0.36–2.06]	0.61 [0.30–1.24]	0.60 [0.29–1.21]
<b>Added Fats and Oils</b> <sup>4</sup>				
Low	1.00	1.00	1.00	1.00
Medium	1.39 [0.79–2.45]	0.67 [0.28–1.57]	0.87 [0.46–1.65]	0.96 [0.45–2.03]
High	1.19 [0.67–2.13]	0.92 [0.39–2.18]	1.31 [0.70–2.45]	1.63 [0.79–3.35]
<b>Fast Foods</b> <sup>4</sup>				
Low	1.00	1.00	1.00	1.00
Medium	0.92 [0.31–2.73]	0.89 [0.25–1.31]	1.07 [0.44–2.59]	1.64 [0.59–4.57]
High	1.40 [0.53–3.66]	1.34 [0.45–4.03]	1.46 [0.67–3.16]	2.05 [0.83–5.08]
<b>Sugar and Sweets</b> <sup>4</sup>				
Low	1.00	1.00	1.00	1.00
Medium	0.72 [0.41–1.27]	0.82 [0.35–1.88]	0.82 [0.44–1.53]	0.50 [0.25–1.17]
High	0.82 [0.47–1.42]	0.75 [0.32–1.73]	0.82 [0.44–1.53]	0.72 [0.39–1.31]
<b>Sugar Sweetened Beverages</b> <sup>4</sup>				
Low	1.00	1.00	1.00	1.00
Medium	2.49 [1.50–4.12]	1.74 [0.78–3.88]	1.77 [1.02–3.07]	1.62 [0.83–3.13]
High	1.36 [0.80–2.29]	1.69 [0.76–3.77]	0.94 [0.52–1.70]	1.19 [0.60–2.37]

<sup>1</sup> Overweight and obesity defined based on sex and age specific +1 and +2 BMI z-scores, respectively [22];

<sup>2</sup> Low, medium and high education levels refer to primary or less, intermediate or high school and above, respectively;

<sup>3</sup> The three categories of physical activity (Low, Moderate, High) refer to the frequency of physical activity outside the school setting (Never, 1–2 times/week; >2 times/week); <sup>4</sup> Food groups' intake based on percent contribution to daily energy intake. Low, medium and high refer to first, second, and third tertiles, respectively.

#### 4. Discussion

Based on a nationally representative survey, this paper reports on the prevalence of overweight, obesity, and abdominal adiposity in Lebanese children and adolescents and provides evidence linking specific dietary, lifestyle, and socioeconomic factors to increased risk of adiposity in this population group. Recognizing that the development of successful obesity prevention strategies should rely on evidence-based public health approaches, the results of this paper could represent a stepping stone for the formulation of effective interventions and policies aiming at curbing the epidemic of obesity in Lebanese youth.

The findings of the present study indicate high prevalence rates of overweight and obesity amongst Lebanese children and adolescents. Using the WHO 2007 BMI criteria, it was found that more than third of 6–19-year-old children and adolescents (34.8%) are overweight (BMI z score >+1), with about one in seven (13.2%) being obese (BMI z score >+2). To allow comparison with findings reported

from selected countries in the region and worldwide, data were re-analyzed according to IOTF and CDC criteria. Based on the IOTF criteria, current prevalence rates of obesity amongst children and adolescents in Lebanon (9.6%) are comparable to those reported from Bahrain (11.3%) [28] and Syria (11.1%) [29], higher than those observed in Qatar (6.3%) [30], while being lower than those reported from the UAE (13.7%) [31]. Based on the CDC 2000 definition, the prevalence of obesity in Lebanese youth (12.6%) appears lower than that reported from the US (18.7%) [32], while being considerably higher than estimates reported from Iran (1.8%) [33] and Saudi-Arabia (5.7%) [34]. When the results of the present study are compared to those provided by the first national survey conducted in 1997 in Lebanon [19,20], an approximate two-fold increase in the prevalence of obesity in 6–19-year-old Lebanese children is noted (7.3% in 1997 vs. 13.2% in 2009, based on WHO 2007 criteria). As such, the observed annual increase (+6.7%) in the prevalence of child obesity in Lebanon exceeds the estimated annual increase in the EMRO region (+5.6%), as determined by Wang and Lobstein (2006) [2]. The prevalence of abdominal adiposity in Lebanese youth has followed a parallel increasing trend between 1997 and 2009, with elevated WC rates increasing from 8.5 to 14% and elevated WHtR rates increasing from 19.1 to 21.3% amongst 6–19-year-old children. Current prevalence rates of abdominal obesity as assessed by WHtR (21.3%) were found to exceed those reported from several other countries including Germany (10.7% in boys and 8% in girls) [35] and Pakistan (16.5%) [16] while being lower than those reported from Italy (29.5%) [36]. The prevalence rates of elevated WC (14%) were similar to those reported from Pakistan (13%) [16] and Germany (17.3%) [37], but were lower than estimates reported from Italy (29%) [36].

Gender differentials in the prevalence of obesity and central fatness were noted in 12–19-year-old adolescents, with the odds of obesity being five times higher in boys compared to girls. Adolescent boys were also approximately two times more likely to be abdominally obese compared to girls based on the WHtR indicator. The higher prevalence of obesity amongst boys in this age group, is in line with previous reports from other countries in the region such as Syria, Qatar, Saudi-Arabia, and Greece [29,30,38,39], and with previous studies conducted in Lebanon [19,40]. This may possibly be resulting from stronger cultural and social pressure on adolescent girls to maintain an acceptable body image in this age group [19]. Gender differentials may also be explained by differences in dietary patterns and food choices. In this study, adolescent boys had a significantly higher intake of fast food, sugar sweetened beverages, and breads and cereals, while having significantly lower intakes of fruits and vegetables compared to girls (data not shown).

Our finding of a positive significant association between paediatric adiposity and parental obesity corroborates those reported from other studies and underscores the importance of genetic factors in the aetiology of body fatness [29,41]. However, strong evidence also suggests that childhood obesity is linked to socio-economic development, changes in environmental factors, such as living and school environments, diet, and physical activity patterns [2]. In the present study, specific dietary habits and food choices were associated with the risk of adiposity in the study sample. In 6–11-year-old children, and in line with several previous studies [42–44], regular breakfast consumption was associated with a significantly lower risk of overweight and obesity. Although mechanisms linking breakfast consumption to lower body weight are unclear, several possible explanations may exist [42]. Skipping breakfast may lead to excess hunger, rebound overeating [42], and consumption of larger portion sizes [45] and higher amounts of discretionary calories at subsequent meals [42]. Breakfast consumption may also be

associated with the selection of more healthful food choices [46], more regular eating habits and increased frequency of eating meals, which is suggested to reduce the efficiency of utilization of metabolizable energy and promote diet-induced thermogenesis and energy expenditure [42,47].

In agreement with previous reports [48,49], fast food consumption was associated with a threefold increase in the risk of overweight amongst 6–11-year-old children. Fast food's poor nutritional quality [50,51] and higher content of fat and saturated fat [52] underline their potential role as contributors to childhood adiposity and weight gain. Previous studies have shown that compared with non-consumers, children who consume fast food were found to have higher total energy, total fat, and saturated fat intakes [53] and higher obesity risk, while having lower intakes of fiber, milk, fruit, vegetables and fiber [48,49,53,54]. Contrary to the observed association between fast food and adiposity in the study sample, the intake of milk and dairy products was found to be associated with lower odds of abdominal adiposity in this age group (6–11-year-old children). In agreement with our findings, several observational studies have illustrated inverse associations between dairy intake and adiposity in children, while suggesting a role for dairy protein in the regulation of body weight [55,56]. Other studies have found that dietary calcium intake, especially from dairy products, may have a protective effect against overweight and obesity [57,58]. Based on a retrospective analysis of several studies, Heaney *et al.* (2002) proposed that a daily increase of 300 mg of calcium, or approximately 1 dairy serving, was associated with a yearly reduction of approximately 1 kg of body fat in children [59]. It is hypothesized that the relationship between calcium and body weight may be mediated by the lower intracellular calcium levels resulting from high calcium intakes, which reduce lipogenesis while increasing lipolysis and decreasing adiposity [60]. Surprisingly, the intake of “added fats and oils” was found to be associated with a protective effect against obesity and abdominal adiposity in 6–11-year-old children. When looking at the types of fats and oils included in this food group, olive oil was found to contribute 78% of added fats and oils, on a weight basis. Monounsaturated fats (MUFAs) and olive oil, which represent one of the distinctive properties of the Mediterranean diet, was suggested to reduce the risk of obesity in childhood [61]. In a one-year longitudinal study conducted on 13–166-month-old children, the risk of weight gain was significantly lower in children who consumed olive oil compared to those who did not [61]. MUFAs may act on the regulation of appetite, on the intestinal absorption of fat, on the lipolytic activity of the adipocyte and on thermogenesis, among other functions and therefore may contribute to the regulation of body weight [61–64].

Amongst 12–19-year-old adolescents, and similarly to the findings documented in 6–11-year-old children, higher intakes of milk and dairy products were associated with lower odds of adiposity. In addition, a positive association was documented between higher consumption of sugar-sweetened beverages and a higher risk of overweight and elevated WHtR amongst adolescents. This is in agreement with findings reported from large cross-sectional studies and several well-powered prospective cohort studies [65], which document a positive association between greater intakes of sugar sweetened beverages and obesity in children. A recent meta-analysis of cohort studies found that a higher intake of sugar-sweetened beverages among children was associated with 55% (95% CI 32%–82%) higher risk of being overweight or obese compared to lower intakes [66]. The high added sugar content, low satiety and the resulting incomplete compensation of energy at subsequent meals are likely mechanism by which sugar-sweetened beverages may lead to weight gain [67].

Through combined effects on energy balance, physical activity and sedentary time were suggested as two important and distinct modulators of obesity risk in children and adolescents [68]. In the present study, a borderline significant association was documented between high physical activity and lower odds of overweight and central fatness in adolescents. Similarly, sedentary time was associated with significantly higher odds of overweight, obesity and abdominal adiposity (elevated WC and WHtR) in the same age group. It is suggested that adolescents usually become more interested in screen-time activities such as computer games or watching TV than their younger peers, and, hence, are more prone to engage in sedentary behaviors [69]. When compared to the findings of the previous national survey conducted in 1997 in Lebanon [19], sedentary behavior among Lebanese children and adolescents (defined as  $\geq 10$  h sedentary time per day) was found to increase from 19.9% in 1997 to 60.5% in 2009, a finding that may mirror the increased reliance of youth on TV and telecommunication technology. Similarly, regression analyses showed that the risk of overweight/obesity and abdominal obesity was higher in children and adolescents living in the capital Beirut as compared to their counterparts residing in other governorates. Beirut, as a city, is characterized by a complete lack of safe greens and public spaces, such as gardens, parks, playgrounds and sports fields which may have direct repercussions on the lifestyle of children and adolescents such as decreased physical activity, increased screen time and television watching and consequently sedentary behavior [6]. In a European sample of 766 children, aged 10 to 12 years, engagement in more moderate to vigorous physical activity and spending less sedentary time were associated with a more favorable weight status in the study sample [68].

The results of this study document significant associations between certain parental socioeconomic characteristics and adiposity amongst 6–11-year-old children, but not amongst adolescents. An inverse association between fathers' education level and child obesity was documented. This finding is in disagreement with that reported from several developing countries [29,70] where a positive association between paediatric obesity and higher parental education was documented. However, our findings are in agreement with those reported from developed countries [71–73]. A study conducted in Italy among 8- to 9-year-old children showed that the prevalence of paediatric obesity was inversely related to the educational level of fathers, thus highlighting the role of paternal education in modulating the family's lifestyle, economic and cultural resources, all of which may bear ramifications on nutritional and behavioral choices and therefore obesity risk in childhood [73]. In contrast, and in agreement with findings reported from various developing countries [29,70], higher maternal education was found to be associated with significantly higher odds of overweight amongst 6–11-year-old children. This finding may be a reflection of the association between maternal employment and adiposity in children as the likelihood of employment of the mother increases as her education level increases. In the 6–11-year-old study sample, children with working mothers were found to carry more than a two-fold increase in the risk of obesity and abdominal obesity (elevated WHtR) compared to their counterparts. Maternal employment may in fact be one of the modulators of the family environment, which can have a direct influence on children's lifestyles, physical activity, and eating habits [74]. A recent longitudinal study in the UK showed that children with working mothers were more likely to be overweight or obese than those of non-working mothers, and children's likelihood of being overweight or obese increased with the mother's working time [72].

The results of this study should be considered in light of the following limitations. The use of cut-offs that are not population-specific may jeopardize the sensitivity and specificity of the indices used to assess overweight, obesity and abdominal adiposity. Another limitation of concern is the fact that children aged above 11 years reported themselves on their dietary intake. Children's recall of food intake may be associated with under-reporting (missing foods), over-reporting [75], as well as incorrect identification of foods due to their lower knowledge of foods and their preparation [76]. It is also important to note that, in our study, dietary information was based on the collection of one 24-h recall, which may not be representative of dietary intakes at the individual level. However, despite its well-known limitations such as reliance on memory and day-to-day variation, the 24-h recall may provide accurate estimates of energy intake at the population level [76]. In the present study, dietary information was collected by the multiple pass 24-h recall approach, which was shown to provide accurate estimates of dietary intake in children [77]. In addition, the recalls were taken by research nutritionists who went through extensive training prior to data collection in order to minimize interviewer errors. Similarly, inter-observer measurement error in anthropometric assessment was minimized by extensive training and follow up to maintain quality of measurement among all research nutritionists. It is important to note that the physical activity questionnaire that was used in this study was not validated. However, the questionnaire was reviewed by a panel of experts including a nutritionist, a physical activity educator and an epidemiologist, and was based on tools used in similar studies.

## 5. Conclusions

This study has documented high prevalence rates of overweight, obesity and abdominal adiposity amongst Lebanese children and adolescents. More importantly, the study's findings pinpointed towards specific socioeconomic, dietary, and lifestyle factors that may increase the risk of adiposity in Lebanese youth. The documented high prevalence of child adiposity raises questions about its implications for psychosocial development and disease burden in the country, given the association of paediatric adiposity with metabolic syndrome, insulin resistance, hypertension, glucose intolerance, and dyslipidaemia [3,4]. With those below 20 years of age, making up close to 50% of the Lebanese population [78], these estimates do not bode well for the health and well-being of the population. Childhood obesity is related to growing up in an obesogenic environment, in which changes in physical activity and diet appear as the main drivers. In countries undergoing the nutrition transition such as Lebanon, children and adolescents represent the age group that suffers the most from adoption of western lifestyle characterized by long hours of television viewing, computer games, and heavy reliance on fast food, all of which are key factors affecting nutritional habits and obesity levels [79]. In the present study, adiposity in children was positively associated with sedentarity, irregular breakfast consumption, and higher intakes of fast food and sugar-sweetened beverages while the consumption of milk/dairies and olive oil were associated with a lowered risk. Parental socioeconomic characteristics, including education level and maternal employment, were documented as risk factors for adiposity in 6–11-year-old children, but not in adolescents. This highlights the importance of the home environment in modulating the child's lifestyle and dietary habits and hence obesity risk early in life. Taken together, these findings call for community-based intervention programs that involve

multisectoral partnerships and that are responsive to the sociocultural norms of the population. The prevention of paediatric overweight and obesity requires systems-level approaches and environmental support across all sectors of society to achieve sustained dietary and physical-activity behavior change [80]. Based on the results of this study, physical intervention strategies should in particular target adolescents who were shown to have higher levels of sedentarity and to be less likely to engage in physical activity compared to their younger peers. Family-focused interventions and behavioral strategies are needed to instil healthy lifestyle and dietary habits early in life. School-based interventions should integrate behavioral and environmental approaches that focus on dietary intake and physical activity using a systems-level approach [80]. Policy and environmental interventions are recommended as sustainable ways to support healthful lifestyles for children and families and to ensure that all youth have the opportunity to achieve and maintain a weight that is optimal for health [80].

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### Authors' Contributions

LN wrote the paper and contributed to data analysis/interpretation; NH and AMS designed and supervised the study and critically reviewed the paper; MCC coordinated field work; FN participated in data analysis and critically reviewed the paper; SK participated in data analysis; CA conducted data analysis and contributed to data interpretation; LN and FN contributed equally to this manuscript. All authors read and approved the final manuscript.

### Conflicts of Interest

The authors declare no conflict of interest.

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## Micronutrient Intakes from Food and Supplements in Australian Adolescents

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**Abstract:** Objective: Low micronutrient intakes in adolescents are frequently reported. We assessed micronutrient intakes in adolescents to determine whether supplement use optimises intakes. Methods: Dietary intake was assessed using a food frequency questionnaire in 17 year old participating in the Western Australian Pregnancy Cohort (Raine) Study ( $n = 991$ ). We calculated median daily micronutrient intakes in supplement users and non-users (from food sources only and from food and supplements), along with the percentage of adolescents meeting the Estimated Average Requirements (EAR) or Adequate Intake (AI) where appropriate. Results: Intakes of calcium, magnesium, folate and vitamins D and E from food only were low. Although supplements significantly increased micronutrient intakes in supplement users, more than half of supplement users failed to meet the EAR or AI for some key micronutrients. Compared with non-users, supplement users had higher micronutrient intakes from food sources with the exception of vitamins D and B12 and were more likely to achieve the EAR or AI for many micronutrients from food only. Conclusions: Intakes of some key micronutrients were low in this population, even among supplement users. Those facing the greatest risk of micronutrient deficiencies were less likely to use supplements.

**Keywords:** adolescents; food intake; micronutrients; dietary supplements; Raine Study

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## 1. Introduction

Low intakes of micronutrients, including calcium, folate, magnesium and potassium, have been previously reported in Australian adolescents [1]. Similarly, adolescent diets in Europe and the United States (US) have been associated with low intakes of calcium, vitamin D, iron, folate and zinc [2–5]. Assessing micronutrient status in adolescents is important due to the contribution of micronutrients to disease prevention [6]. Herbison and colleagues reported that low intake of B vitamins was associated with poor mental health and behaviour in adolescents [7]; calcium and magnesium may play a protective role in type 2 diabetes [8]; and young adults with higher magnesium have a lower risk of developing the metabolic syndrome [9]. Furthermore, adequate calcium and vitamin D levels are essential during adolescence, when approximately 40% of total bone mass is accumulated [10,11].

In order to reliably assess micronutrient intakes, the contribution of nutritional supplements to intake must be taken into account [12]. Nutritional supplement use is increasing in many countries and is popular worldwide in adolescents [5,12–16]. The EPIC study in the United Kingdom (UK) found that the contribution of nutritional supplements to nutrient intakes can be substantial, and miscalculation of nutrient intakes can occur if supplement use is not considered [17]. Therefore, we aimed to assess micronutrient intakes in 17 year old adolescents in Western Australia and to determine whether supplement use optimises micronutrient intakes.

## 2. Experimental Section

### 2.1. Participants

Participants were from the Western Australian Pregnancy Cohort (Raine) Study, which has been described previously [18]. In brief, 2900 pregnant women were recruited through the public antenatal clinic at King Edward Memorial Hospital and nearby private clinics in Perth, Western Australia between May 1981 and November 1991. A total of 2868 children were available for follow-up. The King Edward Memorial Hospital and Princess Margaret Hospital Ethic Committees approved the study protocol. The participant and/or their primary caregiver provided written consent for their participation in the study. In order to increase participation at each follow-up, participants were sent regular newsletters, Christmas cards, birthday cards and received regular updates and results of the study. Participants for the 17 year follow-up were contacted by a research assistant over the telephone. A total of 2168 adolescents were eligible for follow-up at 17 years between July 2006 and June 2009. Of these, 1754 individuals participated and 1009 provided dietary intake data.

### 2.2. Dietary Intake

Dietary intake at the 17 year follow-up was assessed using a self-reported semi-quantitative food frequency questionnaire (FFQ) developed by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Adelaide, Australia [19]. This FFQ has been validated for reliability against a 3-day food record in the same cohort [20] and also in adults [21]. From the FFQ we collected information on 212 foods, mixed dishes and beverages, including beverages and snacks popular among adolescents. An overall estimate of the adolescents' usual dietary intakes in the past year was

established using the portion size in standard household measures, and the number of times the food was eaten per day, per week or per month. Participants were asked to record any additional items that were consumed regularly but were not included in the FFQ. All questionnaires were checked by a research nurse and queries were clarified with the adolescent. Seasonal differences were accounted for by asking how often foods were eaten in summer and winter. Food intake data were entered into a database and verified by CSIRO. Estimated daily micronutrient intakes were provided by CSIRO using nutrient composition derived from four sources: the Australian nutrient database (NUTTAB95) [22]; the British Food Composition Tables [23]; the US Department of Agriculture food tables [24]; and manufacturers' data. Questionnaires were excluded if the daily energy intake reported was implausible (<3000 or >20,000 kJ per day). Micronutrient intakes were calculated for thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, vitamin B12, folate, beta-carotene, vitamins A, C, D, E, calcium, iron, potassium, magnesium, zinc, phosphorus and copper.

### *2.3. Supplement Use*

Participants were asked to record any supplements they used over the last twelve months, including brand, name of product, dose and frequency of use. Composition data for supplements were obtained from the product label or directly from the manufacturer. If the frequency of use was less than daily, the nutrient intake was calculated to reflect daily intake over the last twelve months. When there were insufficient data regarding the brand, name, dose or frequency of use, a standardised default was used based on the most common supplement of that type recorded by the participants. Micronutrient intake from supplements was added to the intake from food sources to give a total daily micronutrient intake for supplement users.

### *2.4. Demographic Characteristics*

Height was measured using a Holtain Stadiometer to the nearest 0.1 cm. Weight was measured using a Wedderburn Digital Chair Scale to the nearest 100 g. Body Mass Index (BMI) was calculated as weight in kilograms divided by height in metres squared. Underweight, normal weight, overweight and obesity were defined according to age- and sex-specific BMI cut-offs [25,26]. Physical activity was assessed using a self-reported questionnaire, based on exercise outside of school hours per week, where exercise was defined as activity causing breathlessness or sweating  $\geq 4$  times per week, 1–3 times per week and <once per week). Television/computer viewing was assessed by the amount of hours watching television or using the computer per day (<2 h per day, 2–4 h per day, >4 h per day). Family income was defined as the gross income before tax and was determined as AUD (per year) <\$35,000, \$35,001–\$70,000 or >\$70,001 (average gross salary in 2009 was AUD \$63,612 [27]). Maternal education level was indexed by whether the mother had completed 12 years of education or not by the time the child was 8 years old.

### *2.5. Statistical Analysis*

Chi-square tests were applied to identify differences in sex, BMI category, maternal education, family income, screen use and physical activity between supplement users and non-users. Median daily

micronutrient intakes in males and female supplement users and non-users were calculated from food sources alone and from food and supplements. The percentage of males and female supplement users and non-users meeting the Estimated Average Requirement (EAR) or Adequate Intake (AI) was calculated. The EAR is defined as the daily nutrient level estimated to meet the requirements of half the healthy individuals in a particular life stage and gender [28]. Where evidence was insufficient or too conflicting to establish an EAR, an Adequate Intake (AI) was set. The AI is defined as the average daily nutrient intake level based on observed or experimentally-determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate [27].

Since most micronutrient intakes were non-parametrically distributed, Mann Whitney-U tests were applied to investigate differences in male and female intakes from food sources in supplement users and non-users. We applied Wilcoxon signed rank tests to identify differences in micronutrient intakes between male and female supplement users, from food sources only and from food sources plus supplements. Chi-square tests were used to determine differences in the percentage of adolescents achieving the EAR from food sources between supplement users and non-users. We used Statistical Package for Social Science for Windows Rel.20.0.0 (Chicago:SPSS Inc., Illinois, IL, USA) and defined statistical significance as  $p < 0.05$ .

### 3. Results

#### 3.1. Characteristics

At the 17 year follow-up, 1009 participants provided dietary intake data; however, 18 of these were excluded due to implausible energy intakes ( $<3000$  or  $>20,000$  kJ per day) [29]. Ultimately, dietary intake data were available for 238 supplement users (24%) and 753 non-users. Supplement users were more likely to be physically active than non-users ( $p < 0.05$ ) (Table 1). No other significant differences between supplement users and non-users were observed.

**Table 1.** Characteristics of 17 year old adolescents providing dietary intake data in the Western Australian Pregnancy Cohort (Raine) Study.

	Total Population ( <i>n</i> = 991) <i>n</i> (%)	Supplement Users ( <i>n</i> = 238) <i>n</i> (%)	Non-Users ( <i>n</i> = 753) <i>n</i> (%)	<i>p</i> value
Sex				0.061
Male	454 (45.8)	96 (40.3)	358 (47.5)	
Female	537 (54.2)	142 (59.7)	395 (52.5)	
BMI category				0.165
Underweight	65 (7.7)	13 (6.2)	52 (8.3)	
Healthy weight	606 (72.1)	158 (75.2)	448 (71.1)	

**Table 1. Cont.**

Overweight	108 (12.9)	30 (14.3)	78 (12.4)	
Obese	61 (7.3)	9 (4.3)	52 (8.3)	
Computer and/or television use				0.273
<2 h per day	33 (3.6)	11 (4.6)	22 (2.9)	
2–4 h per day	486 (53.1)	124 (52.1)	362 (48.1)	
>4 h per day	397 (43.3)	89 (39.8)	308 (40.9)	
Physical activity				
Once per week or less	186 (19.7)	42 (18.4)	144 (20.2)	0.013 *
1–3 times per week	512 (54.4)	110 (48.2)	402 (53.4)	
4+ times per week	244 (25.9)	76 (33.3)	168 (23.5)	
Maternal Education				0.082
<12 years of education	551 (55.7)	122 (51.3)	429 (57.1)	
>12 years of education	438 (44.3)	116 (48.7)	322 (42.9)	
Annual Family income <sup>1</sup>				0.214
<\$35,000	118 (13.0)	34 (15.5)	84 (12.3)	
>\$35,001–\$70,000	228 (25.2)	47 (21.4)	181 (26.4)	
>\$70,001	559 (61.8)	139 (63.2)	420 (61.3)	

<sup>1</sup> Average gross salary in Australia in 2009 was \$63, 612 [26]; \* Significant at  $p < 0.05$ .

### 3.2. Types of Supplements Consumed

The most common supplement was a multivitamin, used by 42% of male supplement users and 33% of female supplement users. This was followed by vitamin C, consumed by 39% of male supplement users and 29% of female supplement users. Only two female supplement users consumed a folate supplement. Protein was only taken by male supplement users. No participants were taking a dedicated vitamin D supplement (Table 2).

**Table 2.** Nutritional supplements consumed by 17 year old adolescents in the Western Australian Pregnancy Cohort (Raine) Study.

Supplement Type	Males	Females
	( <i>n</i> = 96)	( <i>n</i> = 142)
	<i>n</i> (%)	
Vitamin C	37 (39.0)	41 (29.0)
Vitamin B/B complex	4 (4.2)	8 (5.6)
Folate	0 (0.0)	2 (1.4)
Iron	4 (4.2)	35 (24.6)
Calcium	1 (1)	7 (5.0)
Magnesium	5 (5.2)	7 (5.0)
Zinc	8 (8.3)	12 (8.5)
Multivitamin/mineral	40 (42.0)	47 (33.0)
Fish/Cod liver oil	25 (26.0)	37 (26.0)
Primrose/Starflower Oil	4 (4.2)	4 (2.8)

**Table 2.** *Cont.*

Probiotics	2 (2.1)	4 (2.8)
Protein <sup>1</sup>	7 (7.3)	0 (0.0)
Other <sup>2</sup>	9 (9.4)	12 (8.5)

<sup>1</sup> Protein powder, micronized creatine monohydrate, muscle building supplement, lipo 6, mixed amino acids, complete protein; <sup>2</sup> Fibre, cranberry, phytelle, garlic and horseradish, echinacea, garlic, glucosamine, chondroitin, spirulina, olive oil extract, butter menthol, l-lysine.

### 3.3. Median Daily Micronutrient Intakes from Food and Supplements

When micronutrient intakes from food only were compared between users and non-users, male supplement users had significantly higher intakes than male non-users ( $p < 0.05$ ) for all nutrients with the exception of vitamin D (Table 3). Female supplement users had significantly higher intakes of magnesium, potassium, vitamin A, beta-carotene, pantothenic acid, pyridoxine, folate and vitamin C ( $p < 0.05$ ) from food sources than female non-users. In supplement users, all micronutrient intakes were significantly higher ( $p < 0.05$ ) from food and supplements compared with food only.

### 3.4. Adequacy of Intakes

Fewer than 50% of females (both supplement non-users and supplement users) met the EAR for calcium, magnesium, folate or the AI for vitamins D and E (Table 4). Fewer than 50% of male non-users met the EAR for magnesium, potassium, pantothenic acid, folate or the AI for vitamins D and E. From food sources only, in male supplement users, fewer than 50% met the EAR for folate or the AI for vitamins D and E. When the contribution of supplements was accounted for, there was an approximate 20% increase in the number of males and females meeting the EAR for folate and those meeting the AI for vitamins D and E. However, more than 50% of males still failed to meet the AI requirements for vitamin D and more than 50% of females failed to meet the requirements for folate, vitamins D and E as well as calcium and magnesium.

## 4. Discussion

We found that adolescents in Western Australia had intakes below recommendations for calcium, folate and vitamins D and E. Females also had low intakes of magnesium. Low micronutrient intakes were also found in other studies carried out in Australia amongst 16–18 years old, where females reported low intakes of calcium, folate, and magnesium, and males reported low intakes of folate, calcium and potassium [1]. Similarly, in the UK, adolescent males aged 11–18 years had low intakes of magnesium, potassium, zinc, folate, iron and vitamin D, while females of the same age had low intakes of calcium, magnesium, potassium, folate, iron and vitamin D [30]. In 11–14 year old adolescents in Spain, more than 50% of males had intakes below the recommended nutrient intake (RNI), for magnesium, calcium, folate and vitamins A, B6, D and E, while more than 50% of females had intakes below the RNI for magnesium, calcium, folate and vitamins A, B6, D and E [31]. In the US around 90% of adolescent girls have inadequate intakes of calcium, magnesium, potassium and vitamins D and E, and many do not meet the recommendations for, zinc, phosphorous and vitamins A, B6, B12 and C [32].

**Table 3.** Median daily micronutrient intakes from food and supplements in male and female supplement non-users ( $n = 753$ ) and users ( $n = 238$ ) in 17 year old adolescents in the Western Australian Pregnancy Cohort (Raine) Study.

Nutrient	Supplement Non-Users ( $n = 753$ )			Supplement Users ( $n = 238$ )		
	Food Sources			Food Sources		
	Males ( $n = 358$ )	Females ( $n = 395$ )	Median	Males ( $n = 96$ )	Females ( $n = 142$ )	Median
Calcium (mg)	1089.5	842.3		1395.3 *	851.4	1402.1
Iron (mg)	13.8	10.8		16.4 *	11.1	19.4
Zinc (mg)	12.8	10		14.6 *	10.1	18.8
Magnesium (mg)	309.1	248.1		397.8 *	265.7 *	428.2
Potassium (mg)	3421.8	2890		4232.9 *	3167.7 *	4243.5
Phosphorous (mg)	1654.4	1252.9		2064.5 *	1293.8	2064.5
Copper (mg)	1.9	1.5		2.2 *	1.7	2.2
Vitamin A ( $\mu\text{g}$ )	1003.1	886.7		1218.9 *	985.2 *	1447.6
Beta-carotene ( $\mu\text{g}$ )	3229.5	3397.3		3805.4 *	3733.6 *	4113.1
Thiamin (mg)	1.8	1.3		2.1 *	1.3	2.7
Riboflavin (mg)	2.3	1.8		2.9 *	1.9	3.8
Niacin (mg)	38.6	29.6		44.5 *	29.7	54.3
Pantothenic acid (mg)	5.2	4.2		6.3 *	4.6 *	8.7
Pyridoxine (mg)	1.6	1.4		2.0 *	1.5 *	2.9
B12 ( $\mu\text{g}$ )	4.6	3.4		5.1 *	3.4	7.4
Folate ( $\mu\text{g}$ )	252.4	204		313.1 *	224.4 *	399.9
Vitamin C (mg)	146.4	128.3		178.4 *	142.9 *	335
Vitamin D ( $\mu\text{g}$ )	1.7	1.3		1.9	1.2	3
Vitamin E (mg)	7.1	5.8		8.6 *	5.9	11.5
						880.9
						14.8
						11.7
						287.8
						3180.1
						1293.8
						1.7
						1136.7
						4494.5
						1.6
						2.3
						35.3
						5.4
						2.4
						4.3
						280.8
						253.3
						1.5
						6.9

\* Significant difference in micronutrient intakes from food sources between supplement users and non-users ( $p < 0.05$ ).

**Table 4.** Number and percentage [*n* (%)] of adolescents meeting the EAR or AI [27] in male and female supplement non-users (*n* = 753) and users (*n* = 238) in 17 year old adolescents in the Western Australian Pregnancy Cohort (Raine) Study.

Nutrient	EAR/AI		Supplement Non-Users ( <i>n</i> = 753)				Supplement Users ( <i>n</i> = 238)					
	Males	Females	Food Sources		Males ( <i>n</i> = 358)	Females ( <i>n</i> = 395)	Food Sources		Males ( <i>n</i> = 96)	Females ( <i>n</i> = 142)	Food and Supplements	
			n (%)	n (%)			n (%)	n (%)			Males ( <i>n</i> = 96)	Females ( <i>n</i> = 142)
Calcium (mg) <sup>1</sup>	1050	1050	185 (51.7)	116 (29.4)	70 (72.9) *	50 (35.2)	73 (76.0)	56 (39.4)				
Iron (mg) <sup>1</sup>	8	8	336 (93.9)	312 (79.0)	92 (95.8)	114 (80.3)	94 (97.9)	128 (90.1)				
Zinc (mg) <sup>1</sup>	11	6	248 (69.3)	354 (89.6)	79 (82.3) *	126 (88.7)	95 (99.1)	128 (90.1)				
Magnesium (mg) <sup>1</sup>	340	300	153 (42.7)	125 (31.6)	59 (61.5) *	58 (40.8) *	75 (78.1)	67 (47.2)				
Potassium (mg) <sup>1</sup>	3600	2600	163 (45.5)	239 (60.5)	63 (65.6) *	101 (71.1) *	85 (88.5)	101 (71.1)				
Phosphorous (mg) <sup>1</sup>	1055	1055	308 (86.0)	266 (67.3)	90 (93.8) *	102 (71.8)	90 (93.8)	102 (71.8)				
Copper (mg) <sup>1</sup>	1.5	1.1	260 (72.6)	327 (82.8)	82 (85.4) *	126 (88.7)	94 (97.9)	128 (90.1)				
Vitamin A (µg) <sup>1</sup>	630	485	296 (82.7)	356 (90.1)	89 (92.7) *	132 (93.0)	95 (99.0)	134 (94.4)				
Beta-carotene (µg) <sup>1</sup>	n/a	n/a										
Thiamin (mg) <sup>1</sup>	1.1	0.9	332 (92.7)	318 (80.5)	90 (93.8)	125 (88.0) *	94 (97.9)	130 (91.5)				
Riboflavin (mg) <sup>1</sup>	1.1	0.9	339 (94.7)	363 (91.9)	93 (96.9)	137 (96.5)	96 (100)	137 (96.5)				
Niacin (mg) <sup>1</sup>	12	11	358 (100)	391 (99.0)	95 (99.0)	139 (97.9)	96 (100)	139 (97.9)				
Pantothenic acid (mg) <sup>1</sup>	6	4	133 (37.2)	214 (54.2)	54 (56.3) *	93 (65.5) *	94 (97.9)	100 (70.4)				
Pyridoxine (mg) <sup>1</sup>	1.1	1	304 (84.9)	306 (77.5)	90 (93.8) *	122 (85.9) *	95 (99.0)	129 (90.8)				
B12 (µg) <sup>1</sup>	2	2	345 (96.4)	332 (84.1)	92 (95.8)	114 (80.3)	95 (99.0)	118 (83.1)				
Folate (µg) <sup>1</sup>	330	330	45 (12.5)	26 (6.5)	94 (26.3) *	46 (32.4) *	63 (65.6)	61 (43.0)				
Vitamin C (mg) <sup>1</sup>	28	28	349 (97.5)	388 (98.2)	96 (100)	141 (99.3)	96 (100)	141 (99.3)				
Vitamin D (µg) <sup>2</sup>	5 <sup>1</sup>	5 <sup>1</sup>	17 (4.7)	5 (1.3)	11 (11.5) *	3 (2.1)	33 (34.4)	38 (26.8)				
Vitamin E (mg) <sup>2</sup>	10 <sup>1</sup>	8 <sup>1</sup>	90 (25.1)	96 (24.3)	56 (58.3) *	33 (23.2)	56 (58.3)	57 (40.1)				

EAR, Estimated Average Requirement; AI, Adequate Intake [27].<sup>1</sup> EAR,<sup>2</sup> AI; \* Significant difference in the percentage of adolescents meeting the EAR or AI from food sources only between supplement users and non-users (*p* < 0.05).

Approximately 24% of adolescents in this cohort consumed supplements. The prevalence of supplement use varies in other countries from 20% to 26% in adolescents in the US [14,33] and 11%–45% in European countries [5,13,14]. In our adolescent cohort, supplement use significantly increased the intakes of all nutrients in both males and females. However, even among supplement users, intakes of some micronutrients, including calcium, folate, vitamins D and E, remained lower than the recommendations. The low intakes of calcium, folate and vitamin D raises concern since inadequate calcium intake may lead to decreased bone-mineral density and increased risk of developing osteoporosis [16], low folate intake in females may lead to neural tube defects in the baby [34], and low vitamin D levels may affect both skeletal and non-skeletal health [35].

Vitamin D occurs naturally in very few foods, many of which are consumed episodically and contain relatively small amounts of vitamin D [36]. The low dietary supply of vitamin D makes it unrealistic that individuals would achieve the recommended intake of vitamin D. Indeed, the major source of vitamin D is exposure to sunlight and dietary sources of vitamin D are not necessary in the presence of adequate sunlight exposure. The AI for vitamin D assumes no, or minimal, sunlight exposure and individuals who do not meet the AI for vitamin D may have sufficient vitamin D status based on exposure to sunlight. Furthermore, care should be taken when estimating inadequacy based on an AI: although the AI can be used as a goal for individual intake, there is limited certainty about the value [27].

Particular characteristics appear to be associated with supplement use. We found that supplement users were more likely to be physically active than non-users. This finding is supported by Reaves *et al.* 2006 in the CATCH study, where 9–18 year old supplement users were more physically active than non-users and 47% of supplement users participated in team sports compared to 40% of non-users [14]. Adolescent supplement users in the NHANES were more physically active and were more likely to engage in less than 2 h of television/video or computer use per day than non-users [15]. In Finland, leisure time physical activity was the strongest factor associated with supplement use in 12–18 year old adolescents [13]. Grm and colleagues [5] showed that 12 and 17 year old adolescent supplement users were more likely to be members of sports clubs than non-users.

Higher supplement use in physically active adolescents may be due to the perceived benefits of dietary supplements on performance [37]. Indeed, protein supplements were more commonly consumed by males in our study and in others [5,13,38], which may be due to the perceived benefits of protein in increasing or improving sports performance [33]. In general, there appears to be a discrepancy in the type of supplements consumed and the micronutrients that are deficient in the diet. For example, almost all adolescents in this study achieved the recommended intake for vitamin C through food alone, even though vitamin C was the most commonly consumed supplement. In contrast calcium intakes were low in females but calcium supplements were only consumed by 3% of supplement users. It has been suggested that manufacturers develop a multivitamin/mineral that responds to the need for micronutrients that may be inadequate in the diet [39].

Supplement users in this study had higher micronutrient intakes from food sources alone compared with non-users. Therefore, supplements were less likely to be consumed by those who would benefit most from them. This pattern was also observed in the National Health and Nutrition Examination Survey (NHANES), where supplement non-users were more likely to have inadequate intakes of calcium, magnesium, phosphorous, vitamins A and C from food sources, than supplement users [40].

Reaves *et al.* 2006 in the Child and Adolescent Trial for Cardiovascular Health (CATCH) study found that adolescents who followed a healthier dietary pattern were also supplement users and those facing the greatest risk of micronutrient deficiencies were less likely to use supplements [15]. This limits the effectiveness of supplementation as a public health strategy to increase micronutrient intakes.

One strategy to improve micronutrient inadequacies is to promote a balanced diet rich in nutrient dense foods. Another alternative to supplementation is fortification of foods with micronutrients known to be low in the population. For example, in order to increase folate intakes at a population level, all wheat flour for making bread (excluding organic) in Australia is fortified with 2–3 mg of folic acid per kilogram of wheat [41]. In the US and Canada, foods are widely fortified with vitamin D in order to increase intakes and reduce to the risk of vitamin D deficiency [42].

## 5. Conclusions

Finding a balance between inadequate and excessive nutrient intakes is paramount to ensuring healthy development in adolescents. Along with increasing the consumption of nutrient-dense foods, supplement use may help to correct micronutrient imbalances. However, our results suggest that those who use supplements have higher micronutrient intakes from food sources and are less likely to require supplements than non-users. Furthermore, the type of supplements used by adolescents may not match the micronutrient deficiencies in the diet. Professional advice should be sought for correcting micronutrient imbalances using food and/or supplements.

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## Conflicts of Interest

The authors declare no conflict of interest.

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## High Plasma Homocysteine Increases Risk of Metabolic Syndrome in 6 to 8 Year Old Children in Rural Nepal

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**Abstract:** Little attention has been given to the association of plasma homocysteine (Hcy) and metabolic syndrome (MetS) in children. We have evaluated the risk of MetS with plasma Hcy in a cohort of 6 to 8 year old rural Nepalese children, born to mothers who had participated in an antenatal micronutrient supplementation trial. We assessed Hcy in plasma from a random selection of  $n = 1000$  children and determined the relationship of elevated Hcy ( $>12.0 \mu\text{mol/L}$ ) to MetS (defined as the presence of any three of the following: abdominal adiposity (waist circumference  $\geq 85^{\text{th}}$  percentile of the study population), high plasma glucose  $\geq 85^{\text{th}}$  percentile), high systolic or diastolic blood pressure ( $\geq 90^{\text{th}}$  percentile of reference population), triglyceride  $\geq 1.7 \text{ mmol/L}$  and high density lipoprotein  $< 0.9 \text{ mmol/L}$ ) and its components. There was an increased risk of low high-density lipoproteins (HDL), [odds ratios (OR) = 1.77, 95% confidence intervals (CI) = 1.08–2.88;  $p = 0.020$ ], high blood pressure [OR = 1.60, 95% CI = 1.10–2.46;  $p = 0.015$ ] and high body mass index (BMI) [OR = 1.98, 95% CI = 1.33–2.96;  $p = 0.001$ ] with elevated Hcy. We observed an increased risk of MetS (OR = 1.75, 95% CI = 1.06–2.90;  $p = 0.029$ ) with elevated Hcy in age and gender-adjusted logistic regression models.

High plasma Hcy is associated with increased risk of MetS and may have implications for chronic disease later in life.

**Keywords:** metabolic syndrome; homocysteine; Nepal

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## 1. Introduction

Metabolic syndrome (MetS) is a complex disorder comprising abdominal adiposity, high-blood pressure (BP), plasma glucose (PG), dyslipidemia [high-plasma triglycerides (TG) and/or low concentrations of high-density lipoproteins (HDL)]. Insulin resistance and cardiovascular disease (CVD) have gained attention as major manifestations of the syndrome. MetS has been considered an illness of adulthood; however an increase in the prevalence of insulin resistance and MetS has been reported among children recently [1,2]. According to a systematic review by Friend *et al.*, the median prevalence of MetS was 3.3% and 22% among Far East (India, South Korea and China) non-obese and obese children, respectively [3]. Given global trends toward increased adiposity, obesity and diabetes, deaths due to outcomes related to MetS, such as coronary heart disease (CHD) and type-2 diabetes, are expected to rise across the age spectrum [4].

In low income societies, the incidence in MetS among children has also been associated with a pattern of intrauterine conditions leading to low birth weight (<2500 g, LBW), rapid postnatal weight gain and less frequent breastfeeding during early life [5,6]. Given a high prevalence of LBW, rural Asian populations experiencing the nutrition transition to diets higher in fat and processed foods [7] may, therefore, be at particular risk of developing MetS. In the terai of southern Nepal, longitudinal studies have shown the incidence of LBW to be 43% [8] and the prevalence of MetS to be 11.7% at 6–8 years of age [9], with a lower risk among children whose mothers were provided antenatal folic acid supplements [9]. The research described here examines homocysteine (Hcy) as a risk factor for the MetS that was observed in this rural setting.

Homocysteine, an intermediary product of methionine metabolism, is elevated in folate and vitamin B12 deficiencies [10]. Hcy has also been accepted widely as an independent risk factor for CVD such as CHD and stroke [11,12]. Moreover, Hcy has been shown to be a thrombogenic and atherogenic substrate that potentiates atherosclerotic phenomena that may lead to adverse cardiometabolic events [13].

Despite its link with CVD, whether Hcy is more broadly associated with MetS is not well established. It is known that atherosclerosis begins in childhood and CVD can occur if children and adolescents have earlier exposure to risk factors such as elevated Hcy and components of MetS. However, to the best of our knowledge, no study has yet been done to assess the relationship of Hcy to MetS among young children living in rural South Asia. Detection of risk factors for MetS early in life in South Asian populations may suggest means to attenuate the progression of MetS and possible development of type-2 diabetes and CVD later in life [14,15].

Our hypothesis in the present study was that MetS and its components such as excess adiposity, hypertension, dyslipidemia and high glucose are associated with increased plasma Hcy concentrations in children. We addressed this question by determining the prevalence of hyperhomocysteinemia and evaluating its associations with aspects of MetS in a cohort of 6–8 year old children in rural Nepal.

## 2. Experimental Section

Children born to mothers who participated in a 5-arm trial of antenatal (to 3 months postpartum) micronutrient supplementation (1999–2001) in the rural District of Sarlahi, Nepal [8], were revisited and consented in 2006–2008 at 6–8 years of age as part of a large cohort follow-up study [9]. Procedures and primary outcomes of the original trial [8,16] and follow-up assessment [9] have been previously reported. Briefly, from 2006 to 2008, 3524 of 4130 children who were born to mothers enrolled in the micronutrient supplementation trial [8,16] were revisited in their homes to reassess vital status, height (measured by stadiometer; Harpenden, Croswell, UK), weight (measured by electronic scale; Model 881, Seca, Cambridge, MD, USA) and BP, and a 10 mL venous blood sample was collected in a heparinized tube in 3305 children, two-thirds of whom reported being fasted. For BP, the mean of last three values out of four measurements collected at one minute intervals using an automated oscillometric device (BpTRU™ BPM-300 Medical Devices Ltd., Coquitlam, BC, Canada) was taken. Standard test kits (DCA 2000 analyzer MN, Bayer HealthCare LLC, Elkhart, IN, USA) were used to estimate glycosylated hemoglobin (HbA<sub>1c</sub>) from whole blood. Plasma was separated in a field laboratory and total cholesterol, HDL-cholesterol, TG and glucose concentrations were measured using a Cholestech LDX analyzer (Cholestech Corp., Haywood, CA, USA). Low density lipoprotein (LDL) cholesterol was estimated using Friedewald's formula [17]. Frozen plasma was shipped to Johns Hopkins University Bloomberg School of Public Health in liquid nitrogen for further analysis, including plasma insulin, in fasted children only, by ultrasensitive sandwich immunoassay (ALPCO Diagnostic, Salem, NH, USA). Data from these laboratory analyses have been reported previously [9].

Based on criteria of having multiple aliquots of plasma and complete data collected from the initial trial and follow up activity, 2130 children were identified as eligible for further biomarker analysis, forming the sampling frame for this analysis. This group of children was similar to the 1394 children without sufficient plasma aliquots or complete data on a range of personal and household characteristics [9]. From the 2130 children we randomly selected 1000 children (511 boys and 489 girls), balanced across maternal supplementation groups ( $n = 200$  children per group evenly distributed over the duration of the field activity), for a subsequent study of micronutrient status. Hcy was measured in this subset of samples by chemiluminescent immunoassay (Immulite 1000, Siemens Diagnostics, Los Angeles, CA, USA), along with other measures of inflammation and micronutrient status [18]. Out of the 1000 participating children, 30.8% children had cholesterol, 7.2% had HDL, 6.0% had TG, and 3.2% had glucose concentrations that were below the detectable limits for the Cholestech assay. For all those who had values below the detectable limits we used the minimum detectable value of that biomarker as an estimate of biomarker concentration, allowing us to retain complete data in our analyses, but thereby overestimating the actual concentrations of those analytes for those children. LDL was not calculated when other lipid data were out of range. Also, 324 children had not adhered to fasting instructions, in whom insulin was not measured and therefore HOMA not determined. Data for lipids and glucose were analyzed from both fasted and non-fasted participants and included in the final analysis as we have reported previously [9].

Ethical clearance of the study was obtained from Institutional Review Boards at the Johns Hopkins Bloomberg School of Public Health (protocol H.22.06.05.26.A2, 20 September 2006) and the Ethics

Review Committee at Institute of Medicine at Katmandu, Nepal. The study was conducted according to the principles of the Declaration of Helsinki.

### 2.1. Variable Definitions

The definition of hyperhomocysteinemia in healthy children and adolescents is not well defined in the literature. Cutoffs have varied from 8.3 to 13.75  $\mu\text{mol/L}$  and differ by age and ethnicity [19–22]. In adults, hyperhomocysteinemia is commonly defined as concentrations greater than 12.0  $\mu\text{mol/L}$  [23] or 15.0  $\mu\text{mol/L}$  [24]. Because there is no specific cut-off for defining high plasma homocysteine level in children, population-specific 85th percentile values may guide the definition of hyperhomocysteinemia for children [22]. In this analysis the 85th percentile of Hcy was 12.6  $\mu\text{mol/L}$ , close to the widely accepted cutoff for hyperhomocysteinemia in adults, and used in previously published reports [22]. Therefore, we chose a cutoff of 12.0  $\mu\text{mol/L}$  to indicate hyperhomocysteinemia, despite lower cutoffs sometimes used in children, because it better reflected the risk of higher homocysteine observed in Asian populations [22] and because it is conventionally used. We have established and reported previously the cutoffs used for other components of MetS and the definition of MetS [9,25–28], which are summarized in Table 1.

**Table 1.** Variable definitions.

Variables	Definitions
Hyperhomocysteinemia	$>12.0 \mu\text{mol/L}$
Adiposity	BMI: $\geq 85$ th percentile of the entire study population (observed in $n = 150/1000$ children) Waist circumference: $\geq 85$ th percentile of the entire study population (observed in $n = 153/1000$ children)
Hypertension	Systolic blood pressure or diastolic BP $\geq 90$ th percentile of the U.S. reference population adjusted for age, height and sex [25]
Dyslipidemia	TG $\geq 1.7 \text{ mmol/L}$ <sup>(1)</sup> HDL cholesterol $< 0.9 \text{ mmol/L}$ <sup>(2)</sup>
Insulin Resistance	Homeostasis model assessment (HOMA) [to estimate insulin resistance]: Product of FPI (mU/L) and PG (mmol/L) standard factor 22.5; HOMA = (FPIxPG)/22.5 [28] PG: ( $\geq 85$ th percentile of the study population; determined in $n = 150/1000$ children)
MetS	Presence of any three of the following constituents: elevated waist circumference, high PG, high systolic or diastolic BP, high TG and low HDL [9]

<sup>(1)</sup> As described by the NCEP criteria set for adults, because there is no separate recommendation for children [26]; <sup>(2)</sup> As described by the NCEP criteria for cholesterol in children and adolescents [27]; BMI, basal metabolic rate; BP, blood pressure; TG, triglyceride; HDL, high density lipoprotein; PG, plasma glucose; FPI, fasting plasma insulin; MetS, metabolic syndrome.

### 2.2. Statistical Analysis

Anthropometric measures, BP and biomarkers were examined by Hcy ( $\leq 12.0 \mu\text{mol/L}$  vs.  $>12.0 \mu\text{mol/L}$ ) using independent sample t-tests. Values were expressed as mean  $\pm$  SD.

The association of hyperhomocysteinemia ( $>12.0 \mu\text{mol/L}$ ) with MetS and dichotomized individual components of MetS was expressed as odds ratios (OR) determined by separate logistic regressions for each outcome variable, with adjustment for age and gender. We also estimated OR for the combined risk of having multiple MetS components (such as low HDL and high BMI combined) against hyperhomocysteinemia through multiple logistic regression. Since we had previously shown an effect of maternal antenatal micronutrient supplementation, particularly with folic acid, on MetS in these children [9], we also initially adjusted our regression models for maternal intervention groups and birth weight, but this adjustment is not reported as these variables were not statistically significant. Likewise we examined statistical models adjusted for various aspects of socioeconomic status (SES), including ownership of televisions, radios, bicycles, livestock and use of electricity, as well as for seasonal effects. However, adjustment for these variables is not reported as their influence was not statistically significant. Finally, we explored models adjusted for fasting status and observed no effect on the OR; thus, that adjustment was not included in our results. Risks were expressed as OR and associated 95% confidence intervals (CI). All statistical analyses were done with IBM SPSS<sup>®</sup> (Statistical Package for Social Sciences, IBM Corp., Armonk, NY, USA) software version 21 for Windows<sup>®</sup>.

### 3. Results

The prevalence of underweight, stunting and low BMI (below-2 Z-score) [29] in these children was 48.2%, 42.0% and 16.1% respectively. The prevalence of hyperhomocysteinemia was 18.4%. Among the 1000 children, 827 (83%) had low HDL cholesterol ( $<0.9 \text{ mmol/L}$ ), 108 (11%) had high triglyceride ( $\geq 1.7 \text{ mmol/L}$ ), 190 (19%) had high blood pressure ( $>90$ th percentile of reference), 153 (15%) had high waist circumference ( $\geq 85$ th percentile). A total of 86 (8.6%) children met the criteria for MetS.

Table 2 indicates that children with elevated Hcy ( $>12.0 \mu\text{mol/L}$ ) had higher BMI ( $p = 0.002$ ), waist circumference ( $p = 0.043$ ), systolic ( $p = 0.071$ ) and diastolic ( $p = 0.052$ ) blood pressure, and triglycerides ( $p = 0.016$ ) and total lipid concentrations ( $p = 0.048$ ). HDL concentrations were lower in subjects with higher vs. lower Hcy levels ( $p = 0.031$ ). In logistic regression analyses adjusted for age and sex (Table 3), an elevated Hcy level was associated with an increased risk of low HDL cholesterol [OR = 1.77 (95% CI = 1.08–2.88);  $p = 0.022$ ], high BP [OR = 1.65 (95% CI = 1.10–2.46);  $p = 0.015$ ] and MetS [OR = 1.75 (95% CI = 1.06–2.90);  $p = 0.029$ ]. No association was observed between elevated Hcy and high TG, waist circumference or PG.

We detected an increased risk of high BMI ( $\geq 85$ th percentile) [OR = 1.98 (95% CI = 1.33–2.96);  $p = 0.001$ ] with hyperhomocysteinemia. Moreover, we observed that the relative odds of having combined low HDL cholesterol and high BP was 1.76 (95% CI = 1.14–2.70;  $p = 0.010$ ). Similarly the odds ratio for combined low HDL cholesterol and high BMI was 2.31 (95% CI = 1.51–3.53;  $p < 0.001$ ). We didn't see additional risk for combined low HDL, high BP and high BMI with hyperhomocysteinemia ( $p = 0.351$ ) (Table 3).

**Table 2.** Anthropometric measures, blood pressure, and plasma biochemical biomarkers of study children by plasma homocysteine levels <sup>1</sup>.

Variables	Total <i>n</i> = 1000	Homocysteine ≤ 12.0 μmol/L <i>n</i> = 813	Homocysteine > 12.0 μmol/L <i>n</i> = 184	<i>p</i> -Value <sup>2</sup>
Age	7.48 ± 0.65	7.47 ± 0.44	7.52 ± 0.40	0.24
Hcy	9.40 ± 3.50	8.06 ± 1.99	15.02 ± 2.97	<0.001
BMI <sup>3</sup>	14.02 ± 1.04	13.97 ± 1.03	14.24 ± 1.05	0.002
Waist Circumference <sup>3</sup> (cm)	51.40 ± 3.06	51.31 ± 3.13	51.81 ± 2.72	0.043
Systolic BP <sup>3</sup> (mmHg)	95.2 ± 8.3	95.0 ± 8.1	96.2 ± 9.0	0.071
Diastolic BP <sup>3</sup> (mmHg)	63.8 ± 8.5	63.6 ± 8.1	64.9 ± 9.8	0.052
Total Cholesterol (mmol/L)	3.01 ± 0.48	3.01 ± 0.49	3.0 ± 0.45	0.760
TG (mmol/L)	2.55 ± 1.09	2.51 ± 1.06	2.73 ± 1.20	0.016
Total lipids (mmol/L)	5.56 ± 1.26	5.53 ± 1.22	5.73 ± 1.40	0.048
LDL (mmol/L)	1.92 ± 0.43	1.91 ± 0.45	1.92 ± 0.36	0.940
HDL (mmol/L)	0.71 ± 0.22	0.72 ± 0.23	0.68 ± 0.20	0.031
PG (mmol/L)	3.99 ± 1.06	4.00 ± 1.11	3.97 ± 0.77	0.783
FPI (pmol/L)	22.56 ± 23.76	22.62 ± 23.58	22.20 ± 25.02	0.847
HbA <sub>1c</sub> (%)	5.11 ± 0.27	5.12 ± 0.28	5.09 ± 0.25	0.167

<sup>1</sup> Values are means±SD; <sup>2</sup> *p* compares mean values in Homocysteine ≤ 12.0 (μmol/L) vs. Homocysteine (>12.0 μmol/L) groups using independent sample *t*-test; <sup>3</sup> Data were missing for BMI (*n* = 1), waist circumference (*n* = 3), systolic blood pressure (*n* = 8), diastolic blood pressure (*n* = 8), LDL (*n* = 367) and FPI (*n* = 324); BMI, body mass index; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein; PG, plasma glucose; FPI, fasting plasma insulin; HbA<sub>1c</sub>, glycosylate hemoglobin.

**Table 3.** Risk of MetS and its components in 6–8 years old children related to hyperhomocysteinemia (*n* = 1000) <sup>1</sup>.

Outcome	Homocysteine > 12.0 μmol/L OR (95% CI)	<i>p</i> -Value
MetS <sup>2</sup>	1.75 (1.06–2.90)	0.029
Low HDL (<0.9 mmol/L)	1.77 (1.08–2.88)	0.022
High TG (≥1.7 mmol/L)	1.31 (0.80–2.13)	0.276
High systolic OR diastolic BP (≥90th percentile)	1.65 (1.10–2.46)	0.015
High waist circumference (≥85th percentile)	0.98 (0.63–1.53)	0.982
High PG (≥85th percentile)	1.26 (0.83–1.94)	0.275
High BMI (≥85th percentile)	1.98 (1.33–2.96)	0.001
High HOMA (≥85th percentile)	1.26 (0.73–2.16)	0.401
High BP + High BMI	1.36 (0.54–0.346)	0.512
Low HDL + High BP + High BMI	1.63 (0.58–4.60)	0.351
Low HDL + High BP	1.76 (1.14–2.70)	0.010
Low HDL + High BMI	2.31 (1.51–3.53)	<0.001

<sup>1</sup> Values are OR (95% CI) based on separate logistic regression analyses for each set of outcomes, adjusted for age and gender; <sup>2</sup> MetS defined as presence of any three of the following constituents: elevated waist circumference, high PG, high systolic or diastolic BP, high TG and low HDL.

#### 4. Discussion

We observed an increased risk of MetS and its components, specifically dyslipidemia, BMI, and hypertension, with elevated Hcy in young school-aged children in rural Nepal. The distributions of these components of MetS were similar to those reported previously for the larger cohort of 3524 children from whom this sample was derived [9]. That study identified intrauterine exposure to folate as a factor that ameliorated the risk of MetS in childhood; the current study expands on those findings by identifying a more proximal potential risk factor for MetS.

Previous studies have revealed that individuals who meet the criteria of MetS are at increased risk for developing CHD and diabetes mellitus [30], both of which are increasing in South Asia. It has been reported that 20%–25% of South Asians have MetS, with the frequency expected to rise in the future [31]. Nepal is not an exception, with CHD considered the leading cause of death in 2010 followed by stroke, hypertension and diabetes mellitus among other major causes of death [32]. However, data for these reports have been generated from urban areas with no study, so far, carried out to establish the burden of CVD in rural Nepal. Shaik, *et al.* collected hospital admission data in one of the tertiary hospitals which served the terai of Nepal and observed that around two patients out of ten were admitted due to stroke [33]. The extent of CVD in the region surrounding Nepal is not different, as it has been reported that 32% of all deaths in rural India are due to CVD [34].

Not limited to adults, South Asian children are also reported to have increased susceptibility to MetS and CVD, as South Asian children living in the UK showed higher mean heart rate, elevated mean triglyceride and fibrinogen levels, and higher mean fasting and post-glucose load insulin concentrations compared to white children [35]. Moreover, evidence also suggests that Indian children who are born small for gestational age have higher blood pressure, cholesterol levels (total cholesterol and LDL-C), as well as increased adiposity at the age of eight years [36]. Our results are in line with the above-mentioned studies, as we also observed high mean triglycerides and low mean HDL in these children, who were nonetheless thin (prevalence of low BMI 16%), underweight and stunted (>40% prevalence for both) [18]. Our data also suggest a role for elevated homocysteine with hypertension, dyslipidemia, and adiposity.

Although Hcy concentrations have been investigated in children in Western populations [20,37], no previous study has examined this risk factor in young South Asian children. The 95th percentile of Hcy (15.9  $\mu\text{mol/L}$ ), mean concentration Hcy of 9.4  $\mu\text{mol/L}$  and prevalence of hyperhomocysteinemia (18.4%) observed in our study area reflect elevated levels of Hcy in rural Nepal compared to Western populations [20,37]. A high burden of hyperhomocysteinemia in children could be a risk factor for CVD in later life, as elevated Hcy (95th percentile) in Dutch children was associated with a subsequent 4-fold increased risk of ischemic cerebrovascular disease [38]. High levels of Hcy in children of rural Nepal could be due to genetic polymorphisms, environmental exposures (e.g., high blood lead), physical activity patterns, and diet, particularly one that may lead to low vitamin B12 concentrations [39].

Atherosclerosis is known to begin in childhood, as autopsy reports in children and young adults with unexpected death have revealed positive associations between atherosclerotic lesions and risk factors such as LDL-C, triglycerides, systolic and diastolic blood pressure, body mass index and cigarette smoking, making it imperative to maintain healthy lipid profiles, blood pressure [40] and plasma Hcy to minimize the burden of diseases like CVD and MetS. High levels of Hcy and high

cholesterol are both associated with CVD risk. However, very few studies have reported the combined effect of hyperhomocysteinemia and dyslipidemia on the risk of CVD [41]. Since we observed a high prevalence of low HDL (83%) and considerable prevalence of hyperhomocysteinemia (18.5%) in these children, a potential interaction between Hcy and HDL cholesterol seems clinically relevant. Although exact mechanisms relating Hcy and cholesterol are not known, animal studies have revealed that hypomethylation due to hyperhomocysteinemia could be accountable for lipid accumulation in tissues [42]. Moreover, Hcy could also modulate activity of some inhibiting enzymes which play a role in HDL-particle assembly [42].

The association we observed between Hcy and blood pressure also merits discussion. We observed that an increase in Hcy was associated with rise in systolic and diastolic blood pressure, a finding consistent with National Health and Nutrition Examination Survey (1994–1998) and other studies of adult populations in developed countries [43,44]. Possible mechanisms to explain a link of Hcy with increased blood pressure are Hcy-induced arteriolar constriction [45] vascular endothelial damage [46] and decreased vasodilator responsiveness [47].

To the best of our knowledge no previous study has addressed the relationship of Hcy with MetS or components of MetS in children. Those studies which have addressed the relationship of Hcy with MetS in adults present contradictory results. Relationships we observed were similar to data published by Kang *et al.* showing a positive association between Hcy and triglycerides, BMI and systolic and diastolic blood pressure and a negative association with HDL in Korean adults [48]. Likewise, Hcy was positively associated with waist circumference, BMI, blood pressure, LDL-C, and triglycerides, but inversely associated with HDL in a Chinese sample of 1680 adults [49]. On the other hand, there are published reports where authors did not find associations [50] or observed associations with few components of MetS [51]. A lack of association of hyperhomocysteinemia with insulin resistance is consistent with other reports [52,53].

This study had some limitations. Due to the cross-sectional design we cannot establish a temporal or causal relationship between Hcy and MetS. The elevated Hcy and MetS could be results of common pathways such as poor diet, environment, inadequate physical activity [54,55], and genetic polymorphisms. Since no stringent range has been set to define hyperhomocysteinemia in children, our definition of hyperhomocysteinemia may underestimate the prevalence of hyperhomocysteinemia as the cut off  $>12.0 \mu\text{mol/L}$  is generally used for adults. Moreover, we somewhat overestimated the actual mean of total cholesterol, HDL, TG and plasma glucose by assigning values for these analytes equivalent to the lowest detectable concentration of these analytes, although prevalence of abnormal findings would not have been affected. We believe that this study provides unique information about the high prevalence of hyperhomocysteinemia and its role as a risk factor for MetS in a population of otherwise undernourished children residing in rural Nepal. Our findings reveal important issues to be considered further on the relationship of Hcy and MetS in a population which is known to have a high burden of CVD in adulthood.

## 5. Conclusions

We conclude that hyperhomocysteinemia exerts risk towards development of MetS and that high prevalence of hyperhomocysteinemia and MetS in this low income population suggest that Nepalese

children are at a greater risk of developing CVD and diabetes in future. It is therefore necessary to understand the causes of hyperhomocysteinemia, such as dietary habits in this population, in order to attenuate the future development of diabetes and cardiovascular disease symptoms, both of which constitute a growing concern among populations of South Asia.

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### Conflicts of Interest

The authors declare no conflict of interest.

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## Dietary Patterns in Children with Attention Deficit/Hyperactivity Disorder (ADHD)

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**Abstract:** The role of diet in the behavior of children has been controversial, but the association of several nutritional factors with childhood behavioral disorders has been continually suggested. We conducted a case-control study to identify dietary patterns associated with attention deficit hyperactivity disorder (ADHD). The study included 192 elementary school students aged seven to 12 years. Three non-consecutive 24-h recall (HR) interviews were employed to assess dietary intake, and 32 predefined food groups were considered in a principal components analysis (PCA). PCA identified four major dietary patterns: the “traditional” pattern, the “seaweed-egg” pattern, the “traditional-healthy” pattern, and the “snack” pattern. The traditional-healthy pattern is characterized by a diet low in fat and high in carbohydrates as well as high intakes of fatty acids and minerals. The multivariate-adjusted odds ratio (OR) of ADHD for the highest tertile of the traditional-healthy pattern in comparison with the lowest tertile was 0.31 (95% CI: 0.12–0.79). The score of the snack pattern was positively associated with the risk of ADHD, but a significant association was observed only in the second tertile. A significant association between ADHD and the dietary pattern score was not found for the other two dietary patterns. In conclusion, the traditional-healthy dietary pattern was associated with lower odds having ADHD.

**Keywords:** dietary pattern; attention deficit/hyperactivity disorder (ADHD); school-aged children; Korean

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## 1. Introduction

Attention deficit hyperactivity disorder (ADHD) is one of the most commonly diagnosed neurobehavioral disorders in childhood, and it often lasts into adulthood [1]. ADHD prevalence rates vary by age, gender, and ethnicity [2,3]. Boys are more likely to have ADHD than girls, and higher rates of ADHD in younger age groups have been observed in studies of children and adolescents [4]. Worldwide, the overall prevalence of ADHD/hyperkinetic disorder (HD) was found to be 5.29% in a pooled analysis [2]. The prevalence of ADHD is 8.7% in US children aged eight to 15 years [5] and 9.7% in Iranian school-aged children [6]. In Korea, the prevalence of ADHD is 7.6% in elementary school children with a mean age of 9.4 years [7] and upper-grade elementary school children with a mean age of 11.6 years [8]. The etiology of ADHD is multifactorial, and both genetic and environmental factors may be involved in ADHD [9]. Family and twin studies have shown that genes play an important role in the development of ADHD. Genome-wide association studies are inconclusive, but candidate gene studies suggest the involvement of genes related to the receptors and transporters of dopamine and serotonin [10,11]. Proposed ADHD environmental risk factors include heavy metal and chemical exposures such as lead, mercury, organochlorine, organophosphates, and phthalates, as well as nutritional and lifestyle/psychosocial factors [5].

The effect of diet and dietary supplements is unclear, but considerable evidence suggests that dietary factors are associated with childhood behavioral disorders such as ADHD [12,13]. Low levels of copper, iron, zinc, magnesium, and omega-3 fatty acids have been reported in children with ADHD,

and sugar, artificial food colorings, and preservatives are associated with an increased risk of ADHD [12,13]. Recently, the association between dietary pattern and ADHD has been examined in several studies [6,20,21]. As nutrients are consumed in combination and because nutrients are highly interrelated, the study of dietary patterns is useful to further understand the overall role of diet in ADHD. Thus, the purpose of this study was to determine the association between various dietary patterns and ADHD among Korean school-aged children.

## **2. Experimental Section**

### *2.1. Study Population*

We conducted a hospital-based case-control study using elementary school students who visited several university hospitals in Busan, Korea, from April to September, 2013. ADHD cases were recruited from two university hospitals (Dong-A and Inje University). ADHD was diagnosed by psychiatrists based on the Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV). Some children with ADHD have concurrent condition such as tic disorder (motor type), anxiety disorder, oppositional defiant disorder, Tourette's disorder, depression, and learning disability. A total of 117 cases, which consented to participate in research, were recruited, and age- and sex-matched controls were recruited from three university hospitals (Dong-A, Pusan, and Kosin University). Controls who did not have severe chronic diseases, a history of ADHD diagnosis and any related disease, such as mental disorder and tic disorder were recruited. Additional test using ADHD Rating Scale (ARS) for controls was performed to exclude ADHD cases. After excluding seven participants who did not complete the questionnaire, a total of 202 controls were recruited. To exclude the seasonal variation in dietary intake, the dietary survey season was also matched in the analysis. Frequency matching by grade (two years), sex, and season (three months) was conducted. A total of 192 elementary school students aged seven to 12 years (96 students with ADHD and 96 healthy controls) were finally selected. Each participant and their legal guardian were provided with an informed consent form according to the procedures approved by the Institutional Review Board of the National Cancer Center.

### *2.2. Data Collection*

The legal guardians of the participants were asked to complete a self-administered questionnaire, which was used to gather information on demographics, lifestyle, and the medical histories of the participants and their parents. A trained interviewer facilitated the 24-h recalls (24HR) interviews face-to-face, and another two non-consecutive 24HR interviews were conducted by telephone between April and September 2013. Individual food intake was calculated using CAN-PRO 4.0 (Computer Aided Nutritional Analysis Program, The Korean Nutrition Society, Seoul, Korea). Mercury and lead exposure from food was calculated using dietary consumption data and their concentrations in 118 core food items. Consumption of omega-3 fatty acids was estimated as the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

### 2.3. Statistical Analysis

Principal-components analysis (PROC FACTOR) was used to extract the participants' dietary patterns using 32 predefined food groups. We used a varimax rotation to enhance the interpretability of the analyzed factors. We determined how many factors to retain after evaluating the eigenvalue, scree test, and interpretability. The dietary patterns were named according to the factors with the highest scores among the defined food groups for each dietary factor. Each dietary pattern's factor score was categorized by tertile for further analysis. Using a Student *t*-test for continuous variables and a chi-square test for categorical variables, we compared the general characteristics between students with ADHD and controls. The trend test was performed to analyze the associations between each of the dietary patterns and ADHD using a generalized linear model with adjustments for total energy intake. Odds ratios (ORs) and 95% confidence intervals (CIs) for ADHD were calculated across the tertiles of dietary pattern scores using logistic regression models. The lowest tertile of each dietary pattern was used as the reference. To assess the trend across the tertiles, we assigned median values to each tertile of the dietary pattern scores as a continuous variable. We performed the statistical analysis using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). All *P* values were two-tailed ( $\alpha = 0.05$ ).

### 3. Results

The general characteristics of the study population are presented in Table 1. The mean ages of the controls and students with ADHD were 9.1 and 9.0 years, respectively. The total energy intake was higher in controls than in students with ADHD ( $p = 0.008$ ). Father's educational background and occupation significantly differed between ADHD students and controls ( $p < 0.001$  and  $p = 0.001$ , respectively).

**Table 1.** General characteristics of study population <sup>1</sup>.

Characteristics	Controls ( <i>n</i> = 96)	Cases ( <i>n</i> = 96)	<i>P</i>
Age (year)	9.1 ± 1.8	9.0 ± 1.7	
Sex, male (%)	65 (67.7)	65 (67.7)	
Total energy intake (kcal)	2027.3 ± 381.7	1879.2 ± 380.7	0.008
Weight (kg)	34.3 ± 9.6	33.1 ± 10.4	0.427
Body mass index (cm/m <sup>2</sup> )	18.2 ± 2.9	17.5 ± 3.2	0.122
Gestation age (week)	39.0 ± 1.7	39.0 ± 1.7	0.787
Birth weight (kg)	3.3 ± 0.5	3.2 ± 0.5	0.099
Breastfeeding, yes (%)	80 (83.3)	70 (72.9)	0.081
Mother's age (year)	38.6 ± 3.7	39.5 ± 4.1	0.133
Birth order	1.5 ± 0.7	1.4 ± 1.1	0.651
Father's education, <i>n</i> (%)			
<High school	22 (22.9)	45 (47.9)	<0.001
College	53 (55.2)	41 (43.6)	
Graduate school	21 (21.9)	8 (8.5)	
Father's occupation, <i>n</i> (%)			
Professional	26 (27.1)	15 (15.6)	0.001

**Table 1.** *Cont.*

Office/service worker	44 (45.8)	28 (29.2)	
Manual worker	13 (13.5)	28 (29.2)	
Other	13 (13.5)	25 (26.0)	
Father's smoking status, <i>n</i> (%)			
Seldom or Never	37 (39.0)	24 (26.4)	0.188
Current	42 (44.2)	49 (53.9)	
Former	16 (16.8)	18 (19.8)	

<sup>1</sup> All analyses were performed with the data matched for age, sex, and dietary survey season.

PCA identified four major dietary patterns among the 32 food groups, and the associated factor loading scores with absolute values  $\geq 0.20$  are shown in Table 2. The “traditional” dietary pattern was characterized by high intakes of condiments, vegetables, tofu/soymilk, and mushrooms. The “seaweed-egg” dietary pattern included high intakes of seaweeds, fats/oils, sweets, and eggs. The “traditional-healthy” dietary pattern included high intakes of kimchi, grains, bonefish, and low intakes of fast foods and beverages. The “snack” dietary pattern was characterized by high intakes of snacks and processed meat and a low intake of noodles. Lean fish, other seafood, and yogurt were not listed due to their low factor loadings in all examined dietary patterns. Each dietary pattern explained 8.0%, 6.0%, 5.6%, and 5.4% of the variation in food intake, respectively.

**Table 2.** Factor loadings for the four major dietary patterns derived from principal components analysis with orthogonal rotation.

<b>Foods/Food Groups</b>	<b>Traditional</b>	<b>Seaweed-Egg</b>	<b>Traditional-Healthy</b>	<b>Snack</b>
Condiments	0.75			
Vegetables	0.56		0.20	
Tofu, Soymilk	0.53			
Mushrooms	0.49			
Salted fermented seafood	0.34			
Fruits	0.32	-0.22		-0.31
Seaweeds		0.69		
Fats, Oils	0.29	0.68		
Sweets	0.27	0.43		0.33
Egg		0.41		
Potatoes	0.22	0.35		
Processed fruit products		0.33		
Legumes		0.29		
Kimchi			0.58	-0.23
Grains	0.23		0.56	
Bonefish	0.28		0.52	0.26
Fatty fish	0.29		0.23	-0.38
Snack				0.49
Processed meats				0.44
Bread				0.43
Milk				0.30

**Table 2.** *Cont.*

Shellfish			-0.22	
Beverages		0.22	-0.44	
Fast foods			-0.49	
Rice cake		-0.36		
Seeds	0.23			
Dairy products			-0.23	-0.23
Meats			-0.31	-0.31
Noodles		0.24		-0.49
Variance of explained (%)	8.0	6.0	5.6	5.4

Factor loadings with absolute values  $\geq 0.20$  were listed in the table among 32 food groups.

The distribution of characteristics by dietary pattern score tertiles is presented in Table 3. Increasing scores in the traditional and traditional-healthy patterns were correlated with a decreased percent energy from fat ( $P$  for trend = 0.001;  $P$  for trend  $< 0.001$ , respectively), whereas the percent energy from carbohydrate increased as the score of the traditional-healthy pattern increased ( $P$  for trend  $< 0.001$ ). Fatty acids were significantly associated with dietary pattern scores. The traditional pattern score was associated with a high intake of total fatty acids; the seaweed-egg and traditional-healthy pattern scores were associated with high intakes of PUFAs and omega-3 fatty acids, whereas the snack pattern score was negatively associated with the intakes of total fatty acids, PUFAs, and MUFAs. Regarding mineral intake, calcium intake was positively associated with the scores of the traditional, traditional-healthy, and snack patterns, and iron was positively associated with the scores of the traditional and traditional-healthy patterns. Heavy metal exposure via food consumption was also assessed, and mercury was positively associated with the traditional, traditional-healthy, and snack patterns; lead was positively associated with the traditional and snack patterns.

The ORs and 95% CIs of ADHD were analyzed across the tertiles of dietary pattern scores (Table 4). The OR (95% CI) in the highest tertiles of the traditional dietary pattern compared to those in the lowest tertiles in crude model was 0.29 (0.13–0.64), but a significant association was not observed in multivariate model 2 (OR: 0.76, 95% CI: 0.26–2.24). The seaweed-egg pattern was not significantly associated with ADHD in any of the models. The snack pattern score was positively associated with the risk of ADHD, but a significant association was observed only in the second tertile in crude model and multivariate model 1. Students in the highest tertile of the traditional-healthy pattern score had an increased risk of ADHD in the multivariate-adjusted models when compared with those in the lowest tertile (OR (95% CI): 0.32 (0.13–0.82) in multivariate model 1; 0.31 (0.12–0.79) in multivariate model 2).

**Table 3.** Distribution of characteristics by the tertiles of dietary pattern scores.

Characteristics	Traditional			Seaweed-Egg			P Trend
	T1 <sup>1</sup>	T2	T3	T1	T2	T3	
Age (year)	8.7 (1.8)	9.1 (1.7)	9.5 (1.6)	8.9 (1.6)	9.0 (1.8)	9.2 (1.8)	0.270
Sex, female (%)	32 (43.2)	19 (25.7)	11 (25.0)	28 (40.6)	22 (33.9)	12 (20.7)	0.018
BMI (kg/m <sup>2</sup> )	17.9 (3.3)	17.6 (3.0)	18.3 (2.7)	17.6 (2.5)	17.7 (3.1)	18.4 (3.6)	0.171
Education, ≥college (%)	41 (56.9)	51 (68.9)	31 (70.5)	41 (59.4)	40 (63.5)	42 (72.4)	0.132
Total energy intake (kcal)	1757.7 (353)	2005.6 (325)	2194.2 (383)	1898.9 (418)	1897.2 (362)	2080.8 (352)	0.008
Carbohydrate (g)	248.6 (44.7)	284.5 (49.5)	315.4 (59.2)	273.0 (61.6)	272.3 (51.4)	289.5 (53.9)	0.216
Carbohydrate (% energy)	56.6 (6.5)	56.5 (6.2)	57.0 (4.9)	57.4 (6.3)	57.1 (6.0)	55.3 (5.5)	0.173
Protein (g)	67.1 (22.3)	78.2 (14.4)	86.0 (15.2)	71.0 (18.7)	74.0 (21.6)	83.2 (15.3)	0.016
Protein (% energy)	15.0 (3.0)	15.6 (2.2)	15.6 (1.7)	14.9 (2.2)	15.4 (2.8)	16.0 (2.2)	0.005
Fat (g)	56.9 (19.1)	63.3 (18.4)	68.1 (18.4)	59.7 (20.6)	59.4 (18.2)	67.3 (17.4)	0.951
Fat (% energy)	28.3 (5.1)	27.9 (5.1)	27.4 (4.4)	27.7 (5.3)	27.6 (4.9)	28.7 (4.6)	0.792
Total fatty acids (g)	29.2 (12.8)	33.1 (14.2)	33.2 (12.6)	28.9 (13.9)	30.9 (12.7)	35.7 (12.7)	0.080
PUFAs (g)	6.9 (2.5)	8.7 (3.4)	8.5 (2.7)	6.4 (2.6)	7.8 (2.1)	10.1 (3.2)	<0.001
MUFAs (g)	10.9 (5.7)	12.2 (5.9)	12.4 (5.3)	10.8 (5.9)	11.5 (5.4)	13.1 (5.5)	0.245
Omega-3 fatty acids (g)	0.10 (0.26)	0.23 (0.48)	0.34 (0.62)	0.25 (0.57)	0.20 (0.38)	0.15 (0.38)	0.059
Calcium (mg)	491.5 (222)	587.7 (171)	730.0 (224)	565.1 (225)	558.1 (230)	632.8 (206)	0.755
Iron (mg)	10.4 (2.3)	13.5 (4.8)	16.6 (8.4)	12.2 (7.3)	12.8 (5.3)	14.1 (3.4)	0.447
Zinc (mg)	8.2 (2.1)	9.8 (2.0)	10.5 (1.9)	8.9 (2.2)	9.1 (2.3)	10.2 (1.9)	0.063
Mercury (µg/kg bw)	0.19 (0.05)	0.22 (0.06)	0.22 (0.07)	0.20 (0.06)	0.21 (0.06)	0.21 (0.06)	0.965
Lead (µg/kg bw)	0.43 (0.14)	0.50 (0.14)	0.53 (0.17)	0.48 (0.17)	0.47 (0.13)	0.49 (0.16)	0.954

Table 3. Cont.

Characteristics	Traditional-Healthy			Snack			
	T1	T2	T3	T1	T2	T3	P Trend
Age (year)	9.2 (1.7)	8.8 (1.8)	9.1 (1.7)	9.8 (1.7)	8.8 (1.7)	8.8 (1.7)	0.002
Sex, female (%)	31 (41.9)	18 (29.0)	13 (23.2)	13 (25.0)	34 (42.5)	15 (25.0)	0.906
BMI (kg/m <sup>2</sup> )	17.8 (3.1)	17.8 (3.0)	18.0 (3.3)	19.5 (3.7)	17.4 (2.3)	17.0 (2.8)	<0.001
Education, ≥college (%)	47 (63.5)	41 (67.2)	35 (63.6)	32 (61.5)	52 (66.7)	39 (65.0)	0.719
Total energy intake (kcal)	1946.2 (411)	1893.1 (391)	2029.3 (343)	2088.5 (361)	1812.7 (362)	2023.5 (387)	0.354
Carbohydrate (g)	266.5 (56.5)	272.8 (52.1)	298.1 (55.8)	292.7 (58.3)	260.5 (53.4)	287.7 (52.8)	0.566
Carbohydrate (% energy)	54.6 (6.0)	57.7 (5.9)	58.3 (5.5)	55.6 (6.1)	57.2 (6.3)	56.9 (5.5)	0.326
Protein (g)	77.4 (23.4)	71.8 (17.3)	77.9 (15.2)	82.6 (15.6)	71.2 (21.4)	75.8 (18.1)	0.079
Protein (% energy)	15.7 (3.1)	15.1 (1.9)	15.2 (1.9)	15.7 (2.4)	15.5 (2.8)	14.8 (1.9)	0.047
Fat (g)	65.7 (20.5)	58.5 (19.2)	60.6 (16.3)	67.7 (18.0)	56.0 (17.9)	64.7 (19.8)	0.872
Fat (% energy)	29.7 (4.6)	27.2 (5.0)	26.5 (4.6)	28.7 (5.1)	27.3 (5.0)	28.2 (4.6)	0.843
Total fatty acids (g)	30.4 (12.9)	30.6 (12.1)	34.3 (15.1)	38.3 (16.9)	28.3 (10.0)	30.3 (11.9)	0.001
PUFAs (g)	7.6 (3.1)	7.7 (2.8)	8.8 (3.0)	9.1 (3.2)	7.5 (2.5)	7.7 (3.3)	0.018
MUFAs (g)	11.3 (5.5)	11.4 (5.2)	12.8 (6.4)	14.8 (7.5)	10.4 (4.0)	10.9 (4.7)	<0.001
Omega-3 fatty acids (g)	0.13 (0.34)	0.18 (0.36)	0.33 (0.63)	0.29 (0.66)	0.19 (0.38)	0.14 (0.31)	0.124
Calcium (mg)	553.3 (227)	551.3 (195)	658.0 (231)	555.1 (221)	541.2 (206)	663.5 (227)	<0.001
Iron (mg)	12.0 (4.1)	12.8 (5.0)	14.6 (7.6)	13.8 (3.8)	12.0 (4.6)	13.7 (7.8)	0.669
Zinc (mg)	9.3 (2.5)	9.2 (2.1)	9.7 (2.0)	10.1 (2.1)	8.7 (2.1)	9.6 (2.3)	0.293
Mercury (µg/kg bw)	0.19 (0.05)	0.21 (0.06)	0.23 (0.06)	0.19 (0.06)	0.21 (0.06)	0.22 (0.06)	0.001
Lead (µg/kg bw)	0.45 (0.13)	0.49 (0.15)	0.50 (0.18)	0.44 (0.16)	0.47 (0.14)	0.51 (0.16)	0.006

<sup>1</sup> Tertiles of dietary pattern scores; <sup>2</sup> P trend was calculated using generalized linear models for continuous variables and using Mantel-Haenszel chi-squared tests for categorical variables; P trend of nutrient and metal consumption was adjusted for total energy intake; PUFAs: Polyunsaturated fatty acids, MUFAs: Monounsaturated fatty acids.

**Table 4.** Distribution of characteristics by the tertiles of dietary pattern scores <sup>1</sup>.

Dietary Pattern		<i>N</i>	Crude Model	Multivariate Model 1 <sup>2</sup>	Multivariate Model 2 <sup>3</sup>
		Control/Case			
Traditional	T1 <sup>4</sup>	32/42	1	1	1
	T2	32/42	1.00 (0.52–1.92)	1.32 (0.61–2.84)	1.88 (0.80–4.42)
	T3	32/12	0.29 (0.13–0.64)	0.43 (0.18–1.04)	0.76 (0.26–2.24)
	<i>P</i> trend <sup>5</sup>		0.003	0.072	0.615
Seaweed-egg	T1	32/37	1	1	1
	T2	32/33	0.89 (0.45–1.76)	0.66 (0.30–1.44)	0.70 (0.31–1.55)
	T3	32/26	0.70 (0.35–1.42)	0.64 (0.29–1.41)	0.84 (0.36–1.94)
	<i>P</i> trend		0.321	0.271	0.682
Traditional-healthy	T1	32/42	1	1	1
	T2	32/30	0.71 (0.36–1.41)	0.60 (0.27–1.32)	0.57 (0.25–1.29)
	T3	32/24	0.57 (0.28–1.15)	0.32 (0.13–0.77)	0.31 (0.12–0.79)
	<i>P</i> trend		0.113	0.011	0.014
Snack	T1	32/20	1	1	1
	T2	32/48	2.40 (1.17–4.91)	2.93 (1.22–7.05)	2.34 (0.95–5.79)
	T3	32/28	1.40 (0.66–2.98)	1.69 (0.70–4.07)	1.59 (0.65–3.91)
	<i>P</i> trend		0.571	0.451	0.505

<sup>1</sup> All analyses were performed with the data matched for age, sex, and dietary survey season; <sup>2</sup> Adjusted for gestation age, birth weight, mother's age, birth order, father's education, and father's occupation; <sup>3</sup> Model 2 + additional adjustment for total energy intake, omega-3 fatty acids, lead, and mercury consumption; <sup>4</sup> Tertiles of dietary pattern scores; <sup>5</sup> Tests for trend were conducted by assigning the median value to each tertile of heavy metal intake as a continuous variable.

#### 4. Discussion

The present study identified four dietary patterns. The traditional-healthy dietary pattern, characterized by high intakes of kimchi, grains, and bonefish, and low intakes of fast foods and beverages, was associated with lower odds having ADHD. Although the present study focused on dietary factors, significant associations with ADHD were found in father's education and occupation. Socioeconomic status of children is generally related to household income, and parent's educational background and occupation. Children from lower socioeconomic status are more likely diagnosed with ADHD than children from higher socioeconomic status in previous studies [14–17]. Family income [14,15], parent's education [15–17] and occupation [15,16] were significantly associated with ADHD. Education status of mother was highly correlated with that of fathers in this study, and occupation of mother did not vary compared to that of father's. Thus, fathers' educational background and occupation were used as surrogate of socioeconomic status. As those variables were high associated with ADHD, we adjusted them for the analysis.

The role of diet in the behavior of children has been controversial, but associations between several nutritional factors and child behavior such as ADHD have been continually suggested [12,13]. Food additives, sugar, and aspartame are considered negative factors in the development of ADHD, and thus, dietary intervention studies with special diets, including additive-free and sugar elimination diets, have been conducted. A meta-analysis has reported that artificial food coloring is associated with

childhood hyperactivity [18]. However, in a sugar elimination intervention study, there was no evidence that refined sugar affected child behavior [19–24].

The role of polyunsaturated fatty acids (PUFAs), particularly omega-3 fatty acids, in relation to neurodevelopmental disorders has been studied because omega-3 fatty acids play a critical role in brain development and function [25]. Children with ADHD have lower levels of omega-3 fatty acids, and the supplementation of omega-3 fatty acids can reduce the symptoms of ADHD in school-aged children and adolescents [26,27]. However, there was no clear evidence of improvement in ADHD symptoms with omega-3 supplementation in randomized controlled trials, but these findings could be the result of methodological problems [28,29]. The association between dietary pattern score and fatty acid intake was investigated in this study. The traditional, seaweed-egg, and traditional-healthy pattern scores were negatively associated with ADHD, although only the traditional-healthy pattern had a statistically significant association; moreover, they were positively associated with fatty acid intake. By contrast, the snack pattern score showed a positive association with ADHD and was negatively associated with the intake of total fatty acids, PUFAs, and MUFAs. However, additional adjustment for omega-3 fatty acid intake did not change the statistically significant association between the traditional-healthy dietary pattern and ADHD. Thus, the factors associated with the beneficial effects of a healthy dietary pattern might be complex.

Regarding mineral intake, calcium was positively associated with the scores of the traditional, traditional-healthy, and snack patterns, and iron was positively associated with the scores of the traditional and traditional-healthy patterns. Zinc was not associated with any of the four pattern scores. Iron deficiency may be associated with ADHD [30] because iron stores in the brain can influence dopamine-dependent functions [31,32]. A case-control study in India reported that the serum ferritin level was lower in children with ADHD [33], while another study found that ADHD symptoms in children with low serum ferritin levels were alleviated following iron supplementation [34]. In a 19-year follow-up study, the iron status of Costa Rican children was found to be associated with behavioral problems in adolescents [35]. The role of zinc nutrition in ADHD is not clear, but evidence suggests that zinc is beneficial in the treatment of children with ADHD [36,37]. Zinc deficiency is involved in dopamine transporter dysfunction [38], and intervention studies have found that zinc supplementation can reduce ADHD symptoms in children with low zinc levels [39–41]. Both, low iron and zinc levels have been associated with dopamine metabolism, and low levels of iron and zinc are involved in impaired dopamine transmission in subjects with ADHD [42–45].

Heavy metal exposure via food consumption was also investigated. Mercury was positively associated with the traditional, traditional-healthy, and snack patterns, and lead was positively associated with the traditional and snack patterns. The association between lead exposure and ADHD has been widely studied, and a meta-analysis has reported that lead exposure is positively associated with ADHD symptoms [46]. In a study with school-aged children living in two Romanian cities near a metal-processing plant, an association with ADHD was observed only for lead exposure, not aluminum or mercury exposure [47]. An association between the blood mercury level and ADHD in Chinese children in Hong Kong has been observed [48], but a significant association was not found in a cross-sectional study of Romanian children [47] or in a Children's Health and Environment Research (CHEER) study that surveyed elementary schools in six South Korean cities [49,50]. A more clear association with ADHD has been observed for lead exposure, even at low concentrations [49,50].

Prenatal mercury exposure is associated with an increased risk of neurobehavioral disorders, and lead exposure in childhood has been associated with ADHD [51]. In this study, lead and mercury consumption was positively correlated with the traditional-healthy dietary pattern, but it did not alter the beneficial effects of the traditional-healthy dietary pattern on ADHD.

Recently, associations between dietary patterns and ADHD have been examined in several cross-sectional studies [6,20,21]. One study, which included a population-based cohort of adolescents, reported that a Western-style dietary pattern, characterized by high intakes of fat, refined sugars, and sodium and low intakes of fiber, folate, and omega-3 fatty acids, was associated with increased odds of an ADHD diagnosis, whereas a healthy dietary pattern, with high intakes of fiber, folate, and omega-3 fatty acids, was not correlated with the diagnosis of ADHD [20]. In a study of adolescents in China, three major dietary patterns were identified, and dietary patterns characterized by a high intake of snacks or animal-derived foods were associated with higher odds for psychological symptoms [21]. In Iranian school-aged children, four major dietary patterns were identified. The higher scores of the dietary patterns associated with a high intake of sweets and fast food were associated with greater odds for having ADHD, but no significant association was observed for the healthy or Western dietary patterns [6]. In this study, traditional-healthy dietary pattern was positively associated with dietary factors, such as PUFAs and minerals that are known for beneficial effects on ADHD. Another beneficial effect of the traditional-healthy dietary pattern might be associated with the low fast food intake. Junk foods are generally high in fat, sugar, additives, artificial food colorings, and preservatives, which may negatively affect ADHD symptoms [52]. Overall, the traditional-healthy dietary pattern was associated with many dietary factors that affect childhood behavioral disorders, such as ADHD.

The present study has several limitations. As this was a case-control study, it is possible that dietary intake was affected by an individual's health status and social background. Thus, causal inference cannot be determined. Results could differ by ADHD types, but information about ADHD type was not gathered for subgroup analysis due to small sample size. However, such pattern analyses are useful to further understand the diet of ADHD children as a whole rather than classifying it by a single nutrient or food group.

## **5. Conclusions**

The traditional-healthy dietary pattern, which is characterized by high intakes of kimchi, grains, and bonefish, and low consumption of fast foods and beverages, appears to be negatively associated with ADHD in school-aged Korean children.

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## Author Contributions

Conceived and designed the experiments: HDW, DWK, YSH, BMC, JHP, JWK, JHY, HWC, JHL, MJK, YMK, JHS, JK. Contributed to the acquisition of data: BMC, JHP, JWK, JHY, HWC, JHL, MJK, YMK, JHS. Analyzed the data: HDW, DWK, JK. Wrote the paper: HDW, JK.

## Conflicts of Interest

The authors declare no conflict of interest.

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## Low Blood Zinc, Iron, and Other Sociodemographic Factors Associated with Behavior Problems in Preschoolers

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**Abstract:** Previous research supports the link among malnutrition, cognitive dysfunction, and behavioral outcomes; however, less research has focused on micronutrient deficiencies. This study investigates whether micronutrient deficiencies, specifically blood zinc and iron levels, will be associated with increased behavior problem scores, including internalizing and externalizing behaviors. 1314 Children (55% boys and 45% girls) from the Jintan Preschool Cohort in China participated in this study. Venous blood samples were collected and analyzed for zinc and iron when the children were 3–5 years old. Behavior problems were measured with the Child Behavior Checklist (CBCL), which was completed by the parents when children were in their last months of preschool (mean age 5.6 years). General linear multivariate modeling was used, with adjustment for important sociodemographic variables. The results indicate that low zinc levels alone ( $p = 0.024$ ) and combined low zinc and iron levels ( $p = 0.022$ ) are significantly associated with increased reports of total behavior problems. We did not find an association between low iron and behavior problems. With regards to sociodemographics, living in the suburbs is associated with increased internalizing problems, while higher mother's education and being female were associated with decreased externalizing problems. This study suggests that micronutrient deficiencies and sociodemographic facts are associated with behavior problems in preschoolers.

**Keywords:** internalizing; externalizing; total behavior; CBCL; child; micronutrient deficiency; zinc and iron

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## 1. Introduction

The link between early nutrition deficiency and behavior outcomes has been receiving increasing attention [1–3]. At the prenatal level, Neugebauer, Hoek, and Susser [4] found that the male offspring of nutritionally-deprived pregnant women had 2.5 times the normal rate of antisocial personality disorder in adulthood. At the postnatal level, in a longitudinal study from the Mauritius birth cohort [5], it was found that children with malnutrition (protein, zinc, iron and vitamin B deficiencies) at age 3 years, compared to controls, have higher externalizing behavior problems (*i.e.*, antisocial, aggressive, and hyperactive behavior) at ages 8, 11, and 17 years [6]. In another more recent longitudinal study, Galler *et al.* [3] found that children who were malnourished at an early age showed significantly higher parent-reported levels of behavior problems, particularly aggression, and decreased executive functioning at age 9–15 and again at 11–17, independent of baseline age, sex, household standard of living, and maternal depressive symptoms. Finally, at the intervention level [7], a double-blind, placebo-controlled randomized trial from England showed that supplementation of adult prisoners' diet with vitamins, minerals, and essential fatty acids significantly reduced antisocial and violent behavior in prison. These findings have been recently replicated in young prisoners in the Netherlands [8]. This initial evidence supports the relationship between nutrition and behavioral problems; however, more research is still needed.

While increasing studies have showed the association between overall nutritional status and child behavior, few studies have specifically investigated blood zinc and iron status in relation to behavior. In developing countries, low zinc and iron levels are common [9–11]. Indeed, more than 90% of affected individuals live in developing countries, and approximately one-tenth of the worldwide population suffers from iron deficiency [12]. Furthermore, few studies have been conducted in Asian populations. In China, zinc and iron deficiency were previously very common, but over the past two to three decades, reports on zinc and iron intake have been mixed due to socioeconomic reform and rapid economic development that have taken place since 1979. The availability [13,14] and affordability [15] of foods have increased dramatically during this time, and as a result of this increased food production and access to food, the prevalence of malnutrition has decreased, while over-nutrition has increased [16,17]. Studies indicate that iron deficiency is less prevalent than zinc deficiency among Chinese children [18,19], and although the prevalence of anemia has decreased in China, it still exists among children [16,20]. Taken together, this makes the consideration of zinc and iron intake in Chinese samples a relevant issue for better understanding putative risk factors for behavioral outcomes.

Zinc and iron play important roles in children's physical and behavioral health; however, there is a relative lack of attention given to the effects of specific micronutrient (e.g., zinc and iron) deficiency on behavior problems, including internalizing and externalizing disorders. Zinc is a component of enzymes that affect growth in infancy and childhood, sexual maturation, neuromotor development, and immunity. Mental function is improved by zinc's promotion of normal brain development and

physiology [21–23]. Iron similarly boosts mental functioning by serving as a co-enzyme involved in the production and release of neurotransmitters [21,22] and by influencing cognitive function [24,25] and behavioral disorders such as attention-deficit hyperactivity disorder [6,26].

There is now increasing evidence of the relationship between malnutrition and childhood behavior problems [27–30], though more data are needed to address the impact of specific micronutrient deficiencies on both internalizing and externalizing problems separately [2]. The importance of zinc and iron in physiological development seems to warrant particular attention with regard to how these micronutrients relate to behavioral outcomes. Childhood behavioral problems represent an important sub-area of developmental psychopathology [31–34]. Thus, identifying early childhood behavior problems—and, perhaps more importantly, their early risk factors, including nutritional and sociodemographic factors—is important for understanding and preventing problem behaviors later in life [35,36]. The purpose of this study is to assess the association of micronutrients controlled for sociodemographic factors with behavior outcomes. We hypothesize that nutritional deficiencies, specifically zinc and iron deficiencies, will be associated with increased behavior problems.

## **2. Experimental Section**

### *2.1. Participants and Procedures*

The current study was part of a population-based community preschool cohort study of 1656 Chinese children (55.5% boys, 44.5% girls) initially recruited between the Fall of 2004 and the Spring of 2005 from four preschools in the city of Jintan, located in the southeastern coastal region of Mainland China. In China, preschools are called kindergartens and enroll children from ages 3–6 years, after which children enter the elementary school system; to be consistent, we use preschool to refer to our study sample. Detailed sampling and research procedures of this larger cohort study have been described elsewhere [37,38]. Briefly, all children and parents taking part in the original cohort study were invited to participate for assessment of children's behaviors while the children were in the final few months of their senior year in preschool (spring 2005 to spring 2007). At that point, some children dropped out of the study because they changed schools or because data were not fully available. Therefore, only 1385 children in the original sample were followed up in the later waves. There was no statistically significant difference between those who dropped out of the study and those who were retained [37,39].

In the last year of preschool, parents were asked to assess their children with the Chinese version of the Child Behavior Checklist (CBCL/1.5–5). Since some of the children were beyond the age limit of the CBCL/1.5–5, the current analysis only addressed the subset of the original sample that was under age 6 to adhere to the age requirement of the measure. Our final data set for analysis was thus comprised of 1314 preschoolers with a mean age of 66.6 months ( $SD = 5$ , range = 50–71), which is close to the common kindergarten age in the US. Written informed consent was obtained from parents. Institutional Review Board (IRB) approval was obtained from the University of Pennsylvania and the ethical committee for research at Jintan Hospital in China.

## 2.2. Measures

### 2.2.1. Micronutrient Deficiency

Blood specimens were collected in Fall 2004 and Spring 2005 by trained pediatric nurses using a strict research protocol to avoid lead contamination. Approximately 0.5 mL of venous blood was collected in a lead-free EDTA tube for zinc and iron analysis. Samples were frozen and shipped to the Child Development Center, Nanjing Medical University, Nanjing, China, for analysis. Specimens remained frozen at  $-20^{\circ}\text{C}$  until analysis. Blood concentrations of zinc and iron were determined by atomic absorption spectrophotometry (BH model 5.100 manufactured by Beijing Bohu Innovative Electronic Technology Corporation), with duplicate readings taken with an integration time of 2 s. The reliability and validity of the analysis and the detailed analytic procedure have been described previously [40]. Detailed information on blood sample data collection and analysis is given in [39].

Low zinc levels were defined by concentration  $<76.5\ \mu\text{g/dL}$  and low iron by concentration  $<7.5\ \mu\text{g/dL}$  in blood, with cutoffs determined from the middle of the normal range. Combined low zinc and iron were defined as currently low zinc and low iron concentrations, *i.e.*, children in this category have both  $\text{Zn} <76.5\ \mu\text{g/dL}$  and  $\text{Fe} <7.5\ \mu\text{g/dL}$ .

### 2.2.2. Behavior Problems at Ages 5–6

Childhood behavior problems were measured with the Chinese version of the Achenbach System of Empirically Based Assessment (ASEBA) CBCL/1.5–5 [41]. The CBCL is a widely used scale for assessing behavioral and emotional problems in children. In this study, parents were asked to answer the 99 items of the CBCL instrument, which dealt with their children's behavior within the past 12 months, and give a rating from a 3-point scale (0 = not true; 1 = sometimes true, or 2 = often true) [41]. Factor analysis performed on the CBCL/1.5–5 has revealed two broadband factors: Internalizing behaviors and Externalizing behaviors [42]. Separately, factor analysis has produced four syndromes for Internalizing behaviors: Emotionally Reactive, Anxious/Depressed, Somatic Complaints, and Withdrawn; and two syndromes for Externalizing behaviors: Attention Problems and Aggressive Behavior [41,43]. These factor structures have also been validated in our previous study [44]. The internal reliabilities (coefficient alpha) for the scales in our study sample were as follows: Emotionally Reactive (0.71), Anxious/Depressed (0.64), Somatic Complaints (0.58), and Withdrawn (0.73), Attention Problems (0.64) and Aggression (0.87). The strategy we employ in this study is to use these established scales as predictors of latent construct "Internalizing behavior" and "Externalizing Behavior". The sum of the items in the scales was used.

### 2.2.3. Sociodemographic Variables

Sociodemographic information was obtained from the questionnaire filled out by the parents, and included information on gender, parental education, home living conditions, and the age of mother when the child was born. These data were collected as control variables given their potential direct effects on child behavioral problems. As discussed in our previous publication [39], we did not ask for data on household income because it is often not the best indicator of socioeconomic status, therefore

we used information on house size as a proxy for evaluating socioeconomic status. A descriptive summary of these demographic variables is presented in Table 1.

**Table 1.** Baseline characteristics of study population ( $N = 1314$ ).

Characteristic	$N$	%
<b>Gender</b>		
Male	758	55
Female	614	45
<b>Location</b>		
City	959	74
Suburb	188	14
Rural	153	12
<b>Mother's Education</b>		
Low ( $\leq 9$ years)	625	48
Medium (9–12 years)	411	32
High ( $> 12$ years)	264	20
<b>Family Size</b>		
$> 3$ persons/household	713	57
$\leq 3$ persons/household	548	43
<b>House Size (<math>m^2</math>)</b>		
$< 100$	561	45
$\geq 100$	696	55

### 2.3. Statistical Analyses

Descriptive statistics, including frequencies and percentages, are used to characterize categorical demographic subject characteristics (Table 1). Behavioral outcomes measured on a continuum are described using means and standard deviations, and compared by low zinc and iron level groups. Multivariate modeling of behavioral outcomes regressed on low zinc and iron group, with adjustment for important sociodemographic variables (family size, gender, house size, mother's education) is accomplished using general linear modeling. The father's education was not included in the analysis to avoid multicollinearity because the variable was found to be highly correlated with the mother's education. Models also accounted for clustering at the school level. Levine's tests are used to test for homogeneity of variance across all cells. In an attempt to identify multicollinearity, bivariate associations between independent variables are examined using chi-square tests. Statistical significance was taken at the two-sided  $p < 0.05$  level. All the analyses were performed using STATA version 11.0 [45].

### 3. Results

Mean behavior problem scores for total behavior, and internalizing and externalizing behavior problems are described in Tables 2–4 according to Zn level (Table 2), Fe level (Table 3), and to Zn and Fe levels (Table 4).

**Table 2.** Children's behavior problems by Zn level ( $N = 1314$ ).

Variable	Low Zn <sup>†</sup>			Normal Zn		
	<i>N</i>	Mean	SD	<i>N</i>	Mean	SD
Total Behavior Problems	502	21.32	17.64	812	18.8	16.61
Internalizing Behavior Problems	502	6.42	5.91	812	5.74	5.84
Externalizing Behavior Problems	502	9.22	9.69	812	7.95	8.43

<sup>†</sup> Defined as zinc <76.5 µg/dL.

**Table 3.** Children's behavior problems by Fe level ( $N = 1314$ ).

Variable	Low Fe <sup>†</sup>			Normal Fe		
	<i>N</i>	Mean	SD	<i>N</i>	Mean	SD
Total Behavior Problems	307	21.1	17.65	1007	19.36	16.85
Internalizing Behavior Problems	307	6.57	5.94	1007	5.83	5.85
Externalizing Behavior Problems	307	8.78	9.65	1007	8.33	8.73

<sup>†</sup> Defined as iron <7.5 µg/dL.

**Table 4.** Children's behavior problems by Zn and Fe level ( $N = 1314$ ).

Variable	Low Zn and Fe <sup>†</sup>			Others		
	<i>N</i>	Mean	SD	<i>N</i>	Mean	SD
Total Behavior Problems	215	21.41	18.2	1099	19.44	16.8
Internalizing Behavior Problems	215	6.63	5.99	1099	5.88	5.85
Externalizing Behavior Problems	215	9.07	10.18	1099	8.31	8.69

<sup>†</sup> Defined as zinc <76.5 µg/dL and iron <7.5 µg/dL.

### 3.1. Zinc Status and Children's Behavior

Results from our analyses (Table 5) using the model of total behavior score regressed on deficient zinc showed that low zinc status was significantly associated with higher total behavior problems ( $p = 0.024$ ) in children, along with living in the suburbs, whereas high mother's education and being female were associated with lower behavior problems. The model of externalization score regressed on low zinc showed being female and high mother's education are associated with significantly lower externalizing behavior scores. The model of internalizing score regressed on low zinc showed living in the suburbs to be positively associated with internalizing problems.

### 3.2. Iron Status and Children's Behavior

We did not find significant association between low iron status and internalizing, externalizing, or total behavior problems (Table 6). However, we observed that several sociodemographic indicators were significantly associated with behavior problems. The analysis using the model of total behavior score regressed on low iron indicated that being female and having high level of mother's education are associated with significantly lower (better) total behavior scores. Living in the suburbs was also positively associated with total behavior problems. For the model of externalization score regressed on low iron, being female and high mother's education were also associated with significantly lower

externalizing behavior scores. The model of internalizing score regressed on low iron showed living in the suburbs to be positively associated with internalizing problems.

### 3.3. Zinc and Iron Status and Children's Behavior

Results from our analyses (Table 7) using the model of total behavior score regressed on combined low levels of zinc and iron, having low zinc and iron was significantly associated with higher total behavior problems ( $p = 0.022$ ), whereas high mother's education and being female were associated with reduced total behavior score. The model of externalization score regressed on combined low zinc and iron showed that being female and high mother's education are also associated with significantly lower externalizing behavior scores. The model of internalizing score regressed on combined low zinc and iron showed living in the suburbs to be positively associated with internalizing problems.

## 4. Discussion

In this community sample of Chinese pre-school children ( $N = 1314$ ), with micronutrient levels measured at ages 3–5 years and behavioral problems measured at mean age 5.6 years, we found an association between micronutrient deficiency and total behavior problems. Firstly, we found that low zinc concentration is positively correlated with total behavior problems. Secondly, we found that combined low blood levels of zinc and iron is positively correlated with total behavior problems. These effects remained significant after controlling for sociodemographic factors such as gender and mother's education. We did not find a significant association between low iron status and behavior problems.

The finding of the association of zinc deficiency with child behavior problems is consistent with previous findings [46,47]. Zinc is a component of enzymes that affect growth in infancy and childhood, sexual maturation, neuro-motor development, and immunity. Specifically, zinc acts as the integral enzymatic agent in metabolic processes of proteins, carbohydrates, and lipids [48] and is used as a neurotransmitter or neuromodulator in the central nervous system [49]. Mental function is improved by zinc's promotion of normal brain development and physiology [22,23]. A recent study reported a relationship between low zinc and greater levels of hyperactivity, anxiety, and conduct problems [50]. Indeed, animal and human models have suggested a relationship between low serum zinc and anxiety, fear-like behaviors, and depression—implicating the role of the dopaminergic and serotonergic systems [51,52].

**Table 5.** The effect of Zinc on children's behavior problems ( $N = 1314$ ).

Variable	Total Problems ( $R^2 = 0.08$ )			Internalizing ( $R^2 = 0.04$ )			Externalizing ( $R^2 = 0.11$ )		
	$\beta$ (SE)	95% CI	$p$	$\beta$ (SE)	95% CI	$p$	$\beta$ (SE)	95% CI	$p$
Low Zn <sup>†</sup>	2.13 (0.33)	0.70–3.57	0.024*	0.63 (0.31)	-0.71–1.97	0.180	1.04 (0.28)	-0.17–2.25	0.066
Gender:									
Male	-	-	-	-	-	-	-	-	-
Female	-5.27 (1.21)	-10.43–-0.05	0.049*	-0.00 (0.54)	-2.32–2.33	0.994	-4.43 (0.38)	-6.07–-2.78	0.007*
Location:									
City	-	-	-	-	-	-	-	-	-
Suburb	8.44 (1.47)	2.11–14.78	0.029*	3.23 (0.32)	1.87–4.60	0.010*	3.13 (0.91)	-0.78–7.03	0.075
Rural	1.96 (3.61)	-13.58–17.51	0.641	0.55 (0.83)	-3.03–4.13	0.576	0.96 (2.05)	-7.86–9.78	0.686
Mother's Education:									
Low ( $\leq 9$ years)	-	-	-	-	-	-	-	-	-
Medium (9–12 years)	-2.51 (1.83)	-8.44–3.41	0.209	-0.52 (0.83)	-4.07–3.04	0.595	-1.45 (0.37)	-3.05–0.15	0.060
High ( $> 12$ years)	-4.58 (0.50)	-6.71–-2.44	0.012*	-0.74 (0.54)	-3.06–1.57	0.301	-2.79 (0.59)	-5.34–-0.26	0.042*
Family size:									
$\leq 3$	-	-	-	-	-	-	-	-	-
$> 3$	-0.83 (0.59)	-3.36–1.70	0.295	-0.07 (0.13)	-0.63–0.48	0.622	-0.50 (0.26)	-1.63–0.65	0.205
House Size:									
$< 100$ m <sup>2</sup>	-	-	-	-	-	-	-	-	-
$\geq 100$ m <sup>2</sup>	-1.09 (0.91)	-5.01–2.83	0.354	-0.07 (0.14)	-0.69–0.55	0.687	-0.90 (0.46)	-2.90–1.09	0.191

<sup>†</sup> Defined as zinc  $< 76.5$   $\mu\text{g/dL}$ ; \* Statistically significant at two-sided  $p < 0.05$  level.

**Table 6.** The effect of Iron on children's behavior problems ( $N = 1314$ ).

Variable	Total Problems ( $R^2 = 0.07$ )			Internalizing ( $R^2 = 0.04$ )			Externalizing ( $R^2 = 0.10$ )		
	$\beta$ (SE)	95% CI	$p$	$\beta$ (SE)	95% CI	$p$	$\beta$ (SE)	95% CI	$p$
Low Fe <sup>†</sup>	1.34 (0.79)	-2.07–4.74	0.233	0.59 (0.27)	-0.59–1.77	0.166	0.31 (0.39)	-1.36–1.98	0.510
Gender:									
Male	-	-	-	-	-	-	-	-	-
Female	-5.29 (1.18)	-10.35–-0.22	0.046 *	0.00 (0.53)	-2.28–2.28	0.999	-4.43 (0.37)	-6.03–-2.84	0.007 *
Location:									
City	-	-	-	-	-	-	-	-	-
Suburb	8.61 (1.57)	1.84–15.38	0.032 *	3.27 (0.32)	1.89–4.66	0.009 *	3.22 (0.96)	-0.91–7.35	0.079
Rural	2.07 (3.75)	-14.08–18.22	0.636	0.58 (0.87)	-3.15–4.32	0.571	1.01 (2.12)	-8.11–10.14	0.680
Mother's Education:									
Low ( $\leq 9$ years)	-	-	-	-	-	-	-	-	-
Medium (9–12 years)	-2.45 (1.44)	-8.66–3.77	0.232	-0.50 (0.85)	-4.16–3.17	0.619	-1.42 (0.34)	-2.90–0.06	0.054
High ( $> 12$ years)	-4.46 (0.48)	-6.51–-2.41	0.011 *	-0.71 (0.57)	-4.88–1.59	0.336	-2.74 (0.54)	-5.08–-0.40	0.037 *
Family size:									
$\leq 3$	-	-	-	-	-	-	-	-	-
$> 3$	-0.95 (0.55)	-3.29–1.40	0.224	-0.11 (0.12)	-0.62–0.41	0.467	-0.56 (0.24)	-1.961–0.49	0.150
House Size:									
$< 100$ m <sup>2</sup>	-	-	-	-	-	-	-	-	-
$\geq 100$ m <sup>2</sup>	-1.23 (0.93)	-5.24–2.79	0.319	-0.11 (0.17)	-0.83–0.61	0.586	-0.97 (0.46)	-2.94–1.00	0.168

<sup>†</sup> Defined as iron  $< 7.5$   $\mu\text{g/dL}$ ; \* Statistically significant at two-sided  $p < 0.05$  level.

**Table 7.** The effect of Zinc and Iron on children's behavior problems ( $N = 1314$ ).

Variable	Total Problems ( $R^2 = 0.07$ )			Internalizing ( $R^2 = 0.04$ )			Externalizing ( $R^2 = 0.10$ )		
	$\beta$ (SE)	95% CI	$p$	$\beta$ (SE)	95% CI	$p$	$\beta$ (SE)	95% CI	$p$
Low Zn & Fe <sup>†</sup>	1.53 (0.23)	0.53–2.53	0.022 *	0.69 (0.23)	-0.30–1.69	0.095	0.49 (0.23)	-0.752–1.49	0.174
Gender:									
Male	-	-	-	-	-	-	-	-	-
Female	-5.27 (1.18)	-10.31–-0.21	0.046 *	-0.01 (0.53)	-2.28–2.30	0.991	-4.43 (0.37)	-6.02–-2.84	0.007 *
Location:									
City	-	-	-	-	-	-	-	-	-
Suburb	8.62 (1.58)	1.83–15.42	0.032 *	3.28 (0.32)	1.90–4.66	0.009 *	3.22 (0.95)	-0.88–7.31	0.078
Rural	2.09 (3.77)	-14.12–18.31	0.634	0.59 (0.87)	-3.14–4.32	0.564	1.02 (2.12)	-8.12–10.16	0.678
Mother's Education:									
Low ( $\leq 9$ years)	-	-	-	-	-	-	-	-	-
Medium (9–12 years)	-2.45 (1.42)	-8.58–3.67	0.227	-0.50 (0.84)	-4.12–3.13	0.615	-1.42 (0.35)	-2.91–0.07	0.054
High ( $> 12$ years)	-4.46 (0.49)	-6.57–-2.34	0.012 *	-0.71 (0.55)	-3.09–1.68	0.329	-2.73 (0.55)	-5.10–-0.37	0.038 *
Family size:									
$\leq 3$	-	-	-	-	-	-	-	-	-
$> 3$	-0.97 (0.53)	-3.25–1.31	0.209	-0.12 (0.12)	-0.62–0.39	0.424	-0.56 (0.24)	-1.57–0.45	0.140
House Size:									
$< 100$ m <sup>2</sup>	-	-	-	-	-	-	-	-	-
$\geq 100$ m <sup>2</sup>	-1.19 (0.94)	-5.14–2.86	0.333	-0.09 (0.17)	-0.84–0.65	0.649	-0.96 (0.46)	-2.96–1.04	0.174

<sup>†</sup> Defined as zinc  $< 76.5$   $\mu\text{g/dL}$  and iron  $< 7.5$   $\mu\text{g/dL}$ ; \* Statistically significant at two-sided  $p < 0.05$  level.

The association of combined zinc and iron with children's behavior has been reported previously in [53]. It has been postulated previously that zinc and iron are thought to interact with one another for absorption. When levels of both zinc and iron are low, a more severe pattern of nutritional compromise is suggested. In fact, our results showed that blood zinc was positively correlated with blood iron ( $r = 0.31, p = 0.000$ ), consistent with other published reports [54,55]. As previously discussed, zinc and iron both play important enzymatic roles in the dopamine metabolism pathways [56–58], and it has been suggested that zinc and iron deficiencies together can lead to additive effects in dopaminergic system alterations [50]. Interestingly, Oner *et al.* [50] also reported that while combined low serum levels of zinc and iron are correlated with increased hyperactivity, as a single nutrient effect, only zinc deficiency, not iron deficiency, was related to conduct problems and anxiety. As concluded in the paper, Oner *et al.* [50] suggested that zinc and iron deficiencies might be associated with different types of behavior problems. However, it is also possible that the association of combine low zinc and iron with behavior problems is driven by the effect of low zinc alone.

While not the focus of this current paper, the sociodemographic control variables also had effects on our outcome constructs. It is worth noting that the children whose mothers had more than 12 years education exhibited decreased externalizing behavior problems in all of our analyses. As the mothers are postulated to be the primary caregiver of the child, this observation points to the fact that children of mothers who had more education tend to exhibit less behavior problems, possibly as the result of better parenting practices in addition to better nutritional habits as results of increased education concerning nutrition. Furthermore, being female is also associated with decreased externalizing behavior and total behavior problems; such that girls in our sample tended to have lower incidents of externalizing and total behavior problems than boys.

Additionally, living in the suburbs has consistently been shown to be significantly associated with increase in total behavior problems and, even more strongly, in internalizing behavior problems. This result might not be too surprising because there has been ample evidence in the literature supporting that children of economically affluent families tend to develop more internalizing behavior problems, such as anxiety, depression, and substance abuse [59,60]. Globally, an epidemiological study has found depression rates to be higher in developed countries than in others [61]. The results from our data support the literature in that the suburban residents in our sample have the highest parental occupation and parental education levels, above both the urban and rural groups. This finding is not surprising given the suburban preschool is in a “new development zone”, where up-and-coming young parents, generally of a better educational and socio-economic background, are pursuing relatively better and higher-paid occupations. Furthermore, parents who are in the transitional stage of social and economic rise in their lives and careers are likely to have high expectation of their children. Additionally, the parents themselves live very high-pressured lives from their own occupational demands, leading to the possibility that they are more likely to suffer from internalizing problems themselves, such as anxiety and depression. Previous studies have shown that parental symptoms of depression have been associated with children's problem behavior in clinical and community samples [62,63]. We postulate that this finding could be the result of a more stressful suburban parental lifestyle due

to social and occupational stress, factors that may indirectly affect their children by not having enough time to interact with them or through a decreased emphasis on food choice, which can contribute to nutrition status. Nevertheless, future research could include stress and lifestyle factors and their effects on malnutrition.

Limitations of this study should not be overlooked. First, these findings do not establish a causal relationship between zinc, iron and behavioral disturbance. However, results from intervention trials should be considered to elucidate whether a causal relationship truly exists. The nature of the study design also required that behavior be assessed during the last year of preschool while blood micronutrient levels were assessed when the children were ages 3–5 years. Consequently, participants differed in the time between times of micronutrient and behavior assessment. Secondly, nutrition was only assessed at a single point in time, making it difficult to generalize findings and assess the role of sustained nutrition deficiency, or even nutrition deficiency during the prenatal period. As a result, we were unable to separate the effects of chronic versus acute nutrition, which may have different implications on behavior [64]. Thirdly, although we included some sociodemographic factors in our analyses, other factors, such as income, should be considered in future studies. In addition, potential confounders such as the effects of other nutrients (e.g., vitamin D), food intake, and physical activity level should also be considered. Fourthly, examination of an all-Chinese sample in this age range limits application to other cultures, as cultural, ethnic, social, and age factors impact child rearing behaviors, including nutrition and feeding. Finally, while this study only included the parent-report, it would be equally important to consider other informants. Currently, the children in our cohort are at school age, and future studies will include youth self-report of behavior to assess the relationship between micronutrient deficiencies and behavior problems.

## **5. Conclusions**

Few studies have specifically examined the role of zinc and iron status in relation to child behavior. This sample of Chinese preschoolers suggests that low blood zinc is correlated to increased total behavior problems and that, additionally, combined low blood zinc and iron levels are also linked to increased total behavior problems early in childhood. Implications may include more public awareness of the importance of micronutrients. While sociodemographic factors are not easily modifiable, it is possible to encourage parents, children, and baby/child care professionals to make healthy food choices, including foods rich in zinc and iron.

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## Conflicts of Interest

Ethical approval was obtained from the Institutional Review Board at the University of Pennsylvania and Jintan Hospital. None of the authors declare any conflict of interest regarding the data and materials presented in this paper.

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## 5. Nutrition Assessment and Body Composition

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### Development and Pilot Testing of 24-Hour Multiple-Pass Recall to Assess Dietary Intake of Toddlers of Somali- and Iraqi-Born Mothers Living in Norway

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**Abstract:** The aim of this study was to develop, test, and evaluate a 24-h recall procedure to assess the dietary intake of toddlers of Somali- and Iraqi-born mothers living in Norway. A protocol for a 24-h multiple-pass recall procedure, registration forms, and visual tools (a picture library for food identification and portion size estimation) was developed and tested in 12 mothers from Somalia and Iraq with children aged 10–21 months. Five female field workers were recruited and trained to conduct the interviews. Evaluation data for the 24-h recall procedure were collected from both the mothers and the field workers. Nutrient intake was calculated using a Norwegian dietary calculation system. Each child's estimated energy intake was compared with its estimated energy requirement. Both the mothers and the field workers found the method feasible and the visual tools useful. The estimated energy intake corresponded well with the estimated energy requirement for most of the children (within mean  $\pm$  2 SD, except for three). The pilot study identified the need for

additional foods in the picture library and some crucial aspects in training and supervising the field workers to reduce sources of error in the data collection.

**Keywords:** 24-h recall; dietary assessment; infants; toddlers; Somalia; Iraq; immigrants; Norway

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## 1. Introduction

Dietary assessment studies are important for the development of public nutrition policies and interventions because they can identify population groups at risk of nutritional health problems and describe their dietary habits [1]. In Europe, some immigrant groups have a higher risk of developing nutrition-related diseases than the host population, in particular overweight/obesity and diabetes mellitus type 2 [2–4]. This may be due to changes in dietary habits and physical activity patterns influenced by a process of acculturation, urbanization and westernization [2,5]. Only relatively few studies describe dietary habits in immigrant groups, but these indicate diets with increased consumption of processed food after migration that replace healthy dietary components, such as fruits, vegetables, nuts, and whole grains [2,3].

Dietary studies among immigrant groups are hampered by a lack of suitable cultural-sensitive assessment methods and data collection procedures. Missing food composition data on ethnic foods and the use of dietary assessment methods, which are not critically assessed for suitability in these groups, may limit the reliability of dietary intake data among immigrants [2]. Furthermore, various methodological aspects, such as sampling and recruitment, tools and method of administration, among others, often require special attention [6]. In addition, dietary assessment among infants and children in particular has several inherent challenges, and these might be amplified among immigrant groups [1,7]. It is, therefore, important to exercise considerable caution when conducting dietary studies in this study group in order to reduce possible errors and increase validity.

Previous Norwegian national dietary surveys among infants (“Spedkost”, aged six months and 12 months) and toddlers (“Småbarnskost”, aged 24 months) excluded children of mothers born outside Scandinavia [8–10]. There are two main reasons for this exclusion: (1) the dietary assessment method used in the study was a food frequency questionnaire (FFQ) and the food list was not adapted to non-Scandinavian food habits and (2) the FFQ was only available in Norwegian. Thus, the method was less suitable for population groups with atypical food habits, poor Norwegian skills or lower literacy levels. The researchers expressed a need for separate studies among children of immigrant parents using more appropriate methods [11].

The “InnBaKost” study was initiated to address the limited knowledge about dietary habits and health among children in Norway with immigrant backgrounds. Children of Somali- and Iraqi-born mothers were chosen because they are the two non-Western immigrant groups currently with the highest number of births in Norway [12]. The aim of this research project was to collect information about breastfeeding practices and feeding patterns among infants aged six months with follow-up at 12 and 24 months to supplement the Spedkost and Småbarnskost surveys [8–10].

A modified FFQ was used for the data collection at six months of age. However, a structured 24-h recall method was considered more appropriate at ages 12 and 24 months. This because the FFQ requires that variations in food habits must be known and included in the food list to develop suitable FFQs [13,14]. The aim of the present study was to develop, pilot test and evaluate a protocol for a 24-h recall procedure, with registration forms and visual tools, to assess the dietary intake of toddlers of Somali- and Iraqi-born mothers living in Norway.

## 2. Methods

### 2.1. *Subjects and Study Design*

The pilot study was carried out January–June, 2013. Twelve Somali- and Iraqi-born mothers with children aged 10–21 months living in Oslo and Akershus counties, Norway, were recruited through several methods: the Norwegian National Population Register, open kindergartens and by using the snowball method. Inclusion criteria were the mothers' country of birth and the child being approximately 12 months old, born in Norway and with no serious health problem or disease requiring a special diet. The mothers received a bilingual information letter and provided written consent. Respondents received a shop voucher after completing two recalls. The study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics.

To measure the dietary intake among toddlers, a structured 24-h recall method was used [15]. In the 24-h recall method, the mothers were interviewed twice, usually 1–2 weeks apart, by trained field workers about the exact food and beverage intake of their child during the preceding 24 h. If other caretakers were involved, the mother was asked to obtain information about the child's food consumption while under their care. A researcher (C.M., M.B.A., or N.K.G.) was present and observed the interviews. In addition to the food and beverage intake of the child, information about the performance of the interviews and methods was collected through an evaluation form.

### 2.2. *The 24-H Recall Method*

#### 2.2.1. *Picture Library for Food Identification*

To help the mothers and the field workers identify the correct foods given to the child, a library with pictures of food items commonly eaten by children in Norway was developed. A list of food items to be included in the library was made based on knowledge about Norwegian children's dietary intake. In addition, food items identified as eaten by Somali and Iraqi children through an informal qualitative pre-study and food items suggested by the field workers were also included. The foods were photographed in supermarkets and independent shops owned by immigrants in Oslo, with permission from the owners/managers. A Canon Ixus 860 IS digital camera was used, and the pictures were edited in iPhoto on a MacBook Pro (Apple Inc., Cupertino, CA, USA).

The library contained pictures of a wide selection of industrially produced baby foods for children aged 8–15 months, as well as other foods and beverages. Before the pilot study, the field workers suggested adding Nido milk powder, different types of meat, cheese and biscuits. The

“bread scale”, a Norwegian labeling scheme for fiber and wholemeal content of bread, was also included in the library.

To ease retrieval of pictures during the interviews, the 336 unique pictures were categorized in 19 different folders on an iPad (Table 1). The folders contained between 4 and 50 pictures, with the largest number of pictures in the folder “fruits and vegetables”. Because certain foods may be categorized differently by different people, some pictures were placed in more than one folder. For instance, smoothies appeared both in the folders “snacks” and “juice and nectar”. Thus, the final library contained 405 pictures in 19 folders.

**Table 1.** Number of pictures in each folder of the picture library.

<b>Food Folder</b>	<b>Number of Pictures</b>
Baby cereals	16
Snacks	33
Infant formula	16
Ready-made meals	10
Bread spreads	19
Dinner	39
Yoghurt and desserts	29
Oils and butter	13
Dairy products	13
Fruits and vegetables	50
Breads	22
Pasta, rice and beans	17
Supplements	8
Milk	38
Juice and nectar	46
Soda	4
Squash, lemonade, <i>etc.</i>	17
Meat	4
Biscuits	11
<b>Total</b>	<b>405</b>

### 2.2.2. Photographic Booklet and Measuring Equipment for Portion Size Estimation

This study used a photographic booklet for portion size estimation developed for the Spedkost and Småbarnskost surveys [8–10]. It included 17 color photograph series of selected food items representing different, usually four, portion sizes appropriate for toddlers ranging from small (A) to large (D), with up to six different portion sizes for baby cereal. The mother used the booklet as a tool to identify portion sizes eaten by her child. The field workers also brought a kitchen scale and three measuring cups (in deciliters and milliliters) to weigh or measure foods or volume in tableware from the respondents’ homes whenever possible. Frequently, both methods were used to compare the results.

### 2.2.3. 24-H Recall Protocol and Registration Form

The protocol contained instructions on how the field workers should conduct the 24-h recalls based on standard procedures for face-to-face 24-h recall in the literature [16]. A 24-h period was

defined as starting at the time the child woke up the previous day until the time the child woke up the day of interview. The field worker informed the mother about the recall procedure at the beginning of each interview and recorded the answers in specially designed paper-based forms that matched the three-stage, multiple-pass interviewing technique [16]. In the first pass, the mother was asked to give a complete overview of all foods and beverages consumed during the 24-h period. If the mother was still breastfeeding her child, each breastfeeding occurrence was registered. In the second pass, a detailed description of each food and beverage consumed were obtained. This included type of product, brand names, cooking methods, amounts, and food leftovers. In this pass, the picture library was used to identify foods, and the photographic booklet and measuring equipment to estimate amounts. The field workers used standardized probe questions to collect specific details. In the last pass, the field workers summarized and reviewed the information to ensure that all items were recorded correctly. This phase also included a checklist of foods and beverages that are often forgotten, such as water, snacks, and supplements. Representativeness of the day and food allergies/intolerances was also registered. A separate questionnaire covered background information of the mother and child.

### *2.3. Training of Field Workers*

Five female field workers were recruited to conduct the interviews, of which two spoke Somali and three Arabic. All of them spoke fluent Norwegian and one of the Arabic-speaking field workers also spoke Kurdish. Thus, the mothers could choose to speak either Norwegian or their own language during the interviews.

The field workers received 1–2 weeks of training on how to conduct 24-h recalls according to the protocol using the forms and tools. Practice took place in pairs and in plenary using different languages. The training particularly emphasized how to ask follow-up questions to make sure all food items were registered, to identify the correct food items and to estimate portion sizes as accurately as possible.

### *2.4. Pilot Testing of the Procedures for 24-H Recall*

The pilot study enabled a full appraisal of all aspects of the 24-h recall procedure. The field worker and the observer recorded data and answered questions regarding the method after each interview using an evaluation form (Table 2). The mothers were asked for their views on the method, including the visual tools, after the second interview.

The dietary data obtained from the 24-h recalls were manually coded and entered by C.M., M.B.A., and N.K.G. in a software system (KBS, database AE-10) developed at the Department of Nutrition, University of Oslo, Norway. The food database in KBS was mainly based on the official Norwegian food composition table. Breast milk intake among the breastfed children was calculated by multiplying the number of feeding events by an estimated breast milk intake per feed of 124 mL. This amount of breast milk per feed was derived from an estimated daily breast milk intake of 497 mL among 12-month old children in developed countries [17] divided by the average breastfeeding frequency in Norwegian 12-month old breastfed children of 4 times per day [9] (497 mL/4 feeds = 124 mL/feed).

As an objective measure of validity for this pilot test, each child's estimated energy intake (EEI) was compared with its estimated energy requirement (EER). According to the Nordic Nutrition Recommendations, estimated average daily energy requirement for 12-month-old boys is 337 kJ/kg and 333 kJ/kg for girls [18]. We did not measure the children's weight, but recorded the weight registered at clinic for the 12-month health check-up (Table 3). However, eight of the children were interviewed at ages between 13 and 21 months and two were interviewed younger than 12 months of age. One of the children younger than 12 months of age had his body weight measured at the 8-month consultation at the child health center. Thus, for 9 of the 11 children with registered body weight, at least one month had passed between the weighing and the 24-h recall. To adjust for this, an estimate of monthly weight gain was calculated using the World Health Organization's growth standards for children between 0 and 24 months [19]. The estimated weight gain for boys 8–21 months of age varies by month and is highest from 8 to 9 months (3.25%) and decreases gradually to 1.76% from 20 to 21 months. For girls, weight gain from 12 to 13 months was found to be 2.64% and from 13 to 14 months average weight gain is 2.46%. Each child's body weight was thus calculated by adding the monthly estimated weight gain to its weight. For the child with no records of body weight registered, the average weight of 11-month old girls was used. Using the estimated body weight at the time of interview, EER for each child was calculated and compared with the EEI calculated from the 24-h recalls, using the mean intake of the two recalls. Differences between EER and EEI were tested with paired samples *t*-test. Bland-Altman plot [20] was used to visualize the dispersion between EER and EEI. Linear regression analysis was applied to study whether there was any relationship between the mean of the estimates EER and EEI and the difference between the two estimates.

**Table 2.** Evaluation form for the pilot study.

Source of Information	Evaluation Topic	Question Asked
Observation by researchers	Time spent by the field worker	Time spent on picture library (iPad)?
		Time spent on photographic booklet?
		Time spent on measuring equipment?
		Other notes?
Standardisation of methods/field workers	Use of visual tools	Which pictures were used most frequently or not at all?
		Did the field workers ask the questions in the same way?
		Did they follow the protocol? Did they use the visual tools?
Questions to respondents	Clarity of questions	Were any of the questions difficult to answer/unclear? If yes, which and why?
		Did you miss pictures of any foods/beverages?
	Missing pictures	Are there some foods/beverages you give your child often, but not yesterday?
		Are there any other foods/beverages you know Somali/Iraqi children often eat/drink?
Portion sizes	Did the portion sizes in the booklet match the portion sizes your child usually eats?	
	Was it easier to estimate the amount the child had eaten by using the booklet, measuring equipment or by showing it on/in the plate/cup used?	
Questions to field workers	24-h recall protocol	Was the protocol easy to understand? If no, why not?
		How did you experience the different passes during the interview? Was it easy to distinguish these from each other?
	Picture library	How did you experience using the picture library during the interview? Was it user friendly? If no, why not?
		How did you experience using the photographic booklet to estimate portion sizes? Did you miss photos of any foods/beverages?

Measuring equipment	How did you experience to estimate amounts using the measuring equipment? Did you miss any equipment?
Registration form for 24-h recall	How did you experience using the form? Was the order of items logical to you? Was there enough space to write? If no, where did you want more space?

**Table 3.** Estimated energy requirements and energy intake among children (10–21 months) with mothers from Iraq (ID 1–5) and Somalia (ID 6–12) living in Norway.

ID	Sex	Estimated Average Daily Energy Requirements (kJ/kg)	Body Weight at 12 Months (g)	Age at Time of Interview (months)	Estimated Body Weight at Time of Interview (g) <sup>a</sup>	EER at Time of Interview (kJ/day) <sup>b</sup>	EI at Time of Interview (kJ/day) <sup>c</sup>	Percentage Differences between EER and EI (%) <sup>d</sup>
1	F	333	8440	14	8855	2949	2843	–4
2	M	337	11,600	13	11,864	3998	3486	–14
3	M	337	11,083	21	13,268	4471	3649	–20
4	F	333	8719 <sup>e</sup>	11	8719	2903	4415	41
5	M	337	8300	12	8300	2797	3102	10
6	F	333	10,000	13	10,246	3412	4043	17
7	M	337	8200	12	8200	2763	2764	0
8	F	333	9970	14	10,460	3483	3157	–10
9	M	337	10,000	14	10,466	3527	2232	–45
10	M	337	11,000	14	11,513	3880	4635	18
11	M	337	9890 <sup>f</sup>	10	10,509	3542	3556	0
12	F	333	9270	13	9498	3163	4636	38
<b>Mean</b>		<b>335</b>	<b>9706</b>	<b>13</b>	<b>10,158</b>	<b>3407</b>	<b>3543</b>	<b>18<sup>g</sup></b>
<b>SD</b>		<b>2</b>	<b>1146</b>	<b>3</b>	<b>1544</b>	<b>527</b>	<b>773</b>	<b>15<sup>g</sup></b>

<sup>a</sup> Estimated body weight at time of interview calculated based on average growth rate from World Health Organization's growth standards [19] multiplied by number of months between time of weighing and time of interview; <sup>b</sup> Estimated body weight at time of interview multiplied with estimated average requirement per kilogram; <sup>c</sup> Mean estimated energy intake of the two recalls; <sup>d</sup> Calculated as percent difference of mean. Difference between EER and EI tested with paired samples *t*-test:  $p = 0.58$ ; <sup>e</sup> Body weight not registered. Average weight for girls at 11 months of age used as reference [19]; <sup>f</sup> Body weight at 8 months of age; <sup>g</sup> Calculated using absolute values of percentage differences

### 3. Results

#### 3.1. Subjects

A total of 28 Somali-born mothers were asked to participate in the pilot study, and 13 consented. However, only seven of these showed up to the appointed interview. Of the fourteen 24-h recalls, eight were conducted in Norwegian and six in Somali. Likewise, 48 Iraqi mothers were contacted, seven consented, but only five showed up at the interview. Five of the 24-h recalls were conducted in Kurdish, four in Arabic and one in Norwegian. Among the 12 participating mothers, mean age was 31 (range 22–42) and the average number of years lived in Norway was 15 (range 3–24). Three mothers had no education from Norway. Two of them had, however, completed a Norwegian

course. Seven mothers had completed high school education and two had completed higher education. Seven mothers had more than one child.

### 3.2. Results from the Evaluation Form

The mean (minimum-maximum) time spent on the total 24-h recall interviews was 47 (20–75) min. There was a decreasing trend in the time spent on each interview conducted over time in both the Somali and Iraqi groups. The repeat interviews were conducted by the same field worker, except for three of the Somali mothers and one Iraqi mother where two different field workers conducted the interviews.

The field workers spent an average of four minutes during each interview showing pictures on the iPad. The visual tools were used during all interviews. Of the 19 folders in the picture library, eight were used by the Somali mothers to identify foods. The most frequently used folders were “baby cereal” (seven interviews) and “oils and butter” (four interviews). Among the Iraqi mothers, pictures from 13 of the 19 folders were used. The most frequently used folders in this group were “breads” (eight interviews) and “baby cereal” (four interviews). Although the mothers browsed through all folders to identify foods given to the child, none of the Somali or Iraqi mothers used or identified foods from the five folders “ready-made meals”, “fruits and vegetables”, “soda”, “squash, lemonade, *etc.*” or “meat”.

Eleven of the 17 colored photograph series were used by the Somali mothers to estimate portion sizes eaten by the child. The portion sizes of baby cereal and butter were the most frequently used total in 10 and 9 of the 14 interviews, respectively. The Iraqi mothers used 10 of the 17 photograph series of portion sizes to estimate foods consumed by the child. The most frequently used series were the portion sizes for milk and butter, which were referred to in 9 and 5 of the 10 interviews, respectively. The measuring equipment was used together with the photographic booklet in the first interviews, but over time the interviewers favored the photographic booklet over actual measurements. Reasons given for this shift were that measurements were time consuming and difficult to use when the interviews were conducted outside the informants’ homes. When mothers were asked to identify amount with both the photographic booklet and by measurements of actual foods, these seemed to correspond well.

The protocol was mostly only used during the last pass, when the field workers were going through the checklist of foods and beverages often forgotten. When the interviews were conducted in Norwegian, the observers noted that the field workers consistently asked about added foods/ingredients, brands and amounts consumed. It was sometimes difficult for the field workers to write down recipes and cooking methods because of limited space on the forms. However, the amount of food eaten by the child was usually asked about and written down clearly.

The mothers expressed that the picture library was a good tool to be reminded of and to identify the type of foods given to the child. It was especially useful for remembering brand names. Among pictures missing in the picture library, some mothers mentioned different types of rice, fruit purees, bread spreads, breads, butter, baby cereals, and yoghurts, as well as Weetabix and prunes.

One of the topics that emerged repeatedly was how difficult it was to estimate portion sizes. Six mothers mentioned the difficulties in estimating the amount of bread eaten by the child, without

pictures of bread in the booklet. Other pictures of portion sizes mentioned as missing were lasagna, spaghetti and pancakes. Five mothers expressed that pre-packed industrially produced foods were easier to estimate. All mothers found that the portion size options in the photographic booklet matched amounts the child usually ate. Ten mentioned that it was easier to show amounts of foods eaten using the booklet, whereas illustrating amounts of beverages was easier using the measuring equipment.

Most mothers said that the day of interview represented the typical foods given to the child, only three recall days were considered non-representative for the typical foods given. Two foods (bread and bulgur) were mentioned by two mothers as being typical foods given to the child, but not during the days in question. Pancakes (*anjera*), Weetabix and juice were mentioned by two Somali mothers as cultural relevant foods often given to children, while four Iraqi mothers mentioned different types of staple foods and vegetables, such as okra.

All field workers found the protocol easy to understand. Finding pictures in the picture library took time to begin with, but became easier after a few interviews. The photographic booklet was judged as a good tool for estimating portion sizes. However, similarly to the mothers, they missed portion size pictures of pasta and bread.

The registration form was described as clear and easy to understand, but the field workers missed more space to write down recipes.

### 3.3. Results from the 24-H Recalls

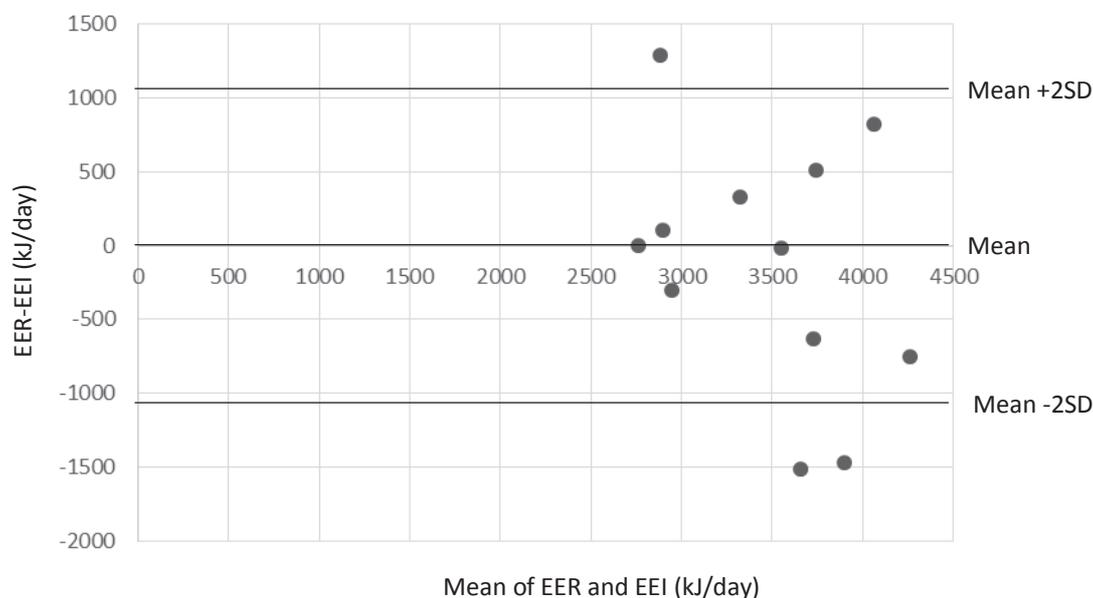
Six mothers were still breastfeeding their children (four Iraqi and two Somali mothers). The average breastfeeding frequency was two times per day (range one to four times per day). Foods given to the children included bread, porridge, different fruit and vegetables, snacks and supplements. The porridge was either industrial produced or home-made using oatmeal or bulgur. A couple of mothers also added margarine, olive oil, salt and/or sugar when preparing the porridge. Some of the foods registered that are not commonly given to Norwegian children were nan bread, feta cheese, Turkish delight, bulgur porridge, pancakes (*anjera*), and seeds. None of the mothers reported using ready-made dinners, as most of them mentioned that they did not trust the contents and the ready-made dinners were not considered to be fresh. The home-made dinners often constituted of different staple foods, vegetables, meat, and fish.

### 3.4. Results from Energy Intake Estimation

Table 3 presents the comparison of each child's EER with its EEI calculated from the 24-h recall. The percent difference between EER and EEI was in the range of  $\pm 0\%$ – $10\%$  for five of the children and in the range of  $\pm 11\%$ – $20\%$  for four of the children, whereas for three of the children the percentage of difference between the two estimates was  $\pm 38\%$ ,  $41\%$  or  $45\%$ , respectively. The mean (SD) for EER and EEI was 3407 (527) kJ/day and 3543 (773) kJ/day, respectively, and the difference was not significant, as tested with paired samples *t*-test. The Bland-Altman plot (Figure 1) showed large individual variations in the differences between EER and EEI but no clear pattern. A linear regression analysis testing the relationship between the mean of the estimates EER

and EEI and the difference between the two estimates was not significant,  $p = 0.24$ . This indicates that the difference between the two estimates is not related to the magnitude of the estimates. Excluding child number 4, for whom there was no registered weight, did not change the results.

**Figure 1.** The difference between estimated energy requirement (EER) and estimated energy intake (EEI), plotted against the mean of EER and EEI ( $n = 12$ ). SD = Standard deviation.



#### 4. Discussion

For the InnBaKost study, we developed a protocol for a 24-h recall procedure, including a picture library to assist in identifying the correct foods eaten. In addition, a photographic booklet was used for portion size estimation. Although the latter approach has become a common method for portion size estimation [21,22], including dietary assessment in children [23,24], the use of a picture library is a rather novel approach. The hypothesis was that the picture library would be a useful tool to identify the correct food and brand, particularly for dietary assessment among immigrant mothers with varying levels of language and literacy skills.

A review conducted by Burrows *et al.* (2010), indicates that weighed food records provide the best dietary estimates for younger children aged 0.5 to 4 years, while 24-h multiple-pass recall that uses parents as reporters is the most accurate method to estimate total energy intake in children aged 4 to 11 years [25]. The weighed food record method requires both motivation and good literacy skills and is often time-consuming. Thus, the method has been considered to be less suitable for dietary assessment in immigrants, as the method has led to misreporting and dropout in immigrant groups due to the burden and time consumption the method carries [26]. The face-to-face FFQs and multiple-pass 24-h recalls are reported to be the two most frequently used methods with immigrant populations in Europe [6]. The 24-h recall is more flexible because it can capture all foods and beverages consumed the preceding day, with no assumptions about the food culture or dependency on literacy levels. In addition, as seen from the few recalls in this pilot, some mothers gave selected atypical foods to their children and mostly made home-made dinners, which may

vary from the general Norwegian population in regards to composition and preparation method. The 24-h recall has therefore been recommended as the most optimal method for many immigrant groups and is considered to provide valid information among children [26,27]. In addition, the interactive nature and the personal contact of the method may contribute to more reliable data collection, although social desirability bias may cause some misreporting [28]. The multiple-pass technique is considered to give the most exact estimates, and limit misreporting, because the probing questions encourage the respondent to remember more of the foods consumed [16]. The respondent burden is usually small compared to weighed records [29].

The protocol was used sparingly during the interviews, because the field workers expressed that they already knew the content in the protocol and that it was difficult to focus on the protocol while registering the child's food consumption. Thus, it was recommended that important guidelines from the protocol could be included in the 24-h recall registration form instead. The decreasing time spent on the second interview with each mother was mostly due to the mother being more prepared and that the background information was already collected. Another reason for the decline in time spent may have been that the field workers became more familiar with the method and navigated the picture library and photographic booklet more easily. The measuring equipment was initially used together with the photographic booklet to see how well both measurements corresponded, but both the field workers and mothers expressed that it was too time-consuming.

Both the mothers and field workers reported the picture library to be a good tool to identify foods given to the child. It was mostly used when the interviews were conducted outside the respondents' homes because the mothers could show foods available in the home. The use of a picture library similar to this has not been described by many; however, the use of photo images has been reported to be useful as a memory aid for respondents during 24-h recalls [30,31]. The picture library seemed to strengthen the mothers' ability to report the correct food and reduce misunderstandings. However, the pilot study revealed many desired additions to both the picture library and booklet.

Portion size estimation is one of the main challenges in dietary assessment studies. Estimating amounts eaten other than direct weighing may contribute to a source of error, both among children and adults [7,22,24,32]. The photographic booklet was considered to be a good tool for estimating portion sizes among the field workers and the mothers, as has also been reported in several other studies [21,24,30,33]. A study by Lillegaard *et al.* (2005) showed that children and adolescents could accurately estimate portion sizes of pre-weighed foods by viewing photographs, approximately 60% of the comparisons were made correctly [24]. The estimations were more accurate when the served portions had the exact appearance as the food portrayed in the photographic booklet [24]. Thus, the arguments can be made that more picture series in our photographic booklet may be favorable rather than using pictures of similar foods. The studies further emphasize the importance of validation studies to test the applicability of photographs for estimating current portions and actual consumption [21,22], especially among immigrant groups [13]. This was not done in this pilot study, but should be considered in the future.

Assessing children's food intake accurately can be difficult for a number of reasons. Infants and toddlers cannot account for their food intake, but parents are seen as reliable sources when

affirming their children's consumption of food [25,34]. Efforts should be made to assess foods eaten outside the home or with other caretakers; for instance, at the kindergarten or with family members. A possible challenge may be that the level of reporting and motivation may vary for each caretaker [7]. In the pilot, one of the Somali fathers was on paternity leave and was in charge of the child's diet at the time; therefore, he was interviewed together with the mother. Among the Iraqi mothers, only one mother reported that her child had spent much of the day with a nanny. Although, this did not apply for many of the mothers in the pilot, it should be taken into consideration for larger studies and dietary assessment of somewhat older children. Potential solutions may be to ask the mothers prior to the interview if the child has other caretakers and if it may be possible to include them to obtain information about their child's food consumption during their supervision.

In regard to EEI, it seemed to correspond well with the EER for most of the children (within  $\pm 2$  SD of the average of the two estimates) except for three. The comparison of EEI and EER has some weaknesses and can only give an indication of whether the method is suitable for capturing habitual energy intake on a group basis. First, each child's EER might not reflect the true energy requirement of the child because an energy requirement is highly variable between children of the same age and weight [35]. There is also intraindividual variation in energy requirement for children, depending on their physical activity level and growth rate [35]. Second, the energy intake measure was simply averaged over the two days without adjustment for intraindividual variation over time. Thus, it may not be representative of habitual energy intake [36]. Although the sample size was small, it was encouraging that there was no consistent over- or underreporting of EEI compared to EER.

Recruitment of study participants in itself was challenging and time consuming in this pilot study, as it was difficult to come in contact with the target group. This was mostly due to wrong contact numbers registered on several mothers when tried to reach by phone. Some reasons for refusals were that they were not interested, skeptical, or had to consult their partner. It was necessary to seek the mothers through several methods and many did not show up to appointed interviews. The use of bilingual field workers was an advantage and enabled the recruitment of mothers who did not speak Norwegian. Challenges related to recruitment when conducting dietary studies with immigrants have previously been reported [6,26]. Most studies conducted with a European immigrant population group have also used nonprobability sampling methods, such as the convenience sampling method [6]. The need for extra effort in recruiting participants has been described, such as using bilingual field workers, involving key leaders and including places of worship and media, to overcome cultural barriers and ensure representativeness [6]. Although the convenience sampling method may lead to the inclusion of highly motivated participants, there seemed to be variations in the background characteristics of the mothers included in the pilot.

Based on the pilot study presented, some suggestions were made for improving the 24-h multiple-pass recall method. Observations of the interviews showed that the field workers were not actively using the protocol, and a possible solution is to incorporate the protocol into the registration form. Other important suggestions were to include more pictures in the library and supply the photographic booklet with portion sizes of bread in particular, but also of foods such as lasagna,

pancakes and other portion sizes of meat, fish, fruits and vegetables. Furthermore, a more thorough training and follow-up of the field workers would be required to increase the quality of the data collection.

## 5. Conclusions

Experiences from the current study indicate that the 24-h multiple-pass recall method with inclusion of visual tools is appropriate method for assessing dietary intake among toddlers of Somali- and Iraqi-born mothers living in Norway. The picture library and photographic booklet were considered to have an added value to the method to aid to identify and describe foods and beverages consumed. However, for the method to be applicable, there is a need for thorough training and follow-up of the field workers during data collection and an update of the picture library and photographic booklet to capture foods, which were not included.

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## Author Contributions

N.K.G., A.M. and L.E.T. designed the research; all authors collaborated in refining the methodology. M.B.A. and C.M. developed the tools and collected the data. N.K.G. wrote the paper and had responsibility for the final content. All authors contributed substantially to the development of the manuscript. All authors read, edited, and approved the final manuscript.

## Conflicts of Interest

The authors declare no conflict of interest.

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## Urinary Metabolite Profiles in Premature Infants Show Early Postnatal Metabolic Adaptation and Maturation

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**Abstract:** Objectives: Early nutrition influences metabolic programming and long-term health. We explored the urinary metabolite profiles of 48 premature infants (birth weight < 1500 g) randomized to an enhanced or a standard diet during neonatal hospitalization. Methods: Metabolomics using nuclear magnetic resonance spectroscopy (NMR) was conducted on urine samples obtained during the first week of life and thereafter fortnightly. Results: The intervention group received significantly higher amounts of energy, protein, lipids, vitamin A, arachidonic acid and docosahexaenoic acid as compared to the control group. Enhanced nutrition did not appear to affect the urine profiles to an extent exceeding individual variation. However, in all infants the glucogenic amino acids glycine, threonine, hydroxyproline and tyrosine increased substantially during the early postnatal period, along with metabolites of the tricarboxylic acid cycle (succinate, oxoglutarate, fumarate and citrate). The metabolite changes correlated with postmenstrual age. Moreover, we observed elevated threonine and glycine levels in first-week urine samples of the small for gestational age (SGA; birth weight < 10th percentile for gestational age) as compared to the appropriate for gestational age infants. Conclusion: This first nutri-metabolomics study in premature infants demonstrates that the physiological adaptation during the fetal-postnatal transition as well as maturation influences metabolism during the breastfeeding period. Elevated glycine and threonine levels were found in the first week urine samples of the SGA infants and emerged as potential biomarkers of an altered metabolic phenotype.

**Keywords:** prematurity; very low birth weight; pediatric nutrition; intervention study; metabolomics; urine; nuclear magnetic resonance spectroscopy; glycine; threonine

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## 1. Introduction

Despite improved perinatal medical care and increased focus on enhanced nutritional support to premature infants, pre- and postnatal growth-restriction still occurs in 60%–100% of infants with a very low birth weight (<1500 g) [1,2]. Premature infants with growth restriction are at risk of impaired cognitive function and adverse metabolic and cardiovascular outcomes later in life [3,4]. Metabolic changes occurring *in utero*, during birth and the postnatal weaning period, seem to be of particular importance for future health [5–7]. Nutritional alterations during these periods are associated with a predisposition to obesity, cardiovascular diseases and associated co-morbidities later in life [5,8]. However, the time frame for these programming effects on long-term disease risk is controversial. Present evidence favors proactive nutritional support in premature infants to promote growth similar to the intrauterine growth rate and to support cognitive development [1,3,4].

This is in contrast with the potentially advantageous effects of relative undernutrition and slower growth on long-term cardiovascular health [8].

Recently, we published results from a randomized, controlled trial comparing the effect of enhanced nutritional supply (intervention) as opposed to a standard (control) diet, on postnatal growth in premature infants with a birth weight < 1500 g [9,10]. The infants in the intervention group, with median nutrient supplies in the upper range of current recommendations (Table 1) [9,11,12], exhibited postnatal growth along their birth percentiles for both weight and head circumference, whereas the control infants fell off their expected growth trajectories from birth to 36 weeks postmenstrual age (PMA). However, a preplanned safety analysis after the enrolment of 50 infants revealed an increased occurrence of late onset septicemia without increased mortality in the intervention group, and it was decided to halt further recruitment [10].

**Table 1.** Daily nutrient supply up to four weeks after birth.

	<b>Intervention (<i>n</i> = 23)</b>	<b>Control (<i>n</i> = 21)</b>	<b><i>p</i></b>
Human milk, mL/kg/day	133 (110–139)	134 (124–141)	0.37
Energy, kcal/kg/day	139 (128–145)	126 (121–128)	<0.001
Protein, g/kg/day	4.0 (3.9–4.2)	3.2 (3.1–3.3)	<0.001
Lipids, g/kg/day	7.3 (6.4–7.6)	5.9 (5.6–6.1)	<0.001
Carbohydrates, g/kg/day	14.4 (13.4–14.8)	14.7 (14.3–15.1)	0.12
Arachidonic acid, mg/kg/day	68 (57–73)	24 (23–25)	<0.001
Docosahexaenoic acid, mg/kg/day	87 (81–91)	36 (34–38)	<0.001
Vitamin A, µg/kg/day	1300 (1105–1442)	252 (238–257)	<0.001

Detailed records of actual nutrient supply was available for 44 infants [9]. Data are presented as medians (interquartile ranges) and compared using the Mann-Whitney U test.

To assess the metabolic status of these premature infants and to explore potentially different responses to the two diets, we used state-of-the-art nuclear magnetic resonance (NMR)-based metabolomics to analyze urine samples obtained during the postnatal period. Metabolomics is recognized as a powerful top-down systems biology approach that explores the genetic-environment-health interaction [13,14]. The approach is to obtain broad snapshots of the metabolism by detecting and quantifying hundreds of small-molecular substances (molecular mass < 1000 Da) in tissues or body fluids, and then link them to disease or development states using multivariate statistical methods such as principal component analysis (PCA) and partial least squares (PLS) regression that handle and integrate large datasets [15,16]. Metabolomics of biofluids is thought to be a promising new tool in neonatology, especially in premature infants, due to its comprehensive and usually non-invasive nature [17]. Metabolomic analysis of urine from the neonatal period may be used to understand metabolic processes linked to early nutrition. It may also be used to identify biomarkers for diagnosis, prognosis and risk prediction of different diseases [5,13,17–19].

The main objectives of the present study were to analyze urine samples from the premature infants of the previous trial in relation to the two different nutritional exposures and to assess the infants' postnatal metabolic maturation. Secondary objectives were to explore potential differences related to age, sex, infections as well as pre- and postnatal growth.

## 2. Materials and Methods

### 2.1. Study Design and Population

The study was part of an open, randomized, controlled clinical trial [9,10], approved by the Regional Committee for Medical and Health Research Ethics and in accordance with the principles of the Helsinki Declaration. Fifty premature infants with birth weight < 1500 g were recruited from the neonatal intensive care units at Oslo University Hospital and Akershus University Hospital, Norway, from 17 August to 21 December 2010; 24 in the intervention group and 26 in the control group. Exclusion criteria were congenital malformations, chromosomal abnormalities, critical illnesses with short life expectancy and clinical syndromes known to affect growth and development. Morbidities were registered according to routine clinical practice and standard definitions [20–23]. Infants were classified as small for gestational age (SGA) if their birth weight was below the 10<sup>th</sup> percentile of a reference population [24], or as appropriate for gestational age (AGA) otherwise. Growth velocity was calculated by the exponential equation described by Patel *et al.* [25].

Two infants in the control group died during the first week of life, leaving 48 infants for the analysis [10]. Demographic and clinical characteristics are presented in Table 2. The significantly higher occurrence of septicemia and electrolyte deviations observed in the intervention group have been reported previously [10].

**Table 2.** Baseline characteristics and clinical outcomes.

	Intervention (n = 24)	Control (n = 24)	p
Gestational age (weeks), mean (range)	28.1 (25.0–33.6)	28.5 (24.0–32.6)	0.60
Birth weight (g), mean (range)	940 (460–1311)	1083 (571–1414)	0.03
Small for gestational age, n (%)	11/24 (46%)	5/24 (21%)	0.12
Sex, boys, n (%)	15/24 (63%)	15/24 (63%)	1.00
Cesarean section, n (%)	16/24 (67%)	19/24 (79%)	0.52
APGAR-score, 5-min, mean (± SD)	7.33 (± 1.7)	7.54 (± 1.7)	0.68
Prenatal steroid treatment, n (%)	22/24 (92%)	24/24 (100%)	0.49
Late onset septicemia, n (%)	15/24 (63%)	7/24 (29%)	0.04
NEC, n (%)	1/24 (4%)	2/24 (8%)	1.00
IVH, grade ≥3, n (%)	2/24 (9%)	2/24 (9%)	1.00
PVL, grade ≥3, n (%)	0/24 (0%)	1/24 (4%)	1.00
ROP (severe grade III/+disease), n (%) <sup>a</sup>	3/23 (13%)	2/23 (9%)	1.00
O <sub>2</sub> at 36 weeks PMA, n (%) <sup>a</sup>	5/23 (22%)	6/23 (26%)	1.00
PDA surgical treatment, n (%)	4/24 (17%)	2/24 (8%)	0.67
Deaths before 36 weeks PMA, n (%)	1/24 (4%)	1/24 (4%)	1.00
Hypophosphatemia 1st week, n (%) <sup>b</sup>	17/22 (77%)	6/23 (26%)	0.001
Hypokalemia 1st week, n (%)	21/24 (88%)	11/24 (46%)	0.005

Student *t*-test or Fisher's exact test was applied as appropriate. NEC: necrotizing enterocolitis; IVH: intraventricular hemorrhage; PVL: periventricular leukomalacia; ROP: retinopathy of prematurity; PDA: persistent ductus arteriosus; PMA: post-menstrual age; Hypophosphatemia < 1.4 mmol/L; Hypokalemia < 3.5 mmol/L. <sup>a</sup> Two more infants died during hospitalization, leaving 46 infants in the analyses of ROP and O<sub>2</sub>-dependency at 36 weeks PMA; <sup>b</sup> Only 45 infants had their phosphate concentrations measured during the first week of life.

## 2.2. Nutritional Intervention

The nutritional intervention was started on the first day of life, after informed consent was obtained [9,10]. The intervention group started with 3.5 g/kg/day of amino acids and 2.0 g/kg/day of intravenous lipids, whereas the control group started with 2.0 g/kg/day of amino acids and 0.5 g/kg/day of lipids. To improve the parenteral supply of the long chain polyunsaturated fatty acids (PUFAs), the intervention group received a lipid emulsion containing marine omega 3 fatty acids (SMOF<sup>®</sup>, Fresenius Kabi Norge AS, Oslo, Norway), whereas the control group received the lipid emulsion used in our units (Clinoleic<sup>®</sup>, Baxter AS, Oslo, Norway). The supply of human milk was increased equally in both groups, and standard fortification with 4.2 g Nutriprem<sup>®</sup> (Nutricia Norge AS, Oslo, Norway)/100 mL human milk was initiated when the infants tolerated 110 mL/kg/day as enteral supply. In addition to standard fortification, the intervention group was given 0.6 g Complete Amino Acid Mix<sup>®</sup> (Nutricia Norge AS, Oslo, Norway)/100 mL human milk, 60 mg/kg/day of docosahexaenoic acid (DHA; 22:6, *n*-3) as well as arachidonic acid (AA; 20:4, *n*-6), and 1500 µg/kg/day vitamin A (Ås Laboratory, Ås, Norway). On average the energy supply was approximately 10% higher and the protein supply 25% higher in the intervention group as compared to the control group [9].

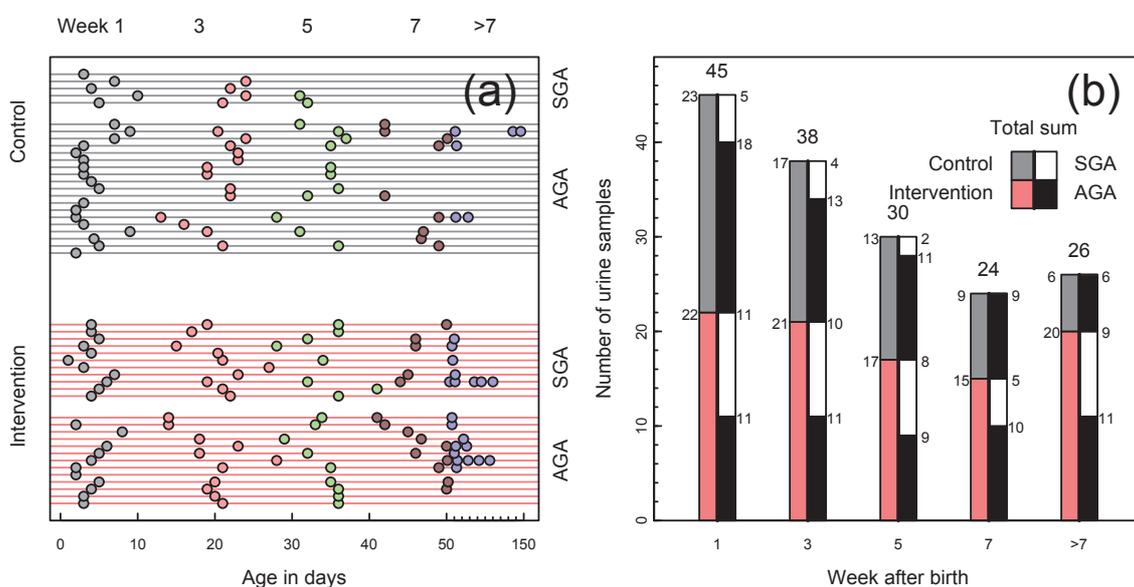
## 2.3. Sample Collection and Preparation

Urine samples were obtained from the infants during the first week of life and thereafter approximately every other week until discharge (Figure 1). We collected 0.5–1.8 mL urine non-invasively by the use of cotton pads, transferred them to Nunc<sup>®</sup> CryoTubes<sup>®</sup> (Nalge Nunc International, Penfield, NY, USA) before they were stored at –80 °C. The urine samples had been thawed once prior to the metabolic NMR profiling when they were acidified for electrolyte analysis by mixing 400 µL of sample with 5 µL of 1 M HCl, resulting in a pH of approximately 3.

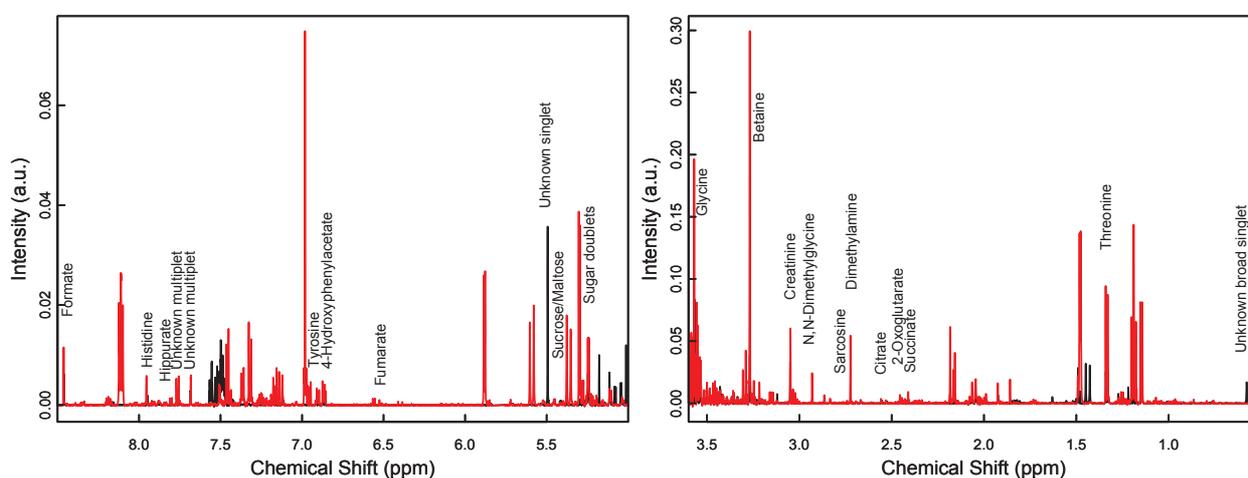
Metabolite profiling in the present study largely followed a protocol described earlier [26]. Briefly, 150 µL of distilled water and 50 µL of a buffer at pH 7.4 containing D<sub>2</sub>O and trimethylsilylpropionate-d<sub>4</sub> (TSP) were added to 350 µL of the samples, which were then centrifuged at 13,400× *g* for 5 min and transferred to 5 mm NMR tubes (Wilmad LabGlass, Vineland, NJ, USA). One-dimensional, water-suppressed proton NMR spectra were acquired at 300.0 K on a Bruker AVI-600 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) equipped with a TCI cryoprobe and a BACS-60 automatic sample changer, under full automation of D<sub>2</sub>O locking, tuning and matching, and gradient shimming using TopSpin 2.1pl6 and iconNMR. Of each sample 32 scans and 4 dummy scans were collected into 64 k data points using the Bruker “noesygppr1d.comp” sequence with a spectral width of 20.6 ppm, 2.65 s acquisition time and a 25 Hz water presaturation during the 4 s relaxation delay. An exponential line broadening of 0.3 Hz was applied. The TSP signal achieved a full width at half maximum of less than 1 Hz after apodization and acted as spectral and concentration reference. The spectra were phase-corrected, a smooth baseline was removed, and the spectra were binned to a spectral width of 0.01 ppm. Signals were assigned to known metabolites using a reference database [27] and the software Chenomx NMR Suite 7.5 professional (Chenomx Inc., Edmonton AB, Canada). Two example

spectra are shown in Figure 2. Pseudo-concentrations were extracted by integrating manually defined spectral regions corresponding to both known and unknown substances, and arranged in a table. Pseudo-concentrations are proportional to absolute concentrations and can be used as such in the statistical analysis. Both the spectra and the table of metabolite pseudo-concentrations were subsequently normalized to the total intensity of the respective spectra, and the metabolite table was log-transformed.

**Figure 1.** (a) Available urine samples by infants' age in days, one infant per line, one sample per symbol. Grouped by intervention and control (red and gray lines, respectively), color-coded by week of life. Age in days was imputed for eight samples where only the week was recorded; (b) Available urine samples by infants' week of life. Bars divided by nutritional intervention vs. control (left half of bar; red and gray, respectively) and further subdivided by small for gestational age (SGA) or appropriate for gestational age (AGA) infants (right half of bar; SGA white, AGA black).



**Figure 2.** Selected regions of two NMR spectra (black for week 1 and red for week 1) of an SGA infant in the intervention group.



## 2.4. Statistical Analysis

We used Student *t*-test, Mann-Whitney U test or Fisher's exact test to evaluate differences in baseline characteristics, clinical outcomes and nutrient supplies between the two study groups [9,10]. Results are presented as frequencies (%) for categorical data, and as means (ranges or standard deviations) or medians (interquartile ranges) for continuous data [9,10]. For the metabolomics study, PCA was applied to mean-centered and unit-variance scaled spectra to explore the major variations in the dataset [28,29]. By definition, a PCA score plot arranges samples based on the similarity of their spectra, thus enabling the identification of natural groupings of and systematic changes between samples. The corresponding loadings reveal which spectral regions, i.e., which metabolites, contribute to the scores. Multivariate PLS regression was used to associate the endpoints in our study to the urine spectra. Again, the spectral variables were mean-centered and scaled to unit variance, and 7-fold cross-validation was applied to evaluate the quality of the resulting statistical models by considering the diagnostic measures  $R^2$  and  $Q^2$  [30], describing the endpoint variation captured in regression model, and the variation reproduced in cross-validation, respectively. Whereas  $R^2$  and  $Q^2$  represent measures of the strength of a multivariate relationship between profiles and endpoints, their ratio  $Q^2/R^2$  is a measure of cross-validation reproducibility. In the present study,  $Q^2/R^2$  ratios above 0.5 were considered indicative of relevant associations, which were then studied further.

Univariate response approaches were used on log-transformed data in the pseudo-concentration table to expand the results from the multivariate analyses. A linear mixed model for repeated measures (first-order autoregressive covariance structure) was used to study the impact of the two diets on the metabolite pseudo concentrations over time (weeks 1, 3, 5 and 7), adjusted for gestational age at birth and SGA status. Linear regression was used to quantify the relations between the metabolite pseudo concentrations at week 1 with SGA status and PMA in weeks, and also between metabolite levels, growth velocity and PMA. Results are reported as fold-change ratios (FC) with respect to back-transformed metabolite levels; ratios below 1 are presented as  $-1/\text{ratio}$ . Bonferroni correction for multiple testing was applied. The analyses were carried out on a Windows PC using SPSS version 20 (SPSS Inc., Chicago, IL, USA) and R 2.12.1, 64-bit (R Foundation, Vienna, Austria), with packages pls 2.2-0 and pcaMethods 1.32.0.

## 3. Results

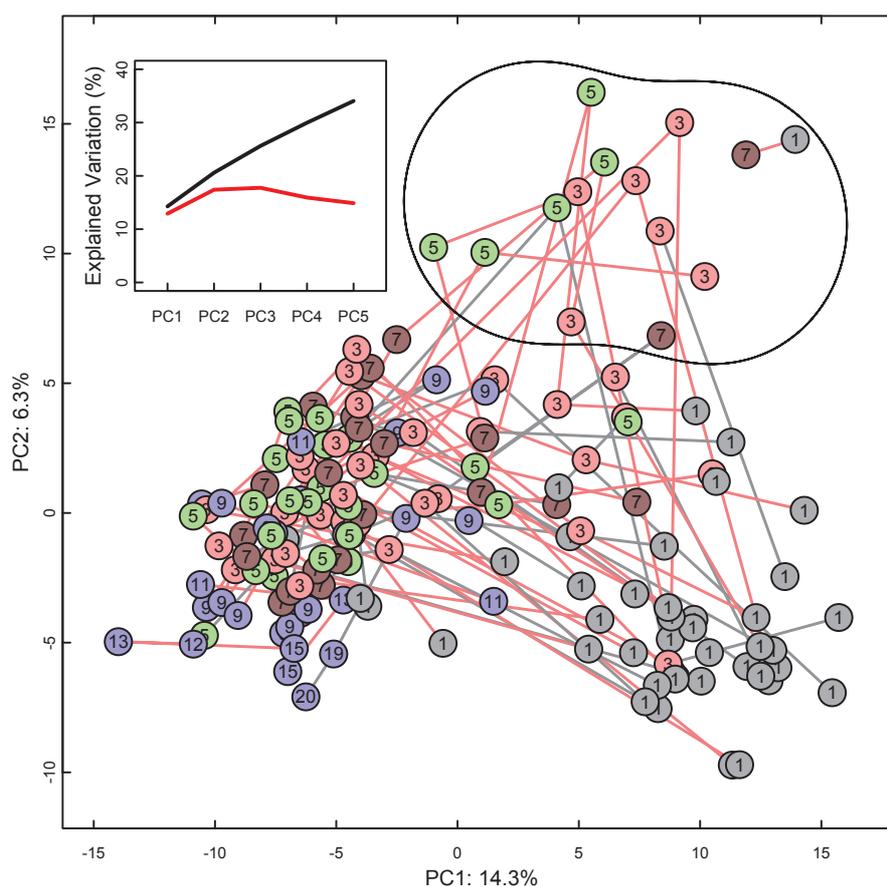
The PCA score plot in Figure 3 presents the overall NMR spectroscopic relations between all the available urine samples, with lines between consecutive samples from the same infant. Urine samples from the first week of life occupy the lower right quadrant of the plot, and mostly progress towards the middle left with increasing age of the infant. There was no obvious difference in distribution or temporal development between the intervention and control group.

Several samples in the upper right quadrant deviated from this general trend and are marked as outliers. The deviation was characterized by strong NMR signals, predominantly in the aromatic region of the NMR spectrum, which could not be identified as known metabolites. Whereas the 10 infants with outlier samples had a somewhat lower gestational age than the others, there were no

significant differences with respect to the nutritional intervention, SGA status, sex or infections or any other of the clinical parameters (data not shown).

The PCA loadings (not shown) revealed that the first principal component (PC1, the *x*-axis) of Figure 3 corresponded to increasing levels of citrate, betaine, glycine and hydroxyproline from right to left, along with decreasing unidentified spectral signals at 0.57 and 5.50 ppm. The second (PC2, the *y*-axis) and the third principal component (not shown) were dominated by the unidentified signals of the outlier samples mentioned above.

**Figure 3.** PCA score plot of NMR spectra of all available urine samples, presented as points marked with infant age in weeks and color-coded as earlier. PCA: Principal component analysis, NMR: Nuclear magnetic resonance, PC: Principal component with percent of explained total variation. Lines connect consecutive samples from one infant; line color red for intervention, gray for control group. Outlier samples marked with a dashed line in the upper right quadrant. Inset: Cumulative explained variation (black) and cross-validation (red) of the first five PCs.



The metabolites were studied in relation to changes over time in a linear mixed model for repeated measures (Table 3). There was no significant interaction between intervention group and time for any of the metabolites, thus interaction terms were not included in the final models. There was no significant effect of the intervention, but for most of the metabolites, there was a significant effect of time. The levels of amino acids and many other metabolites increased between weeks 1 and 3, whereas gluconate and two strong, unidentified signals at positions 0.57 and 5.50 ppm

disappeared. The results were similar irrespective of whether the outliers were kept or removed in the analyses (data not shown).

**Table 3.** Mixed model and change of mean metabolite levels between sampling weeks 1, 3, 5 and 7 ( $n = 48$ ).

Metabolite	Weeks 1→7 Mixed Model			Weeks 1→3	Weeks 3→5	Weeks 5→7
	<i>p</i> Interaction	<i>p</i> Diet	<i>p</i> Time	( $n = 35$ Pairs)	( $n = 28$ Pairs)	( $n = 19$ Pairs)
				FC	FC	FC
Total Integral				-1.2	-1.2	1.2
1-Methylnicotinamide	0.21	0.24	0.02	1.2	1.0	-1.1
2-Oxoglutarate	0.05	0.51	<0.001	4.1	1.4	1.2
4-Hydroxyphenylacetate	0.43	0.11	<0.001	5.1	-1.1	-1.3
Betaine	0.96	0.16	<0.001	1.5	1.2	1.3
Citrate	0.80	0.26	<0.001	5.5	1.9	1.5
Creatinine	0.79	0.52	0.01	1.0	1.1	1.2
Dimethylamine	0.87	0.39	0.003	1.1	1.1	1.0
Fumarate	0.06	0.29	<0.001	3.2	1.7	1.0
Gluconate	-	-	-	x	-	-
Glycine	0.62	0.05	<0.001	1.6	1.2	-1.1
Hipurate	0.56	0.10	0.31	-1.2	1.6	1.0
Histidine	0.70	0.31	<0.001	1.2	1.6	-1.2
myo-Inositol	0.60	0.28	0.08	1.3	1.0	-1.2
<i>N,N</i> -Dimethylglycine	0.23	0.62	<0.001	1.3	1.2	1.3
Succinate	0.42	0.67	<0.001	5.0	1.4	1.0
Sucrose/Maltose	0.12	0.51	<0.001	-8.4	1.1	-1.1
Sugar doublets, 5.23 ppm	0.30	0.08	0.03	1.0	-1.2	-1.2
Threonine	0.58	0.34	<0.001	2.0	1.0	-1.7
<i>trans</i> -4-Hydroxy-L- proline	0.26	0.16	<0.001	3.1	1.3	1.0
Tyrosine	0.33	0.37	<0.001	3.7	1.0	-1.4
Unknown, 0.57 ppm	-	-	-	x	-	-
Unknown, 5.50 ppm	-	-	-	x	-	-
Unknown, 7.68 ppm	0.60	0.48	<0.001	1.2	1.1	1.1
Unknown, 7.76 ppm	0.43	0.9	<0.001	1.6	1.4	1.3

Linear mixed model for intervention (diet) and week of life (time) with adjustment for gestational age (GA) at birth and small for gestational age (SGA) status was used. Statistical significance was assumed for  $p < 0.002$ . Increase or decrease of log-transformed pseudo-concentrations, presented as fold-change (FC; ratios below 1 are presented as  $-1/\text{ratio}$ ). The FC is based on available paired urine samples from the same child at the respective weeks of age. Metabolites marked “x” disappeared entirely; FC is therefore not applicable. Histidine is an uncertain assignment, based on a narrow doublet at 7.9–8.0 ppm. Total integral of the urine spectra was determined early relative to the added internal standard trimethylsilylpropionate-d4 (TSP) and then used to normalize all specified compounds.

Multivariate PLS regression analyses were carried out between the NMR spectra and clinical variables (Table 4). The nutritional intervention, presence of infections, and infants' sex did not influence the urine spectra, whereas SGA status did show an effect on the metabolite profiles: The PLS model of the infants' SGA status based on all spectra except those of the outliers reached a  $Q^2/R^2$  ratio of 0.40. This increased to 0.53 by focusing on spectra from the first week of life, whereas spectra of the urine samples at 36 weeks PMA could not be linked to SGA status. Inclusion of the outliers in the analysis did not change these results (data not shown). PLS regression analysis also indicated that PMA as well as chronological age were associated with the urine spectra.

**Table 4.** Partial least squares regression of variables to sample spectra.

Variable	Samples <sup>a</sup>	A <sup>b</sup>	Q <sup>2</sup>	R <sup>2</sup>	Q <sup>2</sup> /R <sup>2</sup>
Intervention	all		-	-	-
	first		-	-	-
	36 weeks PMA		-	-	-
SGA status	all	3	34%	84%	0.40
	first	1	27%	50%	0.53
	36 weeks PMA		-	-	-
Infections	all		-	-	-
	first		-	-	-
	36 weeks PMA		-	-	-
Age (since birth)	all	2	41%	81%	0.51
	36 weeks PMA		-	-	-
Age (PMA)	all	3	67%	89%	0.75
	first	1	54%	70%	0.76
Sex	all		-	-	-
	first		-	-	-
	36 weeks PMA		-	-	-

R<sup>2</sup>: Endpoint variation contained in regression model. Q<sup>2</sup>: Variation reproduced in cross-validation. Higher numbers, or at least high Q<sup>2</sup>/R<sup>2</sup> ratios mean reliable models. <sup>a</sup> Samples: All except outliers, first urine sampled from subject, and sample from 36 weeks PMA; <sup>b</sup> Number of PLS components resulting in best (highest) Q<sup>2</sup>/R<sup>2</sup> ratio; results with Q<sup>2</sup>/R<sup>2</sup> ratio below 0.3 not shown.

Observations linking the first-week urine samples to SGA status as well as PMA were also studied by linear regression analyses for selected metabolites (Table 5). In simple linear regression analyses, SGA status was associated with increased levels of glycine, histidine and threonine ( $8 \times 10^{-4} \leq p \leq 0.003$ ), as well as creatinine, succinate and *trans*-4-hydroxy-L-proline (hydroxyproline) ( $0.016 \leq p \leq 0.039$ ). PMA was associated with a broader range of metabolites ( $3 \times 10^{-7} \leq p \leq 0.040$ , for all variables in Table 5). The SGA infants had a higher mean gestational age at birth than AGA infants (29.9 vs. 27.5 weeks,  $p = 0.003$ ). When adjusting for PMA in the multiple linear regression analyses, there is an indication that SGA was associated with glycine ( $p = 0.027$ ) and threonine ( $p = 0.033$ ), although not significant at the adjusted significance level.

**Table 5.** Linear regression results for selected metabolites at week 1 with respect to SGA status and PMA.

Metabolite <sup>a</sup>	SGA Alone <sup>b</sup>		PMA Alone <sup>c</sup>		Mutually Adjusted <sup>d</sup>			
	FC	<i>p</i>	FC	<i>p</i>	FC (SGA)	<i>p</i> (SGA)	FC (PMA)	<i>p</i> (PMA)
2-Oxoglutarate	1.6	0.15	1.3	$4 \times 10^{-4}$	-1.1	0.81	1.3	0.001
Betaine	1.4	0.057	1.1	$9 \times 10^{-5}$	1.0	0.95	1.1	$5 \times 10^{-4}$
Citrate	1.9	0.12	1.3	0.001	-1.1	0.87	1.3	0.004
Creatinine	1.8	0.018	1.2	$3 \times 10^{-7}$	1.0	0.86	1.2	$6 \times 10^{-6}$
Dimethylamine	1.5	0.036	1.2	$2 \times 10^{-4}$	1.1	0.69	1.1	0.001
Formate	1.6	0.083	1.2	0.005	1.1	0.66	1.1	0.022
Glycine	1.8	$2 \times 10^{-4}$	1.1	$7 \times 10^{-5}$	1.4	0.027	1.1	0.005
Histidine	2.0	$8 \times 10^{-4}$	1.2	$9 \times 10^{-6}$	1.4	0.091	1.2	$6 \times 10^{-4}$
myo-Inositol	1.5	0.062	1.1	0.001	1.4	0.73	1.1	0.006
<i>N,N</i> -Dimethylglycine	1.0	0.93	1.2	0.020	-1.6	0.18	1.2	0.008
Succinate	2.1	0.039	1.3	$3 \times 10^{-4}$	1.2	0.63	1.3	0.003
Sugar doublets, 5.23 ppm	-1.3	0.11	-1.1	0.002	-1.1	0.66	-1.1	0.012
Threonine	1.8	0.003	1.1	0.040	1.6	0.033	1.0	0.37
<i>trans</i> -4-Hydroxy-L-proline	1.9	0.016	1.3	$5 \times 10^{-7}$	1.1	0.81	1.3	$1 \times 10^{-5}$

Histidine is an uncertain assignment, based on a narrow doublet at 7.9–8.0 ppm. Significance assumed for  $p < 0.002$ .

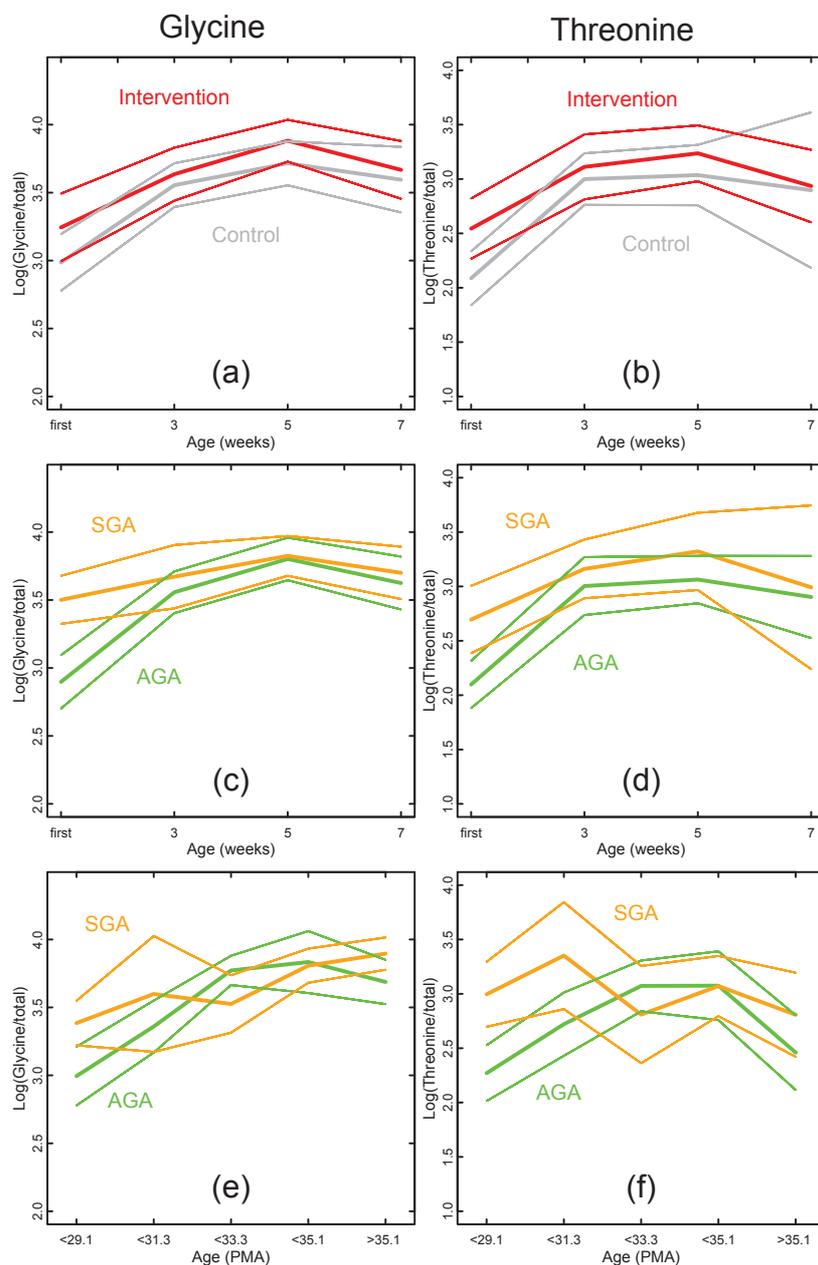
<sup>a</sup> Only selected contributions are shown; <sup>b</sup> Simple linear regression analyses of log-transformed pseudo-concentrations and SGA status; results are presented as fold-change (FC), e.g., glycine levels were 1.8-fold higher in the SGA group and 1.1-fold higher for each week's difference in PMA at week 1 (ratios below 1 are presented as  $-1/\text{ratio}$ );

<sup>c</sup> Corresponding analyses for PMA; FC is per one week difference in PMA; <sup>d</sup> Multiple linear regression including both SGA status and PMA.

The previous considerations are summarized for the urinary metabolites glycine and threonine in Figure 4. There were similar levels of glycine and threonine in the intervention and control group (Figure 4a,b). Glycine and threonine levels appeared to differ between SGA and AGA children in the first week of life, but not at later time points (Figure 4c,d). The same applied when the infants' age was defined as PMA instead of chronological age (Figure 4e,f).

Finally, the metabolite pseudo-concentrations were examined with respect to growth velocity from birth to four weeks of life [9]. In the initial linear regression models, first week glycine and threonine levels and third week glycine and hydroxyproline levels correlated positively with growth velocity. However, when adjusting for PMA in the models, these relations disappeared.

**Figure 4.** Temporal development of glycine and threonine log-pseudo-concentrations (means and 95% CIs) related to nutritional intervention, SGA status and age. (a) Glycine levels by nutritional intervention (intervention red, control gray); (c) Glycine levels by SGA status (SGA orange, AGA green) for samples from weeks 1, 3, 5 and 7; (e) As above, but samples selected by PMA instead of weeks of life; (b, d, f) Corresponding figures for threonine.



#### 4. Discussion

Due to proposed risks of overfeeding, we investigated the impact of enhanced nutrition on the urinary excretion profile during the first weeks of life in premature infants. We did not observe significantly different metabolic trajectories between the intervention group receiving nutritional support in the upper range of recent recommendations as compared to the infants on standard

nutrient supply; neither in the first-week urine profiles nor in the change over time. Furthermore, infants in the intervention group exhibited better overall growth [9]. Together, this suggests that premature infants handle enhanced nutrient supply similarly to a standard diet because the urinary profiles in the intervention group did not indicate an overload of the renal function as compared to the controls.

Our study also revealed that all infants exhibited substantial changes in their urinary profiles during the early postnatal period (Figure 3, Table 3), and these changes correlated with gestational age at birth and with chronological age (Tables 3 and 5). The correlation between PMA and urinary metabolite profiles has been reported previously [17,31,32], and may reflect the degree of organ development and metabolic maturity. Between the first and the third week of life the glucogenic amino acids glycine, threonine, hydroxyproline and tyrosine increased along with metabolites of the tricarboxylic acid cycle like 2-oxoglutarate, citrate, fumarate and succinate. In most mammals, the prenatal-postnatal transition is accompanied by important adaptations in carbohydrate metabolism due to the abrupt change from the placental supply of nutrients to a cyclic supply of nutrients via the breast milk [7]. In rodents this period is characterized by the appearance of gluconeogenic enzymes to maintain glucose homeostasis in the newborn [33]. Thus, the metabolite changes observed in our premature infants might reflect similar metabolic adaptations. The presence of metabolites linked to the tricarboxylic acid cycle may be due to the high metabolic turnover in premature infants. The tricarboxylic acid cycle is important in energy metabolism, providing intermediates for the synthesis of glucose and some amino acids [34].

Hydroxyproline showed a threefold increase in concentration during the initial postnatal period. Urinary hydroxyproline reflects collagen metabolism and is considered a marker of infant growth [35,36]. Although we observed a positive correlation between third week hydroxyproline levels and growth velocity, this correlation disappeared when PMA was introduced as a covariate, suggesting that urinary hydroxyproline is closely related to PMA.

In parallel with the increase of the other metabolites during the first month of life, gluconate and two unidentified metabolites with an NMR signals at 5.50 and 0.57 ppm disappeared. The latter unidentified metabolite has also been observed in the urine of pregnant women [26,37]. Its disappearance shortly after birth suggests that this substance was transferred from the mother to the infant and may reflect a sulfate- or glucuronide-conjugate of pregnanediol or estrogen [26].

Most SGA infants have been exposed to a limited nutrient supply during fetal life, which may cause irreversible metabolic changes (fetal programming). Subsequent catch-up growth, both in early infancy and in childhood, is also associated with later obesity and cardiovascular disease risk [4,5,8]. The so-called mismatch hypothesis proposes that an obesogenic childhood environment increases later cardiovascular disease risk, whereas the postnatal programming or postnatal growth acceleration hypothesis links rapid weight gain in early infancy to later cardiovascular disease risk. In a recent review [3], growth during late infancy and childhood appeared to be the major determinant of later metabolic and cardiovascular disease risk, and not the early postnatal growth. It has also been shown that early postnatal growth has a significant impact on later neurodevelopment [3]. Both these findings support the aggressive nutritional approach in our intervention. We studied metabolic differences between SGA and AGA infants at birth

and over time, and identified glycine and threonine as potential biomarkers of an altered metabolic phenotype.

Glycine has been linked to nutrient restriction of pregnant baboons, where the fetal plasma levels more than doubled compared to control fetuses [38]. A similar increase in fetal glycine levels has been observed in human SGA fetuses [39,40]. Dessi *et al.*, profiled newborn urines one and four days after birth and reported that in addition to the glycine and threonine pathway, prenatal growth restriction also affected metabolic pathways involving hydroxyproline, creatinine and myo-inositol [19]. They interpreted these metabolites as potential early markers of the metabolic syndrome. In line with their study, we found similar differences in the first-week urine profiles between our SGA and AGA infants, although after adjusting for PMA, only threonine and glycine remained independently elevated in the SGA infants. Glycine and threonine are glucogenic amino acids, which may be converted to pyruvate during protein metabolism. Increased levels of plasma glycine may be caused by reduced amino acid oxidation or reduced gluconeogenesis as a strategy to conserve amino acids [38]. We observed that glycine and threonine were linked to SGA status in the first urine sample, but we were unable to detect a persistent difference during the course of time. Although our study did not exhibit similar results for all metabolites as compared to the study by Dessi *et al.* [19], and the elevation of glycine and threonine levels were insignificant after Bonferroni adjustment, it still highlights glycine, threonine and to some extent hydroxyproline as important targets for future research.

Our study has several limitations. In our original intervention trial [10], we planned to recruit 240 infants. The early termination due to increased occurrence of septicemia in the intervention group resulted in a reduced number of infants in our present study. In spite of the fact that the intervention group had a significantly lower mean birth weight and a higher proportion of SGA infants than the control group, we observed increased whole body growth [9], improved white matter maturation and motion perception in the intervention group (manuscript submitted). Moreover, we did not find any significant differences between the metabolic trajectories with regard to the two different diets. The occurrence of septicemia and electrolyte deviations did not seem to influence the urinary metabolite profiles, but the lack of significant differences must be interpreted with caution in view of the relatively small study sample. Similar electrolyte disturbances have been reported in other studies with early and enhanced nutrition to premature infants during the first week of life [41–46]. Thus, a difference in metabolic profile would probably occur during the early postnatal stay, and it raises the question as to whether we would have been able to identify an effect with a more frequent first week monitoring of the urines in our premature infants.

Multiple hypothesis testing was performed using Bonferroni correction. Although samples from such vulnerable patients are challenging to come by, the randomized design of the current trial as well as the strict adherence to the nutritional protocol reduced the number of confounding factors.

## 5. Conclusions

The urinary metabolite profiles were unaltered by the enhanced postnatal nutrition, suggesting that supply in the upper range of current recommendations did not overload renal function. Our data show that both gestational age at birth, *i.e.*, degree of maturation, and postnatal physiological adaptations, may influence metabolism in premature infants during the neonatal period. Several of the first-week urinary metabolites were associated to SGA status and postnatal growth and might be markers for long-term health outcomes.

## Author Contributions

D.S. acquired the NMR spectra of the urine samples. S.J.M. was responsible for the detailed planning of the nutritional protocol, recruitment and treatment of infants, and the collection of clinical parameters and endpoint data. D.S. and S.J.M. jointly carried out the statistical analysis and drafted the manuscript. E.W.B., K.S., B.N. and A.N.A. contributed to the conception and the design of the study, recruitment and treatment of infants, and the collection of clinical parameters and endpoint data. A.C.W., A.R., K.B., M.B.V., P.O.I. and C.A.D. contributed to the conception and the design of the study, and the interpretation of data, M.B.V., in addition to the statistical analysis. F.R. contributed to the design of the study, the metabolic profiling, and the analysis and interpretation of the results. J.P.B. planned the metabolic profiling and contributed to the statistical analysis and the interpretation of the results. All authors revised the manuscript critically for important intellectual comment.

## Conflicts of Interest

The authors declare no conflict of interest.

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## Associations between Infant Feeding Practice Prior to Six Months and Body Mass Index at Six Years of Age

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**Abstract:** Rapid growth during infancy is associated with increased risk of overweight and obesity and differences in weight gain are at least partly explained by means of infant feeding. The aim was to assess the associations between infant feeding practice in early infancy and body mass index (BMI) at 6 years of age. Icelandic infants ( $n = 154$ ) were prospectively followed from birth to 12 months and again at age 6 years. Birth weight and length were gathered from maternity wards, and healthcare centers provided the measurements made during infancy up to 18 months of age. Information on breastfeeding practices was documented 0–12 months and a 24-h dietary record was collected at 5 months. Changes in infant weight gain were calculated from birth to 18 months. Linear regression analyses were performed to examine associations between infant feeding practice at 5 months and body mass index (BMI) at 6 years. Infants who were formula-fed at 5 months of age grew faster, particularly between 2 and 6 months, compared to exclusively breastfed infants. At age 6 years, BMI was on average  $1.1 \text{ kg/m}^2$  (95% CI 0.2, 2.0) higher among infants who were formula fed and also receiving solid foods at 5 months of age compared to those exclusively breastfed. In a high-income country such as Iceland, early introduction of solid foods seems to further

increase the risk of high childhood BMI among formula fed infants compared with exclusively breastfed infants, although further studies with greater power are needed.

**Keywords:** MeSH terms; growth; infant; breastfeeding; weaning; overweight; child

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## 1. Introduction

Childhood obesity is an ongoing public health concern [1]. Infant growth patterns are becoming better understood and the timing and tempo of growth rate appears to be important variables in predicting childhood obesity [2,3]. Rapid growth during the first year of life has been associated with increased risk of overweight and obesity in children [4,5] and differences in weight gain during early infancy are at least partly explained by means of infant feeding [6–8].

The World Health Organization (WHO) recommends exclusive breastfeeding for the first 6 months of life and its benefits are well accepted worldwide [6,9]. However, there is evidence that most mothers in European countries begin to introduce complementary foods before 6 months of age [10]. Studies have shown that formula fed children grow more rapidly than children who are exclusively breastfed [11,12] and are at greater risk of childhood overweight or obesity [6] but less is known about the long-term effects of early solid food introduction in early infancy. In a recent randomized controlled trial, introduction of complementary feeding at 4 months of age did not increase weight gain in infancy and did not appear to affect the risk of overweight or obesity at 18 months or 29–38 months of age compared to infants exclusively breastfed for 6 months [13–15]. The aim in this current analysis was to assess the associations between infant feeding practice in early infancy and body mass index (BMI) at 6 years of age. Furthermore, we aimed to assess whether the introduction of solid foods among partially breastfed or formula fed infants prior to 6 months of age was associated with childhood BMI.

## 2. Experimental Section

### 2.1. Study Design

The study population, recruitment and data collection have previously been described in detail [16]. In brief, a random sample of 250 Icelandic infants born in 2005 was collected by Statistics Iceland. The inclusion criteria were Icelandic parents, singleton birth, gestational length of 37–41 weeks, birth weight within the 10th and 90th percentiles, no birth defects or congenital long-term diseases, and the mother had early and regular antenatal care. In this current analysis, eligible subjects were those with complete data at birth and with a complete dietary record at 5 months, and weight and height measurements at 6 years of age ( $n = 154$ ). Our analysis are mainly based on the feeding practice at the age of 5 months where we compare the growth in infancy between those children who were exclusively breastfed at this time point to those who were either exclusively formula fed or those who had started complementary feeding (defined here as introduction of solid foods in addition to partial breastfeeding or formula feeding) at the age of 5 months. The reason

why we chose to use the 5 month registration for our primary analysis is that this was the earliest detailed food registration available in the present study and it gives information on variations in duration of exclusive breastfeeding. Informed written consent was obtained from all parents. The study was approved by the Icelandic Data Protection Authority (S5099/2011), Local Ethical Committee at Landspítali-University Hospital (1104Ref.16 2011) and the Bioethics committee (VSNb2011010008/037).

### *2.2. Infant and Childhood Growth Data Collection*

Birth information on weight and length was gathered from the maternity wards. Healthcare centers provided the measurements made during infancy at 1, 2, 3, 5, 6, 8, 10, 12 and 18 months of age. As close to the child's sixth birthday as possible (mean  $73.4 \pm 3.2$  months) weight (Marel M series 1100, Iceland;  $\pm 0.1$  kg) and height (Ulmer stadiometer, Professor Heinze, Busse design, Ulm, Germany;  $\pm 0.5$  cm) were measured in a clinical examination at the Landspítali-University Hospital. BMI was calculated as weight (kg)/height ( $m^2$ ). When the infant was 12 months of age, the parent or caregiver was asked to complete a questionnaire regarding information on age, education, and physical characteristics including self-reported height and weight of both parents.

### *2.3. Dietary Assessment*

Information on breastfeeding was gathered monthly during the first 12 months. The parents or caregivers completed a 24-h food record monthly from 5 to 8 months and 10 to 11 months using common household measures such as cups and spoons. At 9 and 12 months of age, weighed food records were kept for three consecutive days on accurate scales (PHILIPS HR 2385, Austria; PHILIPS HR 2385, Hungary;  $\pm 1$  g accuracy).

Average daily consumption of energy and the contribution of energy providing nutrients at the age of 9 and 12 months were estimated using ICEFOOD, a software program used by the Icelandic Nutrition Council. Special infant products, such as cereals and purées were added to the database and nutrient losses due to food preparation were taken into account in the calculations.

### *2.4. Statistical Analysis*

Mean and standard deviation (SD) or proportions (%) were used to describe infant and maternal characteristics. Differences in infant and maternal characteristics were calculated using *t*-tests for continuous variables and chi-squared tests for categorical variables. Linear regression analyses were used to determine associations between infant feeding practice at 5 months of age and BMI at 6 years. All regression analyses were adjusted for sex, birth weight, and maternal education level categorized as completion of elementary school, high school or vocational school, or university. Information on mother's education was missing for  $n = 12$ , thus a total of 142 subjects were analyzed for the linear regression. Changes in growth were calculated from crude differences in measurements at the two different time points. All statistical analyses were carried out using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). The level of significance was set at  $p < 0.05$ .

### 3. Results

Table 1 shows the participants' characteristics among infants at 5 months of age who were exclusively breastfed ( $n = 62$ ), exclusively formula fed ( $n = 12$ ), started on solid foods with partial breastfeeding ( $n = 57$ ) or started on solid foods with formula feeding ( $n = 23$ ).

**Table 1.** Participant characteristics and dietary variables by infant feeding practice at 5 months of age.

Variable	Infant Feeding Practice at 5 Months			
	Exclusively Breastfed ( $n = 62$ )	Excusively Formula Fed ( $n = 12$ )	Solid Foods with Partial Breastfeeding ( $n = 57$ )	Solid Foods with Formula Feeding ( $n = 23$ )
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
<i>Maternal characteristics</i>				
Age (years)	31.5 $\pm$ 4.5	30.4 $\pm$ 4.0	31.0 $\pm$ 4.8	30.1 $\pm$ 4.8
BMI (kg/m <sup>2</sup> )	23.7 $\pm$ 3.2	29.2 $\pm$ 9.1	24.5 $\pm$ 3.8 *	27.0 $\pm$ 6.5
University education [ $n$ (%)]	31 (50)	8 (67)	28 (49)	6 (26)
<i>Infant characteristics</i>				
Girls [ $n$ (%)]	34 (55)	24 (55)	24 (42)	14 (61)
Exclusive breastfeeding (month)	5.0 $\pm$ 0.9	1.7 $\pm$ 1.7 *	3.1 $\pm$ 1.5 *	1.4 $\pm$ 1.5 *
Breastfeeding duration (month)	9.5 $\pm$ 1.9	3.9 $\pm$ 2.7 *	8.3 $\pm$ 2.4 *	2.8 $\pm$ 1.1 *
<i>Food groups introduced at 5 months</i>				
Porridge [ $n$ (%)]	-	-	53 (93)	18 (78)
Fruits and vegetables [ $n$ (%)]	-	-	20 (35)	15 (65)
Dairy products [ $n$ (%)]	-	-	20 (35)	17 (74)
Legumes [ $n$ (%)]	-	-	1 (2)	3 (13)
Meat, organs [ $n$ (%)]	-	-	0	0
Eggs [ $n$ (%)]	-	-	0	0
<i>Dietary composition at 9 months</i>				
Energy (kcal/kg)	101 $\pm$ 36	109 $\pm$ 27	101 $\pm$ 31	101 $\pm$ 48
Protein (g/kg)	3.0 $\pm$ 1.4	3.7 $\pm$ 1.4	3.2 $\pm$ 1.4	3.6 $\pm$ 1.7
<i>Dietary composition at 12 months</i>				
Energy (kcal/kg)	89 $\pm$ 22	80 $\pm$ 16	86 $\pm$ 18	79 $\pm$ 16
Protein (g/kg)	3.1 $\pm$ 0.9	3.0 $\pm$ 0.8	3.6 $\pm$ 1.7	3.2 $\pm$ 0.9

\* Significantly different from exclusively breastfed infants determined by  $t$ -tests for continuous variables and chi-square tests for categorical variables.

Maternal characteristics were not markedly different although mothers who exclusively breastfed their child at 5 months had lower BMI. The total duration of breastfeeding was shortest among infants who were formula fed (both with and without solid food introduction) at the age of 5 months. Among infants who were provided solid foods at 5 months, the food items with the highest frequency of consumption were porridge, dairy products, and fruits and vegetables. Based on the 24-h dietary registration at 5 months, there were no children eating meat, fish, poultry, liver, or eggs although it is possible they were exposed to these foods. Although no difference was

observed in energy intake at 9 or 12 months based on infant feeding practice at the age of 5 months, infants who were receiving formula feeds had higher protein intake per kilogram at 9 months.

There were no differences in birth weight between the different infant feeding groups (Table 2). However, infants who were formula fed at 5 months grew faster after birth, particularly between 2 and 6 months of age compared to exclusively breastfed infants (Table 3).

**Table 2.** Anthropometrics from birth by infant feeding practice at 5 months of age.

Growth Variable	Exclusively Breastfed (n = 62)	Exclusively Formula Fed (n = 12)	Solid Foods with Partial Breastfeeding (n = 57)	Solid Foods with Formula Feeding (n = 23)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Weight (kg)				
At birth	3.8 ± 0.4	3.8 ± 0.4	3.7 ± 0.4	3.7 ± 0.3
2 months	5.6 ± 0.6	5.5 ± 0.7	5.8 ± 0.5	5.4 ± 0.4
6 months	7.8 ± 0.8	8.2 ± 1.0	8.2 ± 1.0	8.5 ± 0.8 *
2 months	9.6 ± 0.9	10.3 ± 1.4	10.1 ± 1.2 *	10.6 ± 1.0 *
18 months	11.3 ± 1.0	11.5 ± 1.2	11.6 ± 1.3	11.9 ± 1.2 *
Length (cm)				
At birth	51.9 ± 1.5	52.0 ± 1.7	51.8 ± 1.6	51.7 ± 1.9
2 months	59.7 ± 1.5	59.4 ± 1.8	59.8 ± 2.0	58.9 ± 1.5
6 months	68.3 ± 2.0	69.4 ± 2.7	69.1 ± 2.3 *	68.4 ± 1.7
12 months	76.2 ± 2.5	77.4 ± 2.9	77.1 ± 2.7	77.6 ± 2.5 *
18 months	83.0 ± 2.5	83.8 ± 3.2	83.9 ± 2.7	83.1 ± 2.5

\* Significantly different from exclusively breastfed infants determined by *t*-tests.

**Table 3.** Changes in weight from birth to 18 months by infant feeding practice at 5 months comparing infants on formula or solid foods,  $\Delta$  (95% CI), to exclusively breastfed infants.

Weight Variable	Exclusively Breastfed (referent) (n = 62)	Exclusively Formula Fed (n = 12)	Solid Foods with Partial Breastfeeding (n = 57)	Solid Foods with Formula Feeding (n = 23)
Changes in weight (g)	Mean ± SD	$\Delta$ (95% CI) <sup>a</sup>	$\Delta$ (95% CI) <sup>a</sup>	$\Delta$ (95% CI) <sup>a</sup>
Birth to 2 months	1859 ± 500	-188 (-531, 155)	156 (-31, 343)	-119 (-369, 132)
2 to 6 months	2208 ± 480	512 (147, 877) *	161 (-63, 385)	787 (472, 1102) *
6 to 12 months	1775 ± 550	172 (-193, 538)	122 (-85, 328)	283 (-8, 574)
12 to 18 months	1649 ± 563	-56 (-450, 338)	-90 (-297, 116)	-314 (-634, 6)
Birth to 6 months	4081 ± 745	308 (-168, 785)	360 (58, 661) *	755 (367, 1143) *
Birth to 12 months	5876 ± 884	510 (-108, 1129)	462 (92, 831) *	1019 (566, 1471) *

<sup>a</sup> Mean difference in weight compared to exclusively breastfed infants (referent); \* Significantly different from exclusively breastfed infants determined by *t*-tests.

Furthermore, the group that had been introduced to solid foods along with formula feeding at the age of 5 months grew significantly faster compared to exclusively breastfed infants, while the difference was smaller and non-significant for the group who had been provided solid foods and were partially breastfed (Table 3). The addition of solid foods along with formula feeding at 5 months predicted greater BMI at 6 years, with BMI being on average 1.1 kg/m<sup>2</sup> (95% CI 0.2, 2.0) higher compared to exclusively breastfed infants at 5 months of age (Table 4).

**Table 4.** Associations between infant feeding practice at 5 months and BMI at 6 years of age ( $n = 142$ ).

<b>Infant Feeding Practice at 5 Months</b>	<b><math>\Delta</math> (95% CI)<sup>a</sup></b>	<b><math>p</math><sup>*</sup></b>
Exclusively breastfed	Referent	-
Formula fed	0.3 (-0.8, 1.4)	0.583
Solid foods with partial breastfeeding	0.5 (-0.1, 1.2)	0.125
Solid foods with formula feeding	1.1 (0.2, 2.0)	0.020

<sup>a</sup> Adjusted difference in BMI at 6 years with respect to exclusively breastfed infants; <sup>\*</sup> Analyses adjusted for sex, birth weight, and maternal education.

#### 4. Discussion

In this current analysis, we found that infants who were provided solids foods in addition to formula feeding at 5 months of age had faster growth during infancy and up to 12 months of age compared to exclusively breastfed infants. Differences in weight were most pronounced between the ages of 2 to 6 months. Furthermore, the addition of solid foods among formula fed infants prior to the age of 6 months predicted greater BMI at 6 years.

The strength in this cohort lies in the longitudinal nature and the detailed information on infant feeding practices. In this way, we are able to describe dietary intake in infancy in greater detail than possible in many other studies in relation to BMI at 6 years of age. It is difficult to separate the effects of exclusive breastfeeding (and total duration of breastfeeding) from the introduction of solid foods at the age of 5 months. However, our findings are in line with existing evidence that show exclusively breastfed infants grow slower compared to infants of the same age who receive formula or solid foods [2,6] and that longer duration of breastfeeding may protect against later obesity potentially due to slower weight gain in infancy [6].

There are several factors that contribute to variations in infant feeding practices. Predictors of early introduction of solid foods include young maternal age, low maternal education and short (<4 weeks) duration of breastfeeding [17]. There were no significant differences with maternal age, and maternal education also did not appear to predict introduction of solid foods at 5 months of age, although the proportion of mothers with higher education was greater among the infants who were exclusively breastfed. We note that the mothers who started their infants on formula feeding had a higher mean BMI compared to mothers of exclusively breastfed infants, although the numbers are too few to detect a significant difference. There is evidence from epidemiological studies that women who are overweight or obese are less likely to breastfeed compared to normal weight women [18,19].

A probable explanation for the observed difference in growth rate between breastfed and formula fed infants is the relatively higher protein content of infant formula compared to breastmilk. High protein intake may have a stimulating effect on insulin-like growth factor 1 which can accelerate growth [20]. Furthermore, higher protein intake during infancy has been associated with faster weight gain and greater adiposity [21] and there is evidence that this is associated with higher BMI in childhood [4,7,22]. Another possible mechanism is that compared to breastmilk, infant formula contains a higher amount of omega-6 fatty acids which may play a role in promoting adipose tissue development and result in greater childhood adiposity [23].

Results from a randomized controlled trial performed in Iceland, showed no significant differences in energy intake [15], growth [14], or risk of being overweight [13] between those exclusively breastfed for 4 vs. 6 months. The reason may be a low amount of energy from solid foods [14,15] mainly consisting of infant cereals (67%) and median protein intake of only 0.9 g/day [14]. In other studies, exposing infants to solid foods prior to the age of 4 months was associated with being overweight or obese in early childhood [24,25], but it was suggested that the risk associated with the timing of introduction of solid foods might be greater among children who were no longer breastfed [24]. In the present study, we saw that partially breastfed infants who had been introduced to solid foods at the age of 5 months did not grow as fast as the infants who were formula fed and consuming solid foods.

Together these findings suggest that in addition to the timing of complementary foods, the type of food, and the protein content of the infant formula introduced may influence infant growth [26]. In this cohort, 70% of infants who had been provided solid foods at 5 months were exclusively breastfed at 2 months of age. Mothers may introduce solid foods earlier if they find their infant appears hungry or worry that breast milk is inadequate for their infant's needs [27]. However, limited data exists on the effects of specific food groups introduced during the complementary feeding period in relation to infant growth and childhood BMI. Further studies are needed in this area to determine whether rapid infant growth leads to children demanding more feedings or whether the introduction of formula feeding or solid foods is the main contributor.

Some limitations exist in this current analysis. The sample size is a possible limitation however, the thorough data on dietary intake and growth variables provides valuable information and the number is sufficient to analyze differences in infant feeding practices and its potential contribution to childhood weight status. Information on the exact timing of solid food introduction would have been useful as well as reasons affecting the duration of breastfeeding, particularly among the infants who had been provided solid foods at 5 months to better understand the observed association with BMI at 6 years. The aim of this present analysis was to determine the association between differences in infant feeding practices and childhood BMI, and even more detailed data related to diet and nutrient composition before the age of 5 months would have been beneficial. Overall, it appears that earlier formula feeding contributes to growth in infancy and adding solid foods along with formula feeding may influence growth in childhood compared to exclusively breastfed infants.

## 5. Conclusions

In this current analysis, we found that infants who were provided solid foods along with formula feeding prior to 6 months of age had faster growth during infancy and greater BMI at 6 years of age compared to exclusively breastfed infants. In a high-income country such as Iceland, early introduction of solid foods seems to further increase the risk of high childhood BMI of formula fed infants compared to exclusively breastfed infants. Furthermore, better breastfeeding promotion strategies may help reduce the incidence of childhood overweight. Further studies with more detailed information on infant feeding practices and statistical power are needed to determine the appropriate composition of infant formula and solid foods especially if mothers are for some reasons unable to breastfeed exclusively for 6 months.

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## Author Contributions

C.M.I. assisted with the design of this current manuscript and performed the statistical analyses and drafting and editing of the manuscript. I.G. assisted with the design, statistical analyses and contributed in data collection, writing and editing the manuscript. B.T. assisted with statistical analyses, did the data collection, and participated in writing and editing the manuscript. T.I.H. participated in the statistical analyses and provided critical revision of the paper. I.T. was the principal investigator and designer of the study and supervised data collection, data analyses and writing and editing of the manuscript.

## Conflicts of Interest

The authors report no conflict of interest.

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## Influence of Posture and Frequency Modes in Total Body Water Estimation Using Bioelectrical Impedance Spectroscopy in Boys and Adult Males

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**Abstract:** The aim of the study was to examine differences in total body water (TBW) measured using single-frequency (SF) and multi-frequency (MF) modes of bioelectrical impedance spectroscopy (BIS) in children and adults measured in different postures using the deuterium (<sup>2</sup>H) dilution technique as the reference. Twenty-three boys and 26 adult males underwent assessment of TBW using the dilution technique and BIS measured in supine and standing positions using two frequencies of the SF mode (50 kHz and 100 kHz) and the MF mode. While TBW estimated from the MF mode was comparable, extra-cellular fluid (ECF) and intra-cellular fluid (ICF) values differed significantly ( $p < 0.01$ ) between the different postures in both groups. In addition, while estimated TBW in adult males using the MF mode was significantly ( $p < 0.01$ ) greater than the result from the dilution technique, TBW estimated using the SF mode and prediction equation was significantly ( $p < 0.01$ ) lower in boys. Measurement posture may not affect estimation of TBW in boys and adult males, however, body fluid shifts may still occur. In addition, technical factors, including selection of prediction equation, may be important when TBW is estimated from measured impedance.

**Keywords:** body fluid; deuterium; dilution technique; impedance technique; prediction equation; accuracy; technical error

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## 1. Introduction

Body composition, including fat mass (FM) and fat-free mass (FFM), is an important variable in the assessment of health status. Obesity has been defined as a state of excessive fat deposition [1,2] and the assessment of body composition assists in identifying individuals with metabolic risks. In addition, while body mass index (BMI:  $\text{kg}/\text{m}^2$ ) and other simple anthropometric indices have been utilized as convenient screening tools for obesity, assessment of body composition reduces misclassification of individuals at risk.

Body composition can be determined using a wide range of techniques. Each technique varies not only in its accuracy and precision, but also in cost, portability, convenience, and requirements for accredited operators. Bioelectrical impedance analysis (BIA) is one of the most commonly utilized techniques as it is simple, portable and cost- and time-efficient. The technique assesses differences in the electrical conductivity between tissues. Tissues that contain water and electrolytes have higher conductivity compared to those with less body fluid. From the measurement of electrical conductivity, resistance (R) and reactance ( $X_c$ ) can be determined. These components can be utilized to calculate impedance (Z) based on their association  $Z^2 = R^2 + X_c^2$  and also a phase angle based on a ratio of  $X_c$  to R [3]. In addition, together with information on the length (L) or height (Ht), a total volume of body water (TBW) can be determined [4,5]. Furthermore, while R has been used most frequently, R,  $X_c$ , and Z have been used to estimate TBW, intra-cellular fluid (ICF) and extra-cellular fluid (ECF) as well as percentage body fat (%BF) of individuals [4].

Existing BIA devices can be divided into single-frequency BIA (SF BIA), multi-frequency BIA (MF BIA) and bioelectrical impedance spectroscopy (BIS). SF BIA devices generally use a frequency of 50 kHz that passes through both ECF and ICF [4]. In comparison, MF BIA uses multiple frequencies in the range of 1 to 1000 kHz and enables one to distinguish between ICF and ECF. A previous study reported that a low frequency, generally below 20 kHz, is used to predict ECF whereas a higher frequency (above 50 kHz) is used to estimate TBW in MF BIA [6]. As a result, ICF can be determined from the difference of the two. Although it has been suggested that MF BIA may overestimate %BF of lean individuals and underestimate that of obese individuals [7], error in estimation of %BF may be minimized compared with SF BIA [8]. BIS is a more sophisticated model that uses a wide range of frequencies and non-linear mathematical algorithm to assess relationships between R and body fluid. This allows estimation of R extrapolated to zero ( $R_0$ ) and infinite ( $R_\infty$ ) frequencies and development of empirically-derived prediction equations [4,5,9]. Although both accuracy and precision of results may vary depending on the characteristics of the study population [9], past studies have reported that BIS provides better estimation of ECF than SF BIA [4,5,10,11] and also has an acceptable accuracy and precision using an animal model [12]. In addition, a technique known as “segmental BIA” is available which determines information

on total body composition through measurements of each segment (*i.e.*, upper and lower limbs and the trunk). A previous review has described a number of advantages and considerations [13], and another study reported that segmental BIA can provide valid information on body composition compared with the four-compartment model [14]. However, most studies have been undertaken on adults and studies of children are relatively scarce. Consequently, little knowledge is available on any differences in TBW estimation between adults and children and the effects of using different frequencies.

In addition, wide variations in measurement posture are commonplace using the impedance technique depending on the device used. Many hand-to-foot models measure in a supine position, the posture recommended in the European Society for Parenteral and Enteral Nutrition (ESPEN) guidelines [10]. However, modern segmental BIAs that are in a scale type are designed to measure in a standing position [13]. A previous study on the influence of posture during measurements reported 2%–5% changes in ECF and 1.8%–8.0% changes in ICF depending on body position [15]. Another study reported no significant differences in %BF using a hand-to-foot device but a significant increase in %BF using a hand-to-hand device [8]. These results suggest that body posture may influence estimation of body fluid status and therefore estimation of %BF using the impedance technique. However, these studies were conducted on adults and no previous research has reported differences between adults and children.

Therefore, the present study aimed to examine influences of frequency modes and measurement posture in estimation of TBW in adult males and boys. The estimated TBW was compared with the result obtained from the reference deuterium ( $^2\text{H}$ ) dilution technique.

## 2. Experimental Section

The study was approved by the Human Research Ethics Committee of Queensland University of Technology and adhered to the principles of medical research established by the National Health and Medical Research Council [16]. Boys aged below 15 years or adult males aged above 20 years with no medical conditions or under medication were included in the study. Participants were recruited through flyers. All participants were given an information package and all signed a consent form prior to their participation. For participants below 18 years of age, parents or legal guardians also signed the consent form. All participants were provided with a \$20 gift voucher after their full participation in the study. In total, 49 participants including 23 boys aged between 6 and 14 years and 26 adult males aged between 23 and 82 years, completed all assessments and were included in the study. All participants were instructed to fast overnight and void their bladders in the morning, prior to measurements being conducted. All assessments on children were conducted by the primary investigator.

### 2.1. Anthropometry

All participants underwent measurements of stature using a stadiometer to the nearest 0.1 cm and body weight using a digital weighing scale to the nearest 0.1 kg. Waist circumference was measured at the narrowest point between the 10th rib and the iliac crest using a steel

anthropometric tape. All measurements were conducted according to the protocol of the International Society for the Advancement of Kinanthropometry (ISAK) [17]. All measurements were taken by a level three anthropometrist accredited by ISAK with an intra-tester technical error of measurements (TEM) of less than 1.0%, below recommended levels [18,19]. From these measurements, BMI and waist-to-height ratio (WHtR) were calculated.

## 2.2. Bioelectrical Impedance Spectroscopy (BIS)

Body fluid status was determined using a BIS device (Imp SFB7, ImpediMed Ltd, Brisbane, Australia) with functionality to switch between multi-frequency (MF) and single-frequency (SF) modes. In the MF mode, TBW was estimated using a wide range of frequencies between 4 and 1000 kHz with 256 data points [20]. In the SF mode, two of the five fixed frequencies (*i.e.*, 50 kHz and 100 kHz) of the device that are commonly selected were used [20].

The device was calibrated before measurements of each participant. All participants rested (lying on a bed) for at least five minutes prior to the measurement. Electrodes were placed on the dorsal surface of the wrist and the ankle as well as at the base of the second or third metacarpal-phalangeal joints of hand and foot after the skin was cleaned with an alcohol wipe. The lead wires were attached to the appropriate electrodes and participants were instructed to abduct their limbs from the trunk. After measurement in the supine position, participants were instructed to stand up and stay in the same position for at least five minutes before measurement in the standing position. The measurements were repeated in both positions after staying in the position at least five minutes. Length of rest time was consistent with the protocol used in a previous study [21]. While the participant was in the same posture, triplicate measurements were conducted and a median value determined. After completion of two sessions of triplicate measurements at each measurement posture, an average of two median values was calculated for each variable.

From each measurement, TBW, ECF, ICF, FFM and FM were calculated for the MF mode using built-in algorithms. For the SF mode, Xc and R were recorded for each frequency. Similar to a previous study [22], all measurements were conducted with resistivity coefficients of 273.9 for zero/extracellular ( $\rho_e$ ) and 937.2 for infinite/intracellular ( $\rho_i$ ), body density of 1.05 g/cm<sup>3</sup> and body proportion of 4.3. From the obtained FM and FFM results, %BF was calculated for the MF mode. For the SF mode, Z was calculated from mean Xc and R using an equation  $\sqrt{R^2 + Xc^2}$ . Calculated Z values were then utilized to estimate TBW of the study groups using age-, gender-, and frequency-specific prediction equations. In the present study, estimation of TBW for boys was conducted using an equation by Davies *et al.* [23] with Z determined from a frequency of 50 kHz ( $Z_{50}$ ):

$$TBW = 0.6 \times (Ht^2/Z_{50}) - 0.5 \quad (1)$$

For males, equations by Deurenberg *et al.* [24] were used to estimate TBW using Z determined from frequencies of 50 kHz ( $Z_{50}$ ) and 100 kHz ( $Z_{100}$ ):

$$TBW = 6.53 + 0.3674 \times Ht^2/Z_{50} + 0.17531 \times \text{body weight} - 0.11 \times \text{age} + 2.83 \quad (2)$$

$$TBW = 6.69 + 0.34573 \times Ht^2/Z_{100} + 0.17065 \times \text{body weight} - 0.11 \times \text{age} + 2.66 \quad (3)$$

### 2.3. Deuterium Dilution Technique

Deuterium ( $^2\text{H}$ ) dilution technique was the reference method used for TBW. Prior to the assessment, a 10% deuterium oxide ( $\text{D}_2\text{O}$ ) solution was prepared by mixing 100 mL of 99.9%  $\text{D}_2\text{O}$  solution (Aldrich Chemistry, Sigma-Aldrich Pty Ltd, Sydney, Australia) and 900 mL of tap water. The solution was sterilized at 120 °C for 10 min using an autoclave. After collecting a pre-dose urine sample, the body weight of participants was measured using a weighing scale. The dose amount of 10%  $\text{D}_2\text{O}$  solution was calculated as  $0.5 \times$  body weight (kg) and weighed using a scale. All participants consumed the weighed 10%  $\text{D}_2\text{O}$  solution and re-weighed the drinking cup to record the precise amount consumed. After consumption of the 10%  $\text{D}_2\text{O}$  solution, participants were instructed to collect a post-dose urine sample after five hours, to ensure that equilibration of  $^2\text{H}$  within the body fluid pool was reached.

Both pre- and post-dose samples were analyzed using an isotope ratio mass spectrometry (IRMS: Hydra 20-20, SerCon Mass Spectrometry, SerCon Limited, Cheshire, CW1 6YY UK). All analyses were conducted at the laboratory at the Institute of Health and Biomedical Innovation (IHBI) of Queensland University of Technology (QUT) in Brisbane, Australia. TBW was calculated using the following equation:

$$\text{TBW (kg)} = ((W \times A/a) \times (\Delta\text{DD}/\Delta\text{BW})) / (1000 \times 1.041) \quad (4)$$

where  $W$  = total weight of water added when making the dose dilution (g),  $A$  = weight of dose taken by the participant (g),  $a$  = weight of dose in diluted dose (g),  $\Delta\text{DD}$  = enrichment of  $^2\text{H}$  in the diluted dose (ppm excess  $^2\text{H}$ ), and  $\Delta\text{BW}$  = enrichment of  $^2\text{H}$  in body water (ppm excess  $^2\text{H}$ ). The value of 1.041 was based on an assumption that the dilution space or the volume of the distribution of  $^2\text{H}$  is 1.041 times greater than TBW [25].

All statistical analyses were conducted using the PASW<sup>®</sup> Statistics package (version 18.0.0, IBM, Chicago, IL, USA). Paired  $t$ -tests were conducted to compare results obtained from different postures (*i.e.*, supine and standing positions). In addition, TBW estimated from different frequency modes were compared with the results from the dilution technique using repeated measures of analysis of variance (ANOVA) and a Bonferroni *post hoc* test. Results were expressed as mean  $\pm$  standard error (SE). In addition, variability of estimated TBW for the study population from different frequency modes were determined using correlation coefficients, limits of agreement (*i.e.*, difference  $\pm 1.96 \times$  standard deviation), and the Bland and Altman plots [26] using the dilution technique as the reference. All statistical tests used significant level of 0.05 unless otherwise stated.

### 3. Results

Physical characteristics of the participants were  $9.8 \pm 0.5$  years,  $144.1 \pm 3.2$  cm and  $36.6 \pm 2.4$  kg for boys and  $36.9 \pm 2.7$  years,  $174.3 \pm 1.4$  cm, and  $76.5 \pm 3.2$  kg for adult males, respectively (Table 1). WHtR, an index of abdominal fat accumulation with the cut-off point of 0.5, were  $0.44 \pm 0.01$  in boys and  $0.49 \pm 0.01$  in adult males, respectively.

**Table 1.** Demographic characteristics of participants.

	Boys ( <i>n</i> = 23) Mean ± SE	Males ( <i>n</i> = 26) Mean ± SE
Age (years)	9.8 ± 0.5	36.9 ± 2.7
Stature (cm)	144.1 ± 3.2	174.3 ± 1.4
Body weight (kg)	36.6 ± 2.4	76.5 ± 3.2
BMI (kg/m <sup>2</sup> )	17.2 ± 0.5	25.0 ± 0.8
WHtR	0.44 ± 0.01	0.49 ± 0.01

Table 2 shows differences in results obtained from supine and standing positions. In both adult males and boys, there were no significant differences in TBW using the MF mode in supine and standing positions. There were no differences in estimated FFM, FM and %BF between measurement postures using the MF mode. Adult males, however, showed a statistically significant ( $p < 0.05$ ) difference in TBW in independent measurement sessions in the supine position ( $44.3 \pm 1.5$  L and  $44.2 \pm 1.5$  L). ECF measured in the standing position was significantly ( $p < 0.01$ ) increased compared with the supine position in both groups (males:  $19.2 \pm 0.6$  L vs.  $18.9 \pm 0.6$  L, boys:  $10.0 \pm 0.6$  L vs.  $9.7 \pm 0.6$  L). There was also a significant ( $p < 0.01$ ) decrease in ICF in the standing position in both groups. Using the SF mode, while no difference in  $Z_{50}$  was observed between measurement postures in males, a significant decrease in  $Z_{50}$  was observed in the standing position in boys ( $665.3 \pm 16.1$   $\Omega$  in the supine position vs.  $656.9 \pm 16.3$   $\Omega$  in the standing position,  $p < 0.01$ ). A similar result was observed using 100 kHz ( $p < 0.05$  in males and  $p < 0.01$  in boys). In addition, adult males showed a significant difference in  $Z$  values in each supine measurement, boys showed significantly different  $Z$  values from standing sessions (data not shown).

**Table 2.** Differences in variables between measurement postures in boys and adult males.

Posture	Boys ( <i>n</i> = 23) Mean ± SE			Adult Males ( <i>n</i> = 26) Mean ± SE			
	Supine	Standing	<i>p</i> -value	Supine	Standing	<i>p</i> -value	
Multi-Frequency	TBW (L)	21.3 ± 1.4	21.2 ± 1.5	0.348	44.3 ± 1.5	44.2 ± 1.5	0.823
	ECF (L)	9.7 ± 0.6	10.0 ± 0.6	<0.001	18.9 ± 0.6	19.2 ± 0.6	<0.001
	ICF (L)	11.6 ± 0.9	11.3 ± 0.8	0.005	25.4 ± 0.9	25.0 ± 0.9	0.005
	FFM (kg)	29.1 ± 2.0	29.0 ± 2.0	0.353	60.5 ± 2.0	60.4 ± 2.1	0.819
	FM (kg)	7.4 ± 0.8	7.6 ± 0.8	0.353	16.0 ± 1.4	16.0 ± 1.5	0.824
	%BF (%)	20.1 ± 1.3	20.5 ± 1.3	0.340	20.1 ± 1.2	20.1 ± 1.3	0.820
Single-Frequency	$Z_{50}$ ( $\Omega$ )	665.3 ± 16.1	656.9 ± 16.3	<0.001	473.1 ± 8.9	472.2 ± 9.4	0.524
	$Z_{100}$ ( $\Omega$ )	635.0 ± 15.8	626.5 ± 16.2	<0.001	447.5 ± 8.6	444.3 ± 9.0	0.021

Using repeated measures ANOVA, the present study also examined the accuracy of the estimated TBW from the impedance technique using the dilution technique as the reference (Table 3). In boys, TBW values estimated from the MF mode were comparable to the results from the dilution technique regardless of the measurement postures. However, in males, the MF mode provided a significantly ( $p < 0.01$ ) greater TBW in both postures compared to results from the dilution technique. When TBW was estimated using the SF mode with a selected prediction equation, no significant difference with the result from dilution technique was observed in males regardless of their measurement posture and frequency used. However, TBW estimated from boys using a

frequency of 50 kHz was significantly ( $p < 0.01$ ) smaller compared to the result from the dilution technique, regardless of their measurement posture.

The variability of TBW estimation using different frequency modes are shown in Table 4. Compared with the dilution technique, results from both MF and SF modes showed high correlation coefficients of 0.956 to 0.988. However, Bland and Altman plots for the supine position showed that TBW of almost all adult males was overestimated when the MF mode was used (Figure 1a) and calculated limits of agreement indicated an average of 2.4 L overestimation with a wide variability of about 3.7 L (Table 4). In comparison, boys showed relatively accurate estimation of TBW but some boys with a larger TBW were overestimated (Figure 1b). Calculated limits of agreement indicated that TBW estimation using the MF mode for boys had an underestimation of 0.38 L with variability of about 2 L. Similarly, TBW from the dilution technique and the SF mode using 50 kHz was compared. In males, the Bland and Altman plot indicated that individuals with relatively low TBW values (less than 40 L) were overestimated by the SF mode whereas the opposite was true for those with relatively high TBW (greater than 40 L). Limits of agreement indicated, on average, overestimated about 0.7 L with a variability of approximately 4 L. In boys, TBW was underestimated in all participants with potentially greater underestimation in individuals with a higher TBW (Figure 2). Limits of agreement showed about 2.7 L of underestimation with a variability of 2.4 L.

**Table 3.** Differences in TBW estimated from different techniques.

	Boys ( $n = 23$ ) Mean $\pm$ SE		Males ( $n = 26$ ) Mean $\pm$ SE	
TBW <sub>(2H dilution)</sub> (L)	21.7 $\pm$ 1.4		41.9 $\pm$ 1.5	
<b>Supine</b>				
Device	Mean $\pm$ SE	$p$ -value	Mean $\pm$ SE	$p$ -value
Multi-Frequency (L)	21.3 $\pm$ 1.4	0.333	44.3 $\pm$ 1.5	<0.001
Single-Frequency Z <sub>50</sub> (L) <sup>†</sup>	19.0 $\pm$ 1.3	<0.001	42.6 $\pm$ 1.4	0.566
Single-Frequency Z <sub>100</sub> (L) <sup>‡</sup>	-	NA	42.2 $\pm$ 1.3	1.000
<b>Standing</b>				
Device	Mean $\pm$ SE	$p$ -value	Mean $\pm$ SE	$p$ -value
Multi-Frequency (L)	21.2 $\pm$ 1.5	0.250	44.2 $\pm$ 1.5	<0.001
Single-Frequency Z <sub>50</sub> (L) <sup>†</sup>	19.3 $\pm$ 1.3	<0.001	42.7 $\pm$ 1.4	0.461
Single-Frequency Z <sub>100</sub> (L) <sup>‡</sup>	-	NA	42.4 $\pm$ 1.4	1.000

<sup>†</sup> TBW was estimated using the estimation equation Deurenberg *et al.* [24] for males and Davies *et al.* [23] for boys;

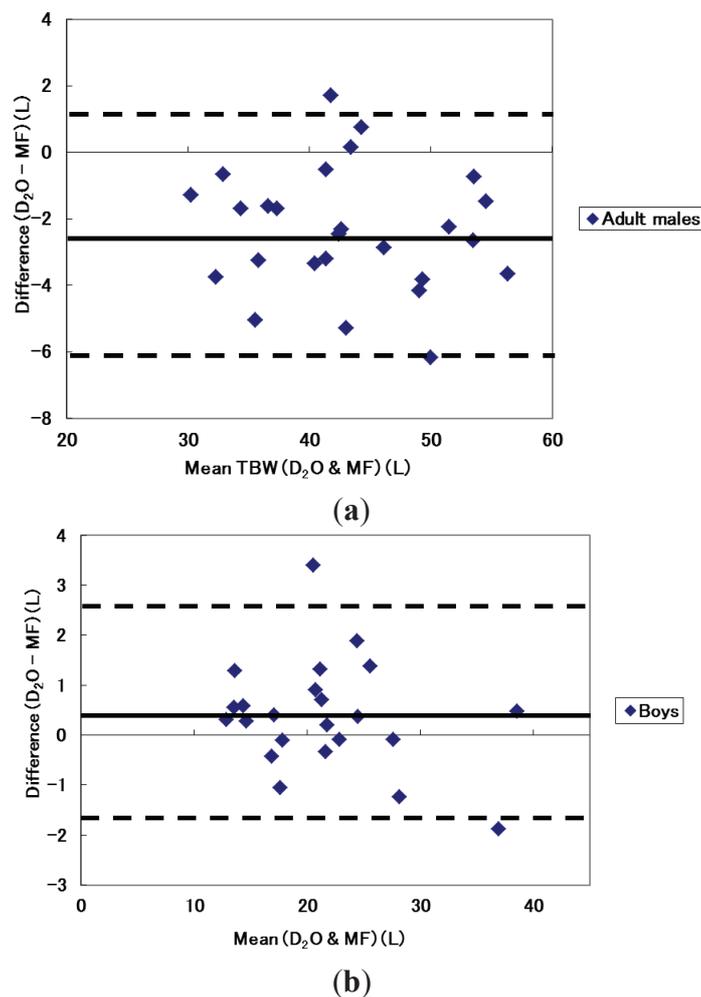
<sup>‡</sup> TBW was estimated using the estimation equation by Deurenberg *et al.* [24].

**Table 4.** Variability of TBW estimation using different frequencies compared with the dilution technique.

		Boys ( $n = 23$ )	Males ( $n = 26$ )
Multi-Frequency	Correlation coefficient	0.988	0.970
	Limits of Agreement	0.378 $\pm$ 2.14 (2.518, -1.762)	-2.361 $\pm$ 3.655 (1.29, -6.015)
Single-Frequency Z <sub>50</sub> <sup>†</sup>	Correlation coefficient	0.985	0.956
	Limits of Agreement	2.6524 $\pm$ 2.357 (5.009, 0.295)	-0.7465 $\pm$ 4.289 (3.543, -5.036)

<sup>†</sup> TBW was estimated using the estimation equation Deurenberg *et al.* [24] for males and Davies *et al.* [23] for boys.

**Figure 1.** Bland and Altman plots between TBW estimated from the dilution technique and multi-frequency mode for (a) adult males and (b) boys.



**Figure 2.** Bland and Altman plots between TBW estimated from the dilution technique and single-frequency mode (50 kHz) for (a) adult males and (b) boys.

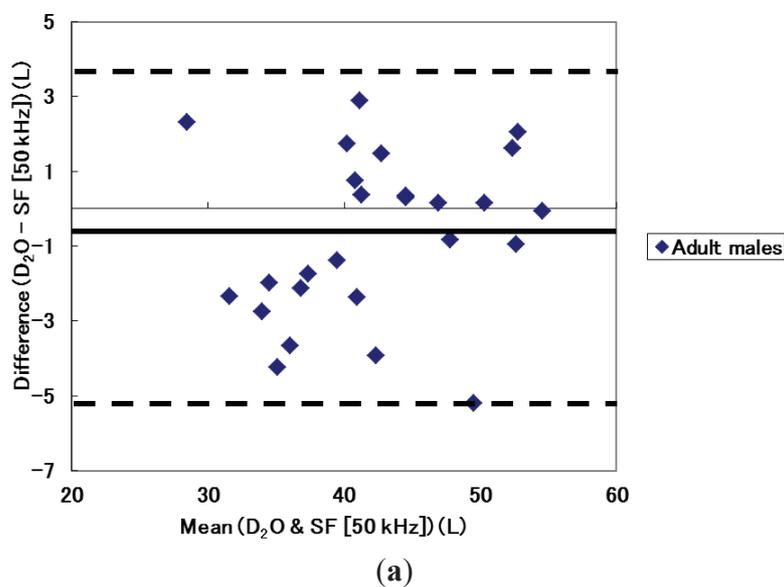
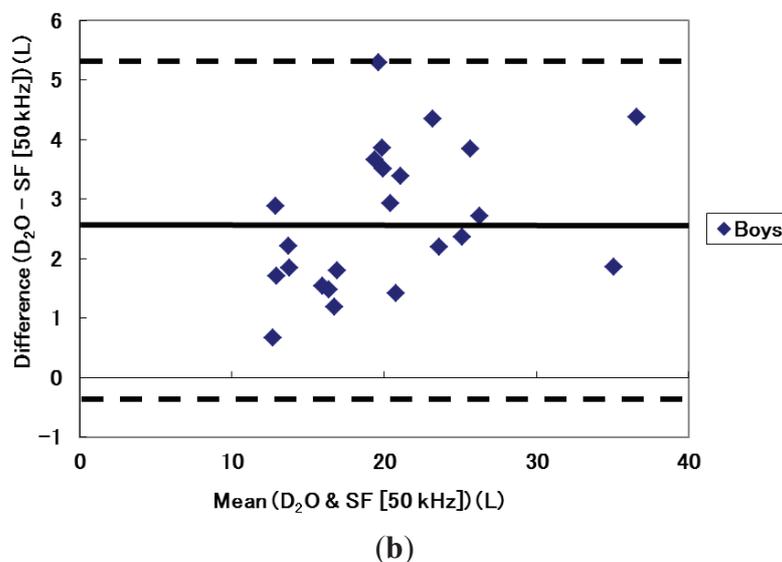


Figure 2. Cont.



#### 4. Discussion

The present study investigated the influence of posture and frequency modes of impedance technique in the estimation of TBW in adult males and boys. Results confirmed that measurement posture had no significant influence on TBW estimation and therefore no influence on estimation of body composition in this convenience sample. The results were consistent with earlier reports using SF devices [21]. This suggests that influence of body posture during measurements using impedance technique has a minimal impact on overall estimation of body composition. However, the present study showed significant changes in ICF and ECF volumes depending on posture during measurements in both adult males and boys. It has been suggested that a change in posture will cause redistribution of ECF. The observed results, although smaller in magnitude, were consistent with a previous study that reported change of ECF and ICF volumes by posture [15]. The result was inconsistent with another study that reported a redistribution of ECF only occurred between body segments (*i.e.*, the limbs and the trunk) with total ECF volume not altering as a function of change in measurement posture [27]. The present findings of change in ECF and ICF with no overall change in TBW may be due to a larger sample size compared to the previous study that examined only 11 males.

In addition to a possible fluid shift as a result of change in measurement posture, the presence of stray capacitance may also have influenced the observed outcomes. Weyer and colleagues [28] suggested that of the two fundamental stray capacitances in the impedance technique, the one formed between the human body and the ground may have considerable impact on the reading. Technical factors such as stray capacitances as well as variables such as body proportions, body density, and resistivity coefficients may be important in interpreting accuracy and quality of results. Boys showed a significant decrease in  $Z_{50}$  measured in the standing position. Since the frequency of 50 kHz can go through both ECF and ICF, a reduction in  $Z$  may indicate a reduction in TBW. However, results from the MF mode did not show a change in TBW between postures. As no

differences in  $Z_{50}$  were observed from males, this may suggest that, together with body fluid shift some technical factors influenced measurements in boys.

While no specific pattern was observed from the MF mode, estimation of TBW in adult males using the SF mode showed a different pattern depending on whether the participant had TBW greater or lesser than 40 L. Compared to adult males, boys showed a better correlation and agreement between the dilution technique and the impedance technique (both SF and MF modes). A smaller difference in results from the dilution technique and both MF and SF modes and narrower limits of agreement indicates the accuracy of the impedance technique. Observed differences in TBW estimation may be associated with a number of technical factors, such as resistivity coefficients, a body density, a body proportion factor and also prediction equations to estimate TBW using measured  $Z$  for the SF mode. The current study used default values for resistivity coefficients, a body density and a body proportion that were derived from healthy Caucasian adults. Although other studies have adopted the same default settings in unhealthy populations (e.g., obese) [22] or children [29,30], the estimation of TBW or  $Z$  values in the current study, particularly in boys, may be the result of technical error. In addition, the estimated TBW from a frequency of 50 kHz showed greater underestimation or noticeable pattern in both males and boys. This may be explained by application of prediction equations to estimate TBW. In this study, TBW for males was estimated by using the equation by Deurenberg *et al.* [24] and TBW of boys were calculated using the equation by Davies *et al.* [23] that was derived from a small group of children ( $n = 26$ ). While the equation by Deurenberg *et al.* [24] was derived from 139 healthy volunteers, the equation by Davies *et al.* [23] was derived from a group of children with particular health conditions, including growth hormone deficiency, inflammatory bowel disease and diabetes. In addition, while the equation was derived from both boys and girls, the equation does not include gender as a variable. Although the age range was matched with the sample of the present study, it may be possible that application of these equations may also affect accuracy and variability of the results. These possibilities suggest the importance of considering the abovementioned technical issues in differentiating biological influence such as fluid shift caused by a change in a measurement posture and also to improve the accuracy of the results, particularly using the SF mode.

## 5. Conclusions

In summary, the present study clarified that estimation of TBW using the MF mode of BIS device is not affected by measurement posture regardless of participants' maturational status or body size. Accordingly, estimation of body composition, including %BF is not affected by change in measurement posture. However, it should be noted that change in posture may be associated with fluid shift within the body that may alter values for ECF, ICF and  $Z$ . In addition, it is important to consider technical factors associated with measurements, including stray capacitances, resistivity coefficients, body proportion factor and also selection of appropriate prediction equations in order to differentiate the effect of measurement posture and technical error. As information on appropriate resistivity coefficients and body proportion factors for children is very scarce future research should consider explore appropriate values for this population. Similarly, as

the current study was based on a relatively small sample size, future research should replicate the study using a larger group in different age categories as well as including females to examine gender differences.

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### Author Contributions

M.K. contributed in study design, recruitment and data collection, sample analysis, data analysis and preparation of the manuscript. C.W. contributed in sample analysis and preparation of the manuscript. A.H. contributed in overall supervision and preparation of manuscript.

### Conflicts of Interest

The authors declare no conflict of interest.

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